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

JOSÉ GABRIEL DA SILVA GOMES

EFEITO CITOTÓXICO E IMUNOGENICIDADE *IN SILICO* DE L-ASPARAGINASE RECOMBINANTE DE *Phaseolus vulgaris* L.

FORTALEZA
2023

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2023

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)

G614e Gomes, José Gabriel da Silva.
Efeito citotóxico e imunogenicidade in silico de L-asparaginase recombinante de Phaseolus vulgaris L. /José Gabriel da Silva Gomes. – 2023.
66 f. : il. color.

Dissertação (mestrado) – Universidade Federal do Ceará, Centro de Ciências, Programa de Pós-Graduação em Bioquímica, Fortaleza, 2023.
Orientação: Prof. Dr. Bruno Anderson Matias da Rocha.

1. Enzima anticancerígena. 2. Feijão. 3. Proteína Recombinante. 4. Citotoxicidade. I. Título.
CDD 572

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Aprovada em 26/07/2023.

BANCA EXAMINADORA

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Prof. Dr. Bruno Anderson Matias da Rocha (Orientador)

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AGRADECIMENTOS

Caro leitor, é de conhecimento geral que trabalhos assim jamais poderiam ser concretizados por uma única pessoa. Não diferente do comum, esta pessoa que redige este texto tem muito a agradecer aos que lhe ajudaram nesse processo veloz e complexo.

Em primeiro lugar, assim como em minha graduação, agradeço aos meus pais Celeneide e Alves que, sem qualquer sombra de dúvida, me apoiaram, e ainda apoiam, nessa jornada em busca de maior grau de estudo. Aprecio ainda mais que se interessem pelo que estudo e escutem com atenção as minhas explicações cheias de contexto e adaptações. Ciência não é só para mim, meus amigos acadêmicos, a própria academia, tampouco a própria ciência. Feliz fico ao compartilhar com vocês os poucos conhecimentos científicos que obtive ao longo desses anos, assim continuo aprendendo a ter paciência e entendimento que a forma que eu entendo não é a forma que outros assimilam.

Não menos importante, agradeço ao Henrique, meu namorado, que esteve presente nessa esburacada viagem comigo desde o momento da inscrição. Obrigado por sempre escutar minhas inúmeras reclamações sobre os dias de trabalho e atribulações. Obrigado por entender que o meu mundo científico não fica no laboratório me esperando até o próximo dia, mas permeia cada segundo do meu viver neste planeta. Obrigado por entender meus momentos ansiosos e me acolher quando necessário. Obrigado por insistir, junto comigo, nesse relacionamento que tanto enfrenta trânsitos e distâncias. Obrigado por ser quem é.

Agradeço de coração ao meu orientador, Bruno Anderson, que me acolheu debaixo de suas asas e me ajudou a crescer como pessoa em muitos aspectos. Nossa relação, ao meu ver, se fortificou ao longo do tempo, e eu já não o vejo apenas como meu orientador. Considero-o um amigo. Talvez isso influencie em atitudes que tomo que podem não ser as mais adequadas, e peço perdão por isso. Que nossa relação se fortifique ainda mais durante meu doutorado e no futuro que nos aguarda.

Segurando-se nesse fio, agradeço aos integrantes do Laboratório de Biocristalografia (LABIC): Misrael, Cássia, Roberta, Francisco, Leonardo, Beatriz, Chay e Micaelle. Obrigado pelos momentos que compartilhamos, sempre com risadas, apesar dos pesares e tensões. Obrigado por qualquer apoio que me deram. Aproveito para me desculpar por qualquer interação possivelmente tóxica, e espero que possamos melhorar juntos nesse caminhar científico. Cada um de vocês é precioso e eu acredito que muito aguarda vocês no futuro. Agradeço também a todos os integrantes do GBME.

Agradeço ao Grupo de Engenharia de Proteínas e Soluções para Saúde da Fundação Oswaldo Cruz – Ceará, coordenado por Gilvan Furtado e Marcos Lourenzoni, pela valiosa parceria que formamos. Agradeço ao professor Gilvan, quem me coorientou mesmo que não registrado no papel. Muito obrigado por confiar em meu trabalho e me auxiliar em tudo. Agradeço por garantir meu acesso aos recursos da instituição e promover minha interação com o maravilhoso e acolhedor ambiente da fiocruz. Espero que nossa parceria ainda dê muitos frutos.

Ainda na fiocruz, eu gostaria de agradecer a todas as pessoas com quem tive contato nesse ambiente de trabalho. Pude aprender muito com cada um e nossos dias no laboratório, e fora dele, foram essenciais para me dar forças para continuar a pesquisa. Em especial, eu gostaria de agradecer à Larissa, quem me acompanhou e me apresentou aos recursos do laboratório, quem muito me ensinou sobre biologia molecular e química de proteínas, quem me auxiliou, junto com Marcus Rafael, a montar meu plasmídeo para que eu pudesse dar início ao meu projeto. Você é uma pessoa maravilhosa, amiga, e cativa muita gente, inclusive eu. Agradeço também ao Igor, quem sempre foi muito gentil e paciente e prestativo na minha primeira semana na fiocruz.

Eu não poderia deixar de registrar minha enorme gratidão à Larisse Cadeira Brandão, minha dupla de experimentos. Um dos presentes que a fiocruz me deu. Amiga, agradeço demais pelos nossos momentos e por ter conhecido você, uma pessoa tão simpática, carismática, cheia de luz e amor. Obrigado por suportar minha miríade de humores ao longo desse tempo que tivemos juntos. Obrigado por suportar minha falta de paciência e tantos outros defeitos. Obrigado por não ligar para minhas cantorias de “ópera”. Deixo registrado aqui que você é, sim, capaz do que você colocar em sua mente, independente de dificuldades. Você é capaz, inteligente e uma cientista, além de professora.

Não menos importante, eu gostaria de agradecer à turminha da 205: Darlyson, Jucilene, Lucas, Maísa e Virgínia. Os momentos que passei com vocês também foram muito preciosos e agradeço muito pela ajuda que cada um me proveu. Obrigado ao Lucas pelos momentos em que ficou comigo até tarde no laboratório me ajudando, pelas risadas e saídas. Obrigado à Jucilene, uma das minhas mães científicas, pelas maravilhosas contribuições que fez ao meu trabalho, em especial nesses últimos tempos.

Aproveito esta oportunidade para agradecer à minha incrível turma de mestrado: Ellen, Gabrielle, Jayssa, Sávio, Fabiano e Isa. Apesar de tudo, acredito que nossa união ajudou cada um de nós a superar as dificuldades da pós-graduação, a passar nas disciplinas e suportar cada momento injusto e difícil. Cada um de vocês tem um lugar no meu coração.

Nessa mesma turma, encontrei um espírito amigo habitado no corpo da Isa. Uma amiga que simplesmente é. Inteligente, carismática, simpática, cafezeira, fornecedora de informações valiosas e, sobretudo, prestativa. Amiga, agradeço demais pela sua pessoa em minha vida e espero que possamos continuar nossa amizade daqui pra frente, pois muito me é valiosa.

Agradeço à minha família de ambas as partes pelo apoio em menor e maior grau. Esse apoio me conduziu até onde estou.

Agradeço aos meus amigos da graduação: Isabel, Leonardo, Ruan, Sara, Ramon, Daniel, Yuri, Mariana. Eu sei que seguimos nossos caminhos e isso dificultou nossos momentos juntos, mas sempre encontramos formas de nos manter amigos e eu amo cada um de vocês. Obrigado por tudo.

Agradeço ao Departamento de Bioquímica e Biologia Molecular e à Universidade Federal do Ceará pela estrutura concedida. Um viva às universidades públicas. Também gostaria de agradecer à Coordenação de Aperfeiçoamento de Pessoal de nível Superior (CAPES) pelo financiamento da bolsa de mestrado.

Agradeço aos membros da banca Claudener Souza e Denise Hissa pelo aceite para participarem da avaliação do meu trabalho e pelas valiosas contribuições que fizeram ao meu projeto.

Por fim, deixo registrado que, apesar de muito gostar da minha vida como cientista e do que a ciência me proporciona, o tempo e energia que tudo isso consome me afastou de mim mesmo. Hoje, eu me sinto menos do que eu era, pois me afastei do meu eu artístico. Hoje, não sou mais o mesmo rio poético que se transpõe. Eu sequei consideravelmente. A seca desidrata e o coração agora dá lugar a tantas cobranças restritivas. Tudo que passo e passei, ainda que arte, não foi expresso. Até agora. Ainda que desidratado, eu...

Sou um mero grão de areia,

Banhado de sal e

Envolto de ondas.

As marés têm substantivo próprio, entretanto

Esqueço todos eles.

Ao olhar para a lâmina,

Que agora me acolhe,

Respiro toda a água que posso,

Pois já fui flor,

Daquelas que desabrocham nas sombras.

Eu me entreguei ao mar e suas aventuras.

Encontro-me aqui, muito mais que simplório espectador,

Muito menos do que força motriz.

E essa dualidade é paz,

Caos,

Razão.

Toda essa cinesia, que me moveu por

Picos e vales,

Precisa de uma pausa.

Então inerte me registro,

Ainda que me mova aos olhos de outrem.

E por se tratar de mim,

Mero grão de areia,

Espero uma próxima onda,

Seguinte maré,

Sequente oceano.

RESUMO

A enzima L-asparaginase desempenha um papel crucial no tratamento da leucemia linfoblástica aguda, um tipo de câncer que afeta principalmente crianças e adolescentes. No entanto, devido à imunogenicidade e atividade glutaminásica, é comum que essas moléculas causem reações adversas ao longo do tratamento. Essas desvantagens estimulam a busca por novas asparaginases que possam mitigar esses problemas. Nessa busca, as plantas têm apresentado ao longo dos anos características promissoras que as tornam candidatas a substituir as enzimas comerciais, que são de origem bacteriana. Nesse contexto, a L-asparaginase recombinante de *Phaseolus vulgaris* carece de uma caracterização completa de suas propriedades bioquímicas, biofísicas e antineoplásicas. Assim, este trabalho objetivou produzir e caracterizar uma L-asparaginase recombinante de *P. vulgaris* (Asp-P). Para tanto, a enzima foi expressa em *E. coli* e purificada por cromatografias de afinidade e exclusão molecular. A atividade enzimática foi medida utilizando reagente de Nessler. Os parâmetros cinéticos e os efeitos de interferentes na atividade enzimática foram determinados. A presença da enzima nas amostras foi analisada por *western blotting* e espectrometria de massas. A estrutura secundária e a estabilidade térmica da proteína foram ambas avaliadas por dicroísmo circular. Além disso, os efeitos de citotoxicidade em células Raji e K562 foram testados pelo método de MTT. Ferramentas de predição *online* foram usadas para determinar a imunogenicidade de Asp-P em comparação com a proteína bacteriana. Os resultados mostraram que Asp-P foi expressa com altos rendimentos e atividade específica de 905 $U.mg^{-1}$, com máxima atividade em pH 9 e 40° C. *Western blots* confirmaram a presença de Asp-P nas amostras, bem como nenhum contaminante nativo de L-asparaginase bacteriana. A espectrometria de massas mapeou 93 % da sequência de Asp-P. A enzima reduziu a porcentagem de células viáveis da linhagem Raji abaixo de 50% apenas na maior concentração testada, mas o mesmo não aconteceu para K562. Por fim, os métodos de predição *in silico* indicaram que Asp-P é menos imunogênica do que as enzimas bacterianas. Juntos, esses resultados mostram que a Asp-P possui propriedades diferentes da enzima nativa e aspectos favoráveis à substituição das enzimas bacterianas.

Palavras-chave: enzima anticancerígena; feijão; proteína recombinante; citotoxicidade

ABSTRACT

The enzyme L-asparaginase plays a crucial role in the treatment of acute lymphoblastic leukemia, a type of cancer that mostly affects children and teenagers. However, due to their immunogenicity and glutaminase activity, it is common for these molecules to cause adverse reactions during treatment. These downsides ignite the search for novel asparaginases that could mitigate these problems. In this quest, plants have shown promising features over the years that turn them into candidates to substitute the commercial enzymes, which are from bacterial source. In this context, *Phaseolus vulgaris* recombinant L-asparaginase lacks a thorough characterization of its biochemical, biophysical and antineoplastic properties. Thus, this work aimed to produce and characterize a recombinant L-asparaginase from *P. vulgaris* (Asp-P). For this purpose, the enzyme was expressed in *E. coli* and purified by affinity and size-exclusion chromatographies. The enzyme activity was measured by the Nesslerization method. The kinetics parameters, thermotolerance and the effects of interferents on enzyme activity were determined. The presence of the enzyme in the samples was analyzed by western blotting and mass spectrometry. The secondary structure and the thermostability of the protein were both assessed by circular dichroism. Also, the cytotoxicity effects of Asp-P on Raji and K562 cells were assayed by MTT method. Online prediction tools were used to determine the immunogenicity of Asp-P in comparison with the bacterial protein. The results showed that Asp-P was expressed with high yields and specific activity of 905 $U.mg^{-1}$, with maximum activity at pH 9.0 and 40° C. Western blots confirmed the presence of Asp-P in the samples, as well as no contaminant native *E. coli* L-asparaginase. Mass spectrometry mapped 93 % Asp-P sequence. Asp-P could reduce Raji viable cells percentage below 50 % only at the highest concentration tested, but the same could not be achieved for K562 cultures. Lastly, *in silico* prediction methods indicated that Asp-P is less immunogenic than bacterial enzymes. Put together, these results show that recombinant Asp-P has different properties when compared to the native enzyme and has favorable aspects that indicate a promising enzyme to substitute the bacterial ones.

Keywords: antineoplastic enzyme; kidney bean; recombinant protein; cytotoxicity assay

SUMÁRIO

1 INTRODUÇÃO

1.1 L-asparagina

A L-asparagina é um aminoácido polar, não carregado e considerado não-essencial para humanos, visto que as células possuem a capacidade e o aparato enzimático para sintetizar a molécula a partir de outros precursores. Este aminoácido, assim como a glutamina, possui em sua cadeia lateral um grupo amida que pode ser hidrolisado para formar L-aspartato. Sua síntese é realizada pela enzima asparagina sintetase, a qual, apesar de possuir maior afinidade por glutamina, também pode utilizar amônia como substrato e, assim, transferir o grupo amida para o L-aspartato (GAUFICHON *et al.*, 2010). Apesar de seu importante papel como componente das estruturas primárias das mais diversas proteínas, o aminoácido em questão possui outras importantes atribuições no metabolismo dos organismos, como ser ponto de glicosilação *N*-ligada em proteínas e ser utilizado como molécula para transporte e armazenamento de nitrogênio em plantas (GARCÍA-CALDERÓN *et al.*, 2017; GAUFICHON; ROTHSTEIN; SUZUKI, 2016; SIECIECHOWICZ; JOY; IRELAND, 1988).

Entretanto, a L-asparagina não se reduz apenas a esses papéis. A molécula está envolvida, também, em diversas reações e mecanismos de interesse humano, principalmente, nas áreas da saúde e da indústria alimentícia. Por exemplo, a presença desse aminoácido em alimentos superaquecidos (acima de 120° C) promove a formação de acrilamida, um composto altamente tóxico, apresentando efeitos cancerígenos e neurotóxicos naqueles que entram em contato com o mesmo. Esse composto prejudicial à saúde humana é formado pela reação de Maillard principalmente em pães, biscoitos, batatas e café, que são alimentos ricos em carboidratos e aminoácidos livres, em especial asparagina (CLAUS; CARLE; SCHIEBER, 2008; DA CUNHA *et al.*, 2019; LEA *et al.*, 2007; WANG *et al.*, 2021).

Nesse contexto, um papel da L-asparagina muito notório nos últimos anos é sua capacidade de ser uma molécula de troca. O aminoácido intracelular é utilizado para o transporte de aminoácidos extracelulares, como serina e histidina, ao ser enviado para o meio. Dessa forma, asparagina, também, exerce a função de regulação dos níveis de aminoácidos intracelulares (KRALL *et al.*, 2016).

A literatura também relata a utilização de L-asparagina como uma forma de prevenir a morte celular em células cancerígenas com baixa nutrição de L-glutamina, sendo este um aminoácido bastante consumido por alguns tipos de células neoplásicas. Nessa situação,

asparagina pode servir como substituta para algumas reações mediadas por glutamina, como a síntese de nucleotídeos (ZHANG *et al.*, 2014).

A participação de L-asparagina em processos neoplásicos, no entanto, não se limita a isso. Sua síntese e presença no microambiente tumoral favorece muito a metástase, enquanto que a inibição de asparagina sintetase e depleção dos níveis séricos do aminoácido em questão reduzem as taxas de metástase de alguns tipos de câncer, apesar de não afetarem o crescimento do tumor primário (KNOTT *et al.*, 2018).

1.2 Leucemia Linfoide Aguda (LLA)

Dentre as neoplasias e suas correlações com a L-asparagina, a mais compreendida e aplicada na atualidade é a Leucemia Linfoide Aguda (LLA). Esta doença é um subtipo de leucemia, uma forma de câncer que é o nono lugar em mortes causadas por neoplasias a nível mundial, ainda que as taxas de sobrevivência mundiais atinjam 90%. No Brasil, no entanto, essa taxa não atinge 70 %, que pode estar associada a problemas no acesso ao tratamento pela população (CECCONELLO *et al.*, 2020; ROSER; RITCHIE, 2019). Sendo do subtipo linfoide, a LLA é caracterizada pela proliferação desenfreada de linfócitos B e/ou T imaturos, os quais, em virtude dessa imaturidade, não desenvolvem suas funções adequadamente, uma vez que o metabolismo não é preservado (INABA; MULLIGHAN, 2020).

Nesse contexto, células B e T possuem alterações diferentes que caracterizam o tipo celular que identifica a neoplasia. A LLA de linfócitos B é a mais comum dentre os casos mundiais, e apresenta mais de 20 subtipos com diferentes perfis gênicos. Dentre as alterações, os três tipos mais importantes são aneuploidia, rearranjos cromossômicos que alteram expressão de oncogenes e fatores de transcrição e mutações pontuais. Estas e outras alterações gênicas são distribuídas entre os vários subtipos com diferentes frequências, coocorrências e prognoses. São essas modificações que afetam diversos processos celulares, como diferenciação, regulação do ciclo celular e da cromatina, além de sinalizações (INABA; MULLIGHAN, 2020; LILLJEBJÖRN *et al.*, 2016; PAULSSON *et al.*, 2015; ROBERTS; MULLIGHAN, 2020).

Por outro lado, a LLA de linfócitos T é menos comum e é mais facilmente identificada por caracterização de expressão gênica. Apesar de haver 10 vias principais afetadas por alterações mais recorrentes nos casos desse subtipo de leucemia, 3 dessas vias são desreguladas com maior frequência: vias de fatores de transcrição específicos da linhagem de células T; sinalização da via de expressão de NOTCH1/MYC e vias de controle do ciclo celular.

A alteração da via de NOTCH1 nesse tipo de neoplasia causa uma expressão extrema desse fator de transcrição, o qual é muito importante para o desenvolvimento de células T. Essa mudança no padrão de expressão, associada à deleção de *loci* supressores de tumor, implica o crescimento descontrolado desse tipo de linfócito (GIANNI; BELVER; FERRANDO, 2020; INABA; MULLIGHAN, 2020; LIU *et al.*, 2017; YUI; ROTHENBERG, 2014).

Apesar de afetar adultos, crianças e adolescentes são os mais acometidos pela doença e apresentam sintomas que, sem devidos exames, podem não ser suficientes para diagnosticar efetivamente o câncer, como hepatoesplenomegalia, infecções recorrentes e persistentes, fadiga e febre. Dessa forma, é necessário um consórcio de exames físicos, hematológicos, bioquímicos e genéticos para um diagnóstico preciso e início do tratamento, por exemplo hematoscopia, mielograma, citogenética da medula óssea e até *Polymerase chain reaction* (PCR) (INABA; MULLIGHAN, 2020; INCA, 2022).

Uma vez diagnosticada, a primeira linha de combate à doença é o tratamento quimioterápico, o qual se divide em fases: indução da remissão; consolidação e manutenção. Ao longo das décadas, aumentou-se gradativamente as taxas de sobrevivência com a melhora do tratamento e sua resposta pelos pacientes. Ainda assim, a quimioterapia apresenta diversos efeitos colaterais associados aos medicamentos utilizados (INABA; MULLIGHAN, 2020; KATO; MANABE, 2018; RADADIYA *et al.*, 2020).

A primeira fase do tratamento, indução da remissão, é uma das mais importantes, uma vez que inicia a tentativa de reduzir a proliferação e provocar apoptose das células cancerígenas, além de restaurar a produção de células hematopoiéticas saudáveis. Com esse intuito, são administrados três medicamentos principais aos pacientes: glicocorticoides (Prednisona ou Dexametasona), vincristina e L-asparaginase. Nessa fase há forte incidência de reações adversas que implicam em alterações de doses ou descontinuação do tratamento convencional (ALDOSS; STEIN, 2018). Em seguida, tem-se a fase de consolidação do tratamento, iniciando uma mudança na medicação administrada ao paciente, a qual é composta por ciclofosfamida, citarabina/metotrexato e mercaptopurina. O principal objetivo da fase de consolidação é manter os baixos níveis de células cancerígenas, impedindo sua proliferação e intrusão do sistema nervoso central e mantendo o paciente em remissão. Por fim, a fase de manutenção aplica doses menores e mais esparsas de metotrexato e diárias de mercaptopurina. Caso haja recidiva do câncer durante as fases de consolidação e manutenção, o paciente é submetido a um intenso tratamento com os medicamentos da fase de indução da remissão, a primeira fase (INABA; MULLIGHAN, 2020; KATO; MANABE, 2018; TOSTA PÉREZ *et al.*, 2023).

Dentre os medicamentos utilizados na indução da remissão, temos a L-asparaginase, uma enzima capaz de hidrolisar a L-asparagina em L-aspartato e amônia. A administração desse remédio é justificada pela característica da maioria das LLAs de não expressarem de forma ativa a enzima asparagina sintetase, responsável pela síntese de asparagina. Dessa forma, essas células neoplásicas se caracterizam pela sua auxotrofia, uma vez que dependem de aminoácidos provenientes do meio extracelular para sobreviverem (CHIU *et al.*, 2020; JIANG; BATRA; ZHANG, 2021).

Visando essa característica, a enzima L-asparaginase, desde sua descoberta e do seu potencial anticancerígeno, vem sendo empregada nos tratamentos quimioterápicos de LLA intuindo diminuir os níveis séricos de L-asparagina e, assim, promover a apoptose das células cancerígenas pela falta desse aminoácido essencial para elas (SHORT; KANTARJIAN; JABBOUR, 2021). Diferentes fabricantes fornecem a enzima comercial com diferentes níveis de pureza e atividade (SCHNUCHEL *et al.*, 2023). No Brasil, não há produção desse medicamento, o qual é adquirido por importação. Essa forma de aquisição, em situações de emergência, pode levar a problemas. Por exemplo, a troca de fabricantes prejudicou pacientes durante um período, visto que a nova marca (Leuginase®), substituindo Aginasa® e Eslpar®, não apresentou atividade considerada terapêutica e uma maior imunogenicidade (MICHALOWSKI *et al.*, 2021).

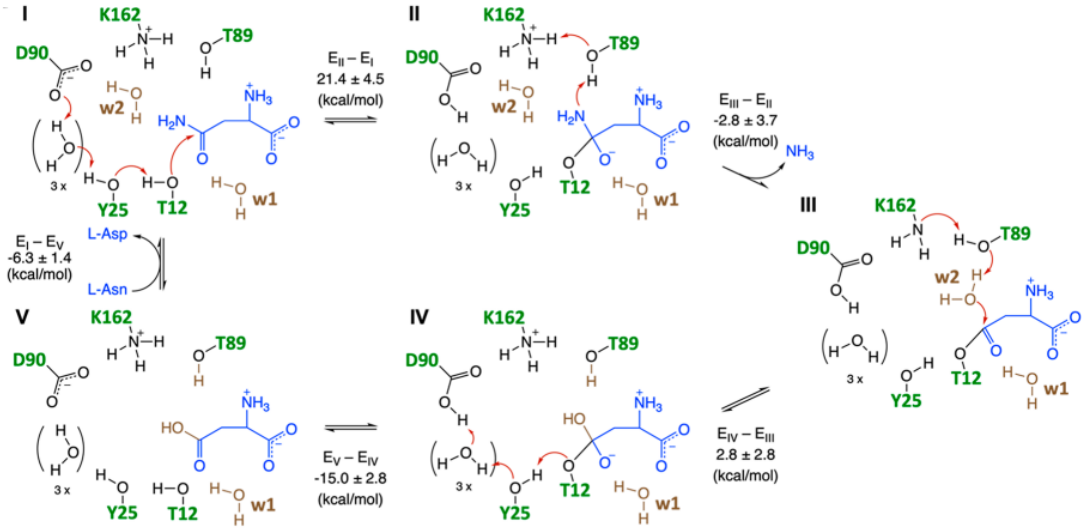
Ainda que importante no tratamento da LLA, várias reações adversas são apresentadas pelos pacientes tratados com essa enzima (hipersensibilidade, pancreatite, hepatite, trombose, entre outros), o que pode levar à alteração de dose ou descontinuação do tratamento com asparaginase (HIJIYA; VAN DER SLUIS, 2016; LANVERS-KAMINSKY, 2017). Porém, já foi demonstrado que a descontinuação da enzima no tratamento quimioterápico é um fator de péssima prognose quanto à taxa de sobrevivência dos pacientes (ISHIDA *et al.*, 2023).

1.3 L-asparaginase

Tão importante nesse contexto, L-asparaginase é uma enzima pertencente ao grupo das amidohidrolases (E.C. 3.5.1.1), sendo responsável pela hidrólise de L-asparagina, liberando no processo L-aspartato e amônia. Seu primeiro relato data do início do século XX, ainda que não fosse nomeada na época. Somente na década de 50 foi demonstrada sua capacidade de inibir a proliferação de linfocarcinoma e carcinoma mamário, uma vez que foi identificada no soro de porcos da índia por Kidd (1953).

Por muitos anos, algumas teorias acerca do mecanismo de catálise das L-asparaginases foram formuladas e testadas através de métodos experimentais e computacionais utilizando, principalmente, a enzima de *E. coli* como modelo de estudo, visto que é uma das mais caracterizadas neste ramo do conhecimento. Até recentemente, duas teorias eram defendidas: a do desacoplamento único, ou direto, e a do desacoplamento duplo (*ping-pong*). A primeira teoria possui uma menor quantidade de passos para a finalização do ciclo catalítico, no qual ambas moléculas de amônia e aspartato são liberadas juntas, sem qualquer ligação forte entre moléculas e enzima. Nesse caso, não há formação de um intermediário (GESTO *et al.*, 2013; SCHALK *et al.*, 2016).

Figura 1 – Mecanismo de reação da L-asparaginase



Nota: Representação do mecanismo de catálise das L-asparaginases bacterianas. (I) doação de um hidrogênio da Thr12 para o Asp90 e conseguinte formação de ligação covalente entre Thr12 e o substrato; (II) ataque nucleofílico realizado pela Thr89, a qual perde um hidrogênio para a amônia formada com o grupo amina do substrato. Esse hidrogênio da Thr89 é reposto com um doado pela Lys162, que fica com carga neutra. (III) a Lys162 recupera seu hidrogênio e a Thr89 obtém um de uma molécula de água. A hidroxila restante dessa molécula se liga ao substrato. (IV) a ligação da hidroxila ao substrato enfraquece a ligação covalente com a Thr12, a qual recupera seu hidrogênio do Asp90 e desfaz a ligação covalente. (V) desfeita a ligação covalente, a enzima encontra-se pronta par outro ciclo de catálise e o produto é liberado do sítio ativo. Fonte: adaptado de Lubkowski *et al.* (2020).

Por outro lado, a principal diferença de uma teoria para outra, ou seja, do desacoplamento direto para o duplo, é a presença de um intermediário acil-enzima. Ao contrário da primeira teoria, amônia e aspartato são liberados do sítio ativo da enzima em momentos distintos. Sendo a mais defendida, a teoria do desacoplamento duplo foi comprovada experimentalmente com estruturas de mutantes da asparaginase II de *E. coli*. Como demonstrado na figura 1, em suma, uma troca de elétrons é realizada entre o Asp90 e Thr12 através de uma cadeia de 3 águas e a Tyr25. Essa troca permite a ligação covalente entre a Thr12 e o substrato. Este, então, adquire um elétron do resíduo nucleofílico, Thr89, e a amônia

é liberada da molécula para o meio, formando, assim, o intermediário acil-enzima. Agora, sem um átomo de hidrogênio, esse resíduo de treonina fica livre o suficiente para adquirir uma conformação que o permite receber um hidrogênio da Lys162, a qual fica com carga líquida neutra por instantes. Nesse momento, uma água entra no processo, doando um elétron para a Thr89, que devolve aquele da Lys162, e a hidroxila restante da molécula de água é ligada ao substrato. Essa ligação enfraquece a ligação covalente com a Thr12, a qual obtém seu elétron novamente do Asp90 pela mesma cadeia de água e Tyr25. Isso retorna o resíduo de aspartato ao seu estado de carga líquida negativa e libera o aspartato do sítio ativo. Dessa forma, restaurada é a enzima para um novo ciclo de catálise (LUBKOWSKI *et al.*, 2020; PALM *et al.*, 1996).

Por muitos anos, as L-asparaginases foram classificadas como do tipo bacteriana, vegetal e *Rhizobium etli*, cada uma com características únicas. L-asparaginases do tipo bacteriana eram comumente associadas àquelas com semelhanças à enzima tipo 2 de *E. coli*, EcAII, expressa no periplasma e com altíssima afinidade pelo substrato. Por outro lado, as enzimas inclusas na classe dos vegetais, apesar de não possuírem alta afinidade como as bacterianas, também apresentam atividade catalítica contra β-isoaspartil dipeptídeos, além de sofrerem uma autoclivagem única em seus sítios ativos. Por fim, as enzimas do tipo *R. etli* foram classificadas mais recentemente e usam a proteína desta bactéria como arquétipo para identificar o grupo (CECCONELLO *et al.*, 2020; LOCH *et al.*, 2021).

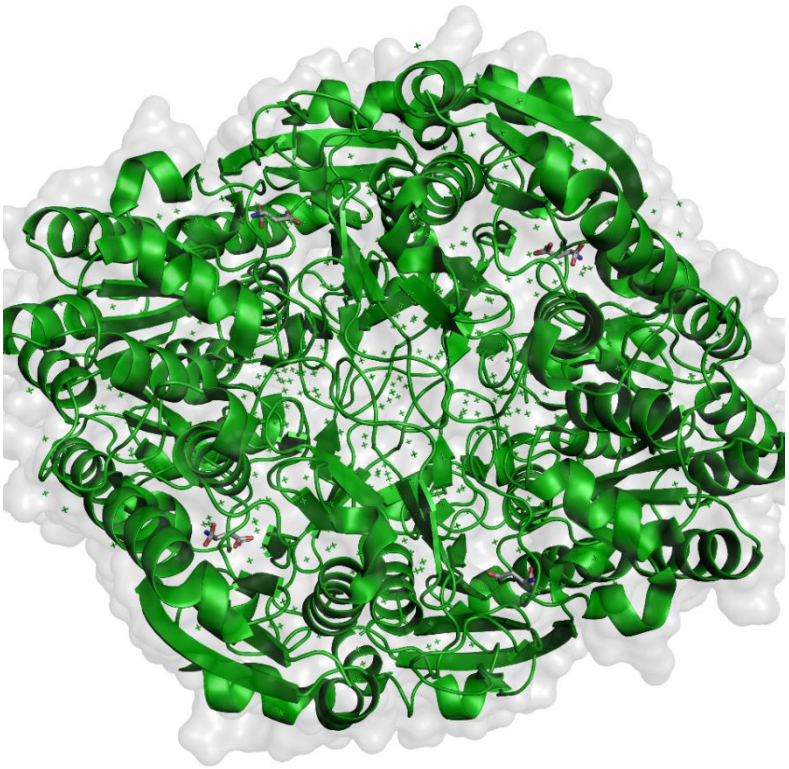
Apesar dessa classificação ter sido aceita pela comunidade científica, por muitos anos, sua nomenclatura não era inclusiva. Dessa forma, em todas as três classes supracitadas, havia representantes de altas distâncias filogenéticas, compreendendo *taxa* que não condiziam com o nome dado à determinada classe. Um exemplo claro disso é a L-asparaginase EcAIII de *E. coli*, a qual possui características do tipo vegetal e, por isso, era classificada como tal. Pensando nisso, recentemente houve uma revisão da nomenclatura dessas classes, as quais passaram a ser chamadas simplesmente de classes 1, 2 e 3 (DA SILVA *et al.*, 2022).

Desde a identificação de seu potencial anticancerígeno, diversos estudos foram realizados com o intuito de desvendar novas fontes e os mecanismos de ação da L-asparaginase, de forma que pudessem ser utilizadas no sistema de saúde mundial (BATOOL *et al.*, 2016; CACHUMBA *et al.*, 2016). Até a atualidade, no entanto, apenas duas enzimas microbianas demonstraram-se capazes de serem efetivamente utilizadas no tratamento de câncer: as L-asparaginases de *Escherichia coli* e *Dickeya chrysanthemi* (BECKETT; GERVAIS, 2019).

Estas L-asparaginases bacterianas são enzimas homotetraméricas (figura 2), cada monômero contendo um sítio catalítico. A asparaginase de *E. coli* (EcAII) possui 326 resíduos

por monômero, os quais, por sua vez, se associam em uma estrutura quaternária de ~140 kDa (tetrâmero). Essas características são, então, compartilhadas por outras asparaginases homólogas pertencentes à classe 1, inclusive *D. chrysanthemi* (BATOOL *et al.*, 2016; SWAIN *et al.*, 1993).

Figura 2 – Estrutura tridimensional de L-asparaginase tipo 2 de *Escherichia coli*



Nota: Fonte: elaborado pelo autor com auxílio de PyMol (PDB: 3ECA) (SWAIN *et al.*, 1993). Tetrâmero da L-asparaginase de *E. coli* com o produto da reação catalítica presente no sítio ativo. O aspartato está representado na figura com carbonos em branco. Cada monômero possui um sítio ativo, e é possível visualizar que cada um possui uma molécula de L-aspartato presente.

Ambas enzimas bacterianas apresentam ótima atividade catalítica e são rotineiramente integradas na combinação medicamentosa utilizada no tratamento quimioterápico de LLA. Entretanto, diversos efeitos colaterais são atribuídos à administração dessas L-asparaginases. Essas reações são causadas por dois fatores principais: a imunogenicidade dessas enzimas e suas atividades enzimáticas, também, contra L-glutamina. Esses motivos influenciam no tratamento e resposta do paciente ao tratamento, visto que a gravidade dos efeitos colaterais pode inviabilizar o uso das enzimas (BARBOSA *et al.*, 2019; BECKETT; GERVAIS, 2019; BURKE; ZALEWSKA-SZEWCZYK, 2022).

Nesse contexto, a busca por substitutas que não induzam reações de hipersensibilidade e outros efeitos colaterais levou a formulação da PEG-Asparaginase de *E. coli*, a qual apresenta relativamente menor imunogenicidade, mas ainda não suficiente para erradicar as reações adversas apresentadas pelos pacientes (HIJIYA; VAN DER SLUIS, 2016; LANVERS-KAMINSKY, 2017). Por isso, a comunidade científica vem pesquisando diversas outras fontes de L-asparaginase que possam fornecer enzimas que substituam as comercializadas atualmente (BEULAH; HEMALATHA, 2019; DIAS; SANTOS AGUILAR; SATO, 2019; IZADPANAH QESHMI *et al.*, 2018).

1.4 L-asparaginases vegetais

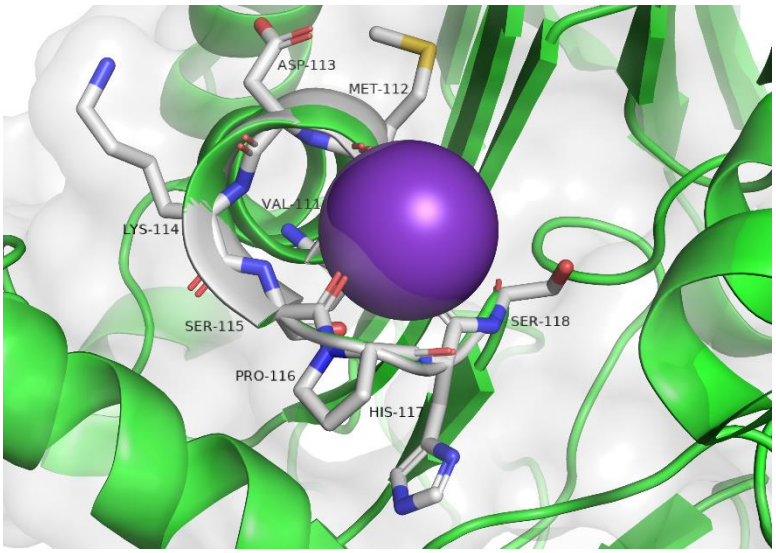
Nesse contexto, o reino vegetal passou a ser um dos alvos da procura por novas L-asparaginases. Ao longo dos anos, espécies vegetais foram alvos de pesquisa básica e aplicada num esforço de encontrar enzimas capazes de substituir as de origem bacteriana. Ainda que não tenha sido relatada, até o momento, uma candidata ideal, revelaram-se características únicas dessas biomoléculas, as quais são integrantes da classe 2 das L-asparaginases (BATOOL *et al.*, 2016; BEULAH; HEMALATHA, 2019; BRUNEAU; CHAPMAN; MARSOLAIS, 2006; SODEK; LEA; MIFLIN, 1980).

As L-asparaginases vegetais podem ser divididas pela sua dependência, ou não, do íon potássio (K^+). Apesar do nome sugerir, essa dependência não é total, porém a presença do cátion aumenta consideravelmente a atividade catalítica dessas enzimas, diminuindo drasticamente o valor de Km da biomolécula. Por outro lado, aquelas que não apresentam dependência de potássio não são afetadas pela presença dele no meio, entretanto apresentam um Km ligeiramente maior em relação à asparagina e maior afinidade por β-aspartil-dipeptídeos (BRUNEAU; CHAPMAN; MARSOLAIS, 2006; CREDALI *et al.*, 2011, 2013; HEJAZI *et al.*, 2002; SODEK; LEA; MIFLIN, 1980).

São características estruturais que determinam essa ligação com um cofator, configuradas pela presença de uma sequência de ativação e, principalmente, pela presença de uma serina numa região conservada das estruturas primária, secundária e terciária. Em estudos cristalográficos prévios, foi demonstrada a coordenação dos íons metálicos potássio (K^+) e sódio (Na^+) com a estrutura de uma asparaginase vegetal. Revelou-se, dessa forma, que o tamanho e o tipo do íon metálico influenciam nas mudanças conformacionais do chamado *loop* de ativação, o qual compreende 8 resíduos (Val111 – Ser118) (Figura 3). Importa ressaltar que essa interação do íon com os resíduos de aminoácidos se dá pelos carbonos α (cadeia principal),

não pelas cadeias laterais dos resíduos, as quais se orientam para o lado contrário. Essas mudanças conformacionais afetam a chamada alavanca catalítica, composta pelos resíduos His117 (pertencente também ao *loop* de ativação), Arg224 e Glu250. Esta alavanca se orienta de forma que ancora o substrato no bolso do sítio catalítico, melhorando a eficiência da catálise. Essa orientação, portanto, é adotada a partir da interação do íon metálico, em maior eficiência potássio, com o *loop* de ativação. Comprovou-se, também, que o último resíduo dessa região, Ser118, é muito importante para essa coordenação, e sua mutação pode induzir a independência de potássio (AJEWOLE *et al.*, 2018; BEJGER *et al.*, 2014).

Figura 3 – *Loop* de ativação de L-asparaginase vegetal



Nota: Fonte: elaborado pelo autor com auxílio de PyMol a partir de PDB 4PU6 (BEJGER *et al.*, 2014). Representação gráfica da coordenação do íon K^+, em roxo, com os resíduos que formam o *loop* de ativação das L-asparaginases vegetais dependentes de potássio, representados com carbonos brancos. É possível notar que a coordenação é feita a partir da interação do metal com as cadeias principais dos resíduos, e não pelas cadeias laterais, as quais estão orientadas para o lado oposto.

Outra característica única das L-asparaginases vegetais é que elas pertencem ao grupo das Ntn-hidrolases, enzimas capazes de sofrer uma autoclivagem para formar diferentes cadeias polipeptídicas. Nesse caso, essas proteínas são expressas como um polipeptídeo precursor, o qual sofre uma autoclivagem *cis* ou *trans* no próprio sítio catalítico, de forma que, caso não haja essa quebra da ligação peptídica, não há atividade catalítica. Numa clivagem *cis*, a própria molécula é a responsável por sua clivagem. A treonina, resíduo do sítio ativo muito conservado em todas as classes de asparaginases, através de torções e formações de pontes de hidrogênio com outros resíduos, cliva sua ligação com o seu antecessor na estrutura primária. Por sua vez, a clivagem *trans* acontece quando um precursor é clivado por uma outra molécula

da mesma enzima (MICHALSKA; BUJACZ; JASKOLSKI, 2006; MICHALSKA; HERNANDEZ-SANTOYO; JASKOLSKI, 2008).

A partir da clivagem desse precursor, são geradas as subunidades α e β, as quais formam um dímero. Apesar do sítio ativo da enzima estar na porção N-terminal da subunidade β, a α exerce importante papel na estabilização do conjunto e, quando há dependência, na interação com o íon de potássio e ativação da atividade enzimática. Entretanto, a grande maioria das L-asparaginases vegetais possui como conformação quaternária nativa um heterotetrâmero, o qual é formado por um dímero de dímeros, $\alpha\beta\beta_2\alpha_2$. Uma vez que uma subunidade de um dos dímeros estabiliza a subunidade do outro, a literatura relata que essas enzimas apenas apresentam atividade total quando o tetrâmero é devidamente formado (BEJGER *et al.*, 2014; DA SILVA *et al.*, 2022; MICHALSKA; BUJACZ; JASKOLSKI, 2006; MICHALSKA; JASKOLSKI, 2006).

Com o passar dos anos, L-asparaginases vegetais, apesar dos poucos estudos, mostram resultados promissores que as encaminham rumo ao estabelecimento como medicamento quimioterápico, assim como as enzimas comerciais atuais. Apesar da problemática acerca da afinidade dessas proteínas vegetais pelo substrato estar na faixa do milimolar, os testes quanto a sua imunogenicidade podem contrapor essa desvantagem, possibilitando uma maior dose caso necessária (AL-HAZMI; NAGUIB, 2022; MOHAMED *et al.*, 2016).

1.5 *Phaseolus vulgaris* e sua L-asparaginase

Dentre as espécies vegetais estudadas até a atualidade, o feijão comum (*Phaseolus vulgaris* L.*)*, e suas diversas variedades ao redor do mundo (como feijão carioca, feijão preto, feijão vermelho), possui certo destaque na literatura sobre L-asparaginases. Essa espécie de planta é mundialmente cultivada, com variedades selecionadas artificialmente e muito presentes na alimentação de países emergentes. Seu alto teor de proteínas, carboidratos e lipídeos tornam o feijão um alimento de baixo custo e produção, além da riqueza de nutrientes (REZENDE *et al.*, 2017).

Como dito anteriormente, L-asparagina é um aminoácido importante para transporte e estoque de nitrogênio em plantas. Dessa forma, atividade da enzima L-asparaginase se torna importante para disponibilizar esse nitrogênio para outras vias metabólicas. Em *Phaseolus vulgaris*, a expressão e atividade de asparaginase é regulada pelo fotoperíodo, aumentando durante períodos escuros e decaindo com as horas de exposição à luz, atingindo o

pico nas últimas horas da noite e primeiras horas da manhã. Essa elevação na atividade asparaginásica entra em consonância com o acúmulo de L-asparagina horas antes do aumento dessa atividade enzimática, de forma que os níveis do aminoácido decaem nos mesmos períodos mencionados (PAJAK *et al.*, 2023).

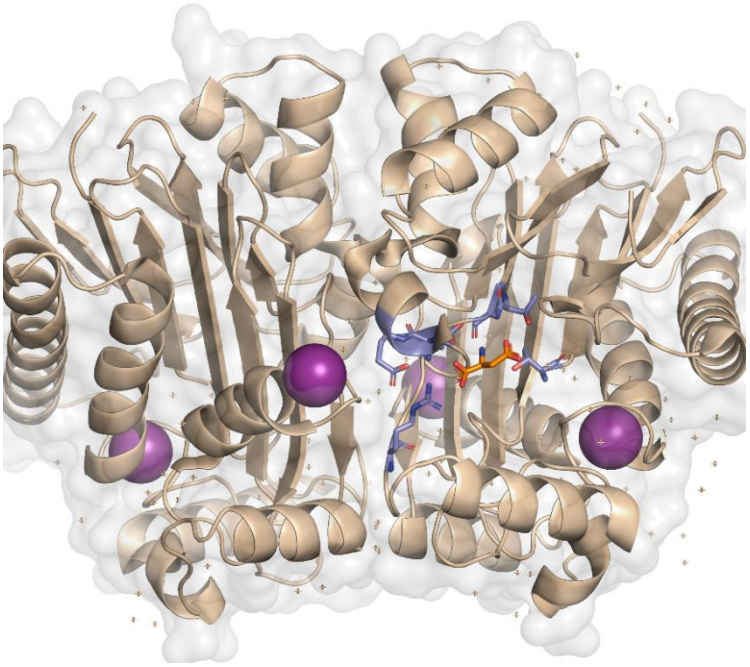
Sem diferir das características das asparaginases vegetais, a L-asparaginase de *Phaseolus vulgaris* é uma proteína heterotetramérica, composta por dois heterodímeros. Cada dímero, então, é constituído de uma subunidade α e uma β. Entretanto, como citado anteriormente, assim como outras enzimas homólogas, a estrutura primária inicial dessa enzima é um único polipeptídeo precursor de 326 resíduos. É justamente no resíduo nucleofílico do sítio ativo, Thr196, onde acontece a autoclivagem característica das asparaginases de classe 2. Dessa forma, o que antes era a parte mais N-terminal, torna-se a subunidade α, com 195 resíduos e aproximadamente 21 kDa, enquanto que a porção mais C-terminal se torna a subunidade β, com 131 resíduos, aproximadamente 13,6 kDa e contendo o sítio ativo da enzima. Com a formação do heterotetrâmero, a proteína atinge aproximadamente 70 kDa (AJEWOLE *et al.*, 2018; BEJGER *et al.*, 2014). A estrutura tridimensional do heterotetrâmero e a interação dos resíduos do sítio ativo com o produto da reação estão representadas nas figuras 4 e 5, respectivamente.

Não obstante, a literatura também possui algumas informações sobre sua purificação e caracterização a partir das sementes da subvariedade Giza 6. São relatadas características bioquímicas que favorecem a enzima nativa como possível substituta das enzimas comerciais. Somado a isso, também é relatado um teste comparativo contra células cancerígenas da linhagem Jurkat (clone E6-1; leucemia linfoide aguda de células T) entre a enzima de *P. vulgaris*, *E. coli* e sua forma conjugada com polietilenoglicol (PEG). Com os resultados, a IC_{50} da enzima vegetal foi menor do que as enzimas comerciais, denotando o grande potencial da asparaginase de feijão (MOHAMED *et al.*, 2016, 2015).

Diante desses dados, é notória a capacidade da L-asparaginase de feijão comum de substituir as enzimas comerciais. Visto que suas sementes são frequentemente consumidas ao redor do mundo, acreditava-se que poucos, ou nenhum, epítopos fossem encontrados em sua estrutura, de forma que essa enzima tenha uma menor imunogenicidade. Essas hipóteses foram corroboradas em certo nível com testes *in silico* e *in vivo*. Nesses estudos, foi possível determinar que a enzima vegetal possui menos epítopos e menor imunogenicidade quando comparada com as enzimas comerciais. Os poucos epítopos sugeridos pelos programas usados não se assemelham àqueles das proteínas bacterianas. Além disso, ao serem administradas em camundongos, as enzimas vegetal e comerciais mostraram diferenças significativas nos níveis

de anticorpos (IgG, IgM e IgE) e mMCP-1, favorecendo a asparaginase de feijão, a qual apresentou níveis semelhantes aos animais sensibilizados com hidróxido de alumínio (MOHAMED *et al.*, 2016; YAZDI *et al.*, 2020).

Figura 4 – Estrutura Tridimensional da L-asparaginase de *Phaseolus vulgaris*



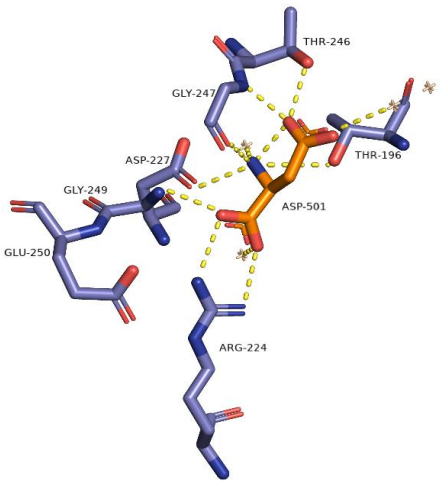
Nota: Fonte: elaborado pelo autor com auxílio de PyMol a partir de PDB (4PU6) (BEJGER *et al.*, 2014). Representação tridimensional da estrutura da L-asparaginase de feijão comum. Em roxo, os íons K^+ em coordenação com os *loops* de estabilização (Leu58 – Arg68) e ativação (Val111 – Ser118). Em azul, estão representados os resíduos que compõem o sítio ativo da cadeia B (Thr196; Arg224; Asp227; Thr246; Gly247; Gly249 e Glu250) interagindo com o produto da reação, representado em laranja, L-aspartato.

Apesar disso, os dados disponíveis na literatura acerca da L-asparaginase de *Phaseolus vulgaris* são insuficientes para determinar sua verdadeira capacidade de se tornar um medicamento para o tratamento quimioterápico de câncer. São necessários estudos mais aprofundados acerca da sua cinética enzimática, mecanismo de catálise, estabilidade térmica, estabilidade em diferentes pHs, testes de atividade com possíveis substratos e inibidores, atividade cancerígena contra outras linhagens tumorais, entre outros. Todos esses estudos podem fornecer um melhor entendimento acerca da bioquímica e biofísica da L-asparaginase de *Phaseolus vulgaris*, além de revelar rumos a serem seguidos para uma efetiva substituição das L-asparaginases comerciais.

Nesse contexto, é importante frisar que proteínas recombinantes podem diferir das nativas quanto às suas características bioquímicas, biofísicas e quanto à atividade biológica em

ambos os sentidos, melhora ou piora. A diferença do meio em que são sintetizadas, o qual difere substancialmente do meio em que se originam, favorece essas divergências biológicas. Dessa forma, a velocidade de tradução, as condições do citosol, as chaperonas que auxiliam no enovelamento e as modificações pós-traducionais influenciam consideravelmente nas características supracitadas (BEYGMORADI *et al.*, 2023; BRITO E CUNHA *et al.*, 2019; ESPEJO-MOJICA *et al.*, 2015; MÉNDEZ-LÍTER *et al.*, 2017).

Figura 5 – Representação dos resíduos do sítio ativo da L-asparaginase de *P. vulgaris* e suas interações com o L-aspartato



Nota: Fonte: elaborado pelo autor com auxílio de PyMol a partir de PDB 4PU6 (BEJGER *et al.*, 2014). Na figura, estão representados, com carbonos em azul, os resíduos Thr196 (resíduo nucleofílico), Arg224, Asp227, Thr246, Gly247, Gly249 e Glu250. Com carbonos em laranja, tem-se o produto da reação, L-aspartato. As pontes de hidrogênio formadas entre o produto e os resíduos do sítio catalítico são representadas em amarelo.

Diante do exposto, nota-se a importância de caracterizar efetivamente uma proteína recombinante, ainda que sua caracterização tenha sido realizada com sua forma nativa, pois diferenças podem, e muito provavelmente irão, surgir. Dessa forma, apesar de haver dados que caracterizem a L-asparaginase de *P. vulgaris*, estes são um misto entre proteína nativa e recombinante. Pensando nisso, o presente trabalho visa caracterizar a L-asparaginase recombinante de *P. vulgaris* e avançar no conhecimento de suas propriedades (AJEWOLE *et al.*, 2018; BEJGER *et al.*, 2014; MOHAMED *et al.*, 2015, 2016; PAJAK *et al.*, 2023).

2 OBJETIVOS

2.1 Objetivo geral

Avaliar a atividade enzimática, o potencial anticancerígeno e a imunogenicidade da L-asparaginase recombinante de *Phaseolus vulgaris*.

2.2 Objetivos específicos

- Obter a L-asparaginase recombinante de *P. vulgaris* (Asp-P);
- Identificar e sequenciar a Asp-P;
- Determinar o grau de pureza da proteína recombinante;
- Determinar os parâmetros de pH e temperatura ótimos para atividade enzimática;
- Determinar os parâmetros de cinética enzimática;
- Avaliar o efeito de agentes desnaturantes na atividade catalítica;
- Determinar o conteúdo de estrutura secundária e a termoestabilidade;
- Avaliar o potencial citotóxico da Asp-P nas linhagens tumorais Raji e K562;
- Avaliar e comparar a imunogenicidade das L-asparaginases bacteriana e de *P. vulgaris.*

3 ARTIGO DA DISSERTAÇÃO

Cytotoxic effect and *in silico* immunogenicity of recombinant L-asparaginase from *Phaseolus vulgaris*

Cytotoxic effect and *in silico* immunogenicity of recombinant L-asparaginase from *Phaseolus vulgaris*

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Abstract

The enzyme L-asparaginase plays a crucial role in the treatment of acute lymphoblastic leukemia, a type of cancer that mostly affects children and teenagers. However, due to their immunogenicity and glutaminase activity, it is common for these molecules to cause adverse reactions during treatment. These downsides ignite the search for novel asparaginases that could mitigate these problems. In this quest, plants have shown promising features over the years that turn them into candidates to substitute the commercial enzymes, which are from bacterial source. In this context, *Phaseolus vulgaris* recombinant L-asparaginase lacks a thorough characterization of its biochemical, biophysical and antineoplastic properties. Thus, this work aimed to produce and characterize a recombinant L-asparaginase from *P. vulgaris* (Asp-P). For this purpose, the enzyme was expressed in *E. coli* and purified by affinity and size-exclusion chromatographies. The enzyme activity was measured by the Nesslerization method. The kinetics parameters, thermotolerance and the effects of interferents on enzyme activity were determined. The presence of the enzyme in the samples was analyzed by western blotting and mass spectrometry. The secondary structure and the thermostability of the protein were both assessed by circular dichroism. Also, the cytotoxicity effects of Asp-P on Raji and K562 cells were assayed by MTT method. Online prediction tools were used to determine the immunogenicity of Asp-P in comparison with the bacterial protein. The results showed that Asp-P was expressed with high yields and specific activity of 905 $U.mg^{-1}$, with maximum activity at pH 9.0 and 40° C. Western blots confirmed the presence of Asp-P in the samples, as well as no contaminant native *E. coli* L-asparaginase. Mass spectrometry mapped 93 % Asp-P sequence. Asp-P could reduce Raji viable cells percentage below 50 % only at the highest concentration tested, but the same could not be achieved for K562 cultures. Lastly, *in silico* prediction methods indicated that Asp-P is less immunogenic than bacterial enzymes. Put together, these results show that recombinant Asp-P has different properties when compared to the native enzyme and has favorable aspects that indicate a promising enzyme to substitute the bacterial ones.

Keywords: antineoplastic enzyme; kidney bean; recombinant protein; cytotoxicity assay

1. INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a type of cancer that affects one or both lymphoid cell lines, the lymphocytes B and T. This hematologic neoplasia primarily affects children and teenagers, who are treated with chemotherapy upon diagnostic (GIANNI; BELVER; FERRANDO, 2020; INABA; MULLIGHAN, 2020; ROBERTS; MULLIGHAN, 2020; STELIAROVA-FOUCHER *et al.*, 2017). This kind of treatment utilizes a myriad of drugs to combat the disease. However, it is during the first phase, induction of remission, that L-asparaginase is employed due to the inability of these tumour cells to express a functional asparagine synthetase, thus relying on exogenous L-asparagine to survive. Therefore, the enzyme L-asparaginase selectively induces apoptosis of these neoplastic cells by asparagine starvation (ALDOSS; STEIN, 2018; CHIU *et al.*, 2020; INABA; MULLIGHAN, 2020; LOPES *et al.*, 2017; TOSTA PÉREZ *et al.*, 2023).

Despite several years of research, only two L-asparaginases are used in the treatment of ALL, both from bacterial sources: *Escherichia coli*, and its pegylated form, and *Dickeya sp.* L-asparaginases (SALZER; SEIBEL; SMITH, 2012; SHORT; KANTARJIAN; JABBOUR, 2021; SURESH; ETHIRAJ; RAJNISH, 2022). These enzymes are commercially available and play an important role in establishing the path to cure for patients, yet they have drawbacks. Adverse reactions are associated with the administration of these drugs and relates to their immunogenicity and glutaminase activity. In this context, patients often suffer from hypersensitivity reactions, thrombosis, hepatitis, pancreatitis, among others (BURKE; ZALEWSKA-SZEWCZYK, 2022; HIJIYA; VAN DER SLUIS, 2016; JONES *et al.*, 2022).

Although L-asparaginase is a crucial component of chemotherapy, the reactions patients present tend to diminish the dose or even prevent the use of the drug. On the other hand, the discontinuation of L-asparaginase administration is related with a poor prognosis and lower survival rates (CHEN *et al.*, 2021; ISHIDA *et al.*, 2023; LANVERS-KAMINSKY, 2017). Therefore, it is urgent the need for new enzyme sources that present no glutaminase activity and lower immunogenicity, which has been a challenge (BECKETT; GERVAIS, 2019; CECCONELLO *et al.*, 2020).

Despite the lack of studies, plant asparaginases show unique characteristics that set them apart from classical type II bacterial enzymes. L-asparaginases from vegetables belong to the Ntn-hydrolases, a characteristic that confers the ability to go through an autoproteolytic cleavage on their active site, turning a polypeptide precursor into two subunits, α and β, which often have different molecular masses. Another interesting asset of these enzymes is that they can be divided into potassium (K^+) dependent and independent (BRUNEAU; CHAPMAN;

MARSOLAIS, 2006; CREDALI *et al.*, 2013; MICHALSKA; HERNANDEZ-SANTOYO; JASKOLSKI, 2008; VAN KERCKHOVEN *et al.*, 2017). Throughout the years, a few studies were able to characterize native and recombinant plant L-asparaginases, showing that some of them have the potential to substitute the commercial enzymes, since they have shown cytotoxicity against neoplastic cells and lower immunogenicity (AL-HAZMI; NAGUIB, 2022; MOHAMED *et al.*, 2016; YAZDI *et al.*, 2020).

Among the studied species, *Phaseolus vulgaris*, commonly known as kidney bean, has been highlighted. *P. vulgaris* asparaginase potassium dependence and the metal coordination with the protein structure has been assessed with the recombinant enzyme (AJEWOLE *et al.*, 2018; BEJGER *et al.*, 2014). Conversely, the native protein has been purified and showed cytotoxicity against Jurkatt clone E6-1 cell line, as well as lower immunogenic responses in mice when compared to the commercial enzymes (MOHAMED *et al.*, 2015, 2016).

Despite the available data about *P. vulgaris* L-asparaginase and its promising antineoplastic potential, it is known that purification from native sources does not have a high yield and is more laborious in many ways. For the effective substitution of bacterial enzymes with plant ones, it would be needed to produce it through heterologous expression, a technique that can possibly alter the protein's characteristics (BEYGMORADI *et al.*, 2023; BRITO E CUNHA *et al.*, 2019; ESPEJO-MOJICA *et al.*, 2015). Since the available data for the recombinant enzyme is not complete (AJEWOLE *et al.*, 2018; BEJGER *et al.*, 2014; PAJAK *et al.*, 2023), a thorough characterization is needed. Thus, this work aims to provide a kinetics characterization of the recombinant L-asparaginase from *Phaseolus vulgaris* and assess its potential as a substitute for the commercial bacterial enzymes.

2. MATERIALS AND METHODS

2.1. Plasmid, bacterial strain and transformation

The plasmid pET-28a containing the codon optimized gene for *Phaseolus vulgaris'* L-asparaginase (GenBank gene ID: XM_007160822.1) was purchased from GenOne®. 5 μg of plasmid DNA was solubilized in 100 μL of molecular grade water. *Escherichia coli* BL21 (DE) Rosetta was used for the heterologous expression of the enzyme of interest. The plasmid bearing the gene of L-asparaginase was transformed into the bacterial strain by heat shock transformation as described before (DE-SOUZA; BRÍGIDO; MARANHÃO, 2016) with adaptations, which consisted of an incubation in ice bath for 30 minutes, then 45 s in 42° C and 45 s in ice bath. The transformed strain was inoculated in LB-agar plates containing 40 $\mu g.mL^{-1}$ Kanamycin and 34 $\mu g.mL^{-1}$ Chloramphenicol and incubated for 16 h at 37° C. A seed culture

was prepared using a loopful of inoculum containing the antibiotics in the concentrations mentioned above and incubated for another 16 h at 37° C, 250 rpm.

2.2. Expression and purification of L-asparaginase

Previous protocols were followed with adaptations (GREEN; SAMBROOK, 2012). The heterologous expression of *Phaseolus vulgaris'* L-asparaginase (Asp-P) was achieved at the optimum conditions of 0.3 mM β-D-Thiogalactopyranoside (IPTG) at 20° C, 200 rpm, for 16 h. The medium used was Luria-Berthani containing 40 $\mu g.mL^{-1}$ Kanamycin and 34 $\mu g.mL^{-1}$ Chloramphenicol. After expression, the cells were pelleted by centrifugation at 8000 x g for 10 min and resuspended in lysis buffer composed of 50 mM Tris-HCl, pH 8, 300 mM NaCl, 20 mM Imidazole, 1 mM Phenylmethanesulfonyl fluoride (PMSF) and 1 % Triton X-100. The culture was then sonicated for 20 min with a pulse of 20 s on, 40 s off. The lysed cells were centrifugated at 10000 x g for 30 min, 4° C. The supernatant was incubated for 1 h with 1.5 mL of Ni^{2+}-Sepharose column (Promega®) equilibrated with lysis buffer at 10° C, 100 rpm. Once incubated, a wash was performed with the lysis buffer and a step with 60 mM Imidazole to remove unwanted proteins. Hence, the enzyme was eluted with 250 mM Imidazole. The collected fractions were analyzed by SDS-PAGE (12.5 %) and dialyzed against 50 mM Tris-HCl, pH 8, containing 0.3 M KCl. For the circular dichroism experiment, the fractions obtained from affinity chromatography were concentrated and applied onto Superdex 200 10/300 collumn (Cytiva®) for complete purification. The buffer used to elute the proteins was 50 mM Tris-HCl, pH 8, 0.3 M KCl. The purified enzyme was stocked at -80° C for further use when needed.

2.3. Western blotting

Western blotting was used to confirm the presence of the protein of interest in the samples tested by marking them with an anti-His tag antibody. Furthermore, the technique was also used to evaluate if Asp-P would be recognized by anti-bacterial asparaginase (*E. coli*). In order to do so, a previous protocol was followed with adaptations (DE-SOUZA; BRÍGIDO; MARANHÃO, 2016). The samples were run on SDS-PAGE (10 %) and the bands in the gel were transferred to nitrocellulose membranes using BioRad® Trans-Blot Turbo equipment using the following parameters: mixed MW/1.3 A/25 V/12 min. Subsequently, the membrane was blocked for 16 h with Phosphate buffer, pH 7.4, 0.05 % Tween 20 and 1 % skimmed milk.

Hence, the membrane was washed 3 times with Phosphate buffer and 0.05 % Tween 20 (PBS-T) for 10 min. Then 10 mL of PBS-T solution containing a 1:2500 dilution of anti-His conjugated with alkaline phosphatase antibody (Sigma-Aldrich®, A5588) was used to incubate the membrane in for 1 h. After incubation, the membrane was washed 2 times with PBS-T for

10 min and one more time with PBS for 10 more min. Finally, marked proteins were revealed by adding 600 μL of Western Blue Stabilized Substrate Alkaline Phosphatase (Promega®), which was recirculated until complete revelation.

For the anti-bacterial asparaginase, procedures stated above were followed, except that membrane was incubated with 10 mL of PBS-T solution containing 1:1000 dilution of anti-L-asparaginase antibody (Biorbyt®) for 2 h. Right after, the membrane was incubated with a 1:20000 dilution of anti-IgG antibody conjugated with peroxidase (Biorbyt®). Lastly, the result was revealed by adding 500 μL of luminol and 500 μL of peroxide and incubation for 3 min.

2.4. L-asparaginase activity assay

For the measurement of L-asparaginase activity, the Nesslerization method was employed based on previous reported protocols with a few adaptations (MAGRI *et al.*, 2018; SHIFRIN; PARROTT; LUBORSKY, 1974). The Nessler reagent (Dipotassium tetraiodomercurate (II)) determines the amount of NH_4^+ released after the conversion of L-asparagine into L-aspartate. For the reaction step, 700 μL of Tris-HCl 50 mM, pH 8, containing 0.3 M KCl was mixed with 250 μL of 94.5 mM L-asparagine and 50 μL of sample. This solution was then incubated for 30 min at 37° C and the reaction was stopped with the addition of 100 μL of 1.5 M Trichloroacetic acid (TCA). The mixture was centrifuged for 5 min at 10000 x g, 4° C to remove precipitated proteins. 125 μL of supernatant was added to a solution containing 1.75 mL of distilled water and 125 μL of Nessler reagent. The concentration of ammonium was determined spectrophotometrically at 436 nm by using a plate reader (EnVision 2105 multimode plate reader - PerkinElmer, Inc). The readings were compared to a calibration curve made by the reaction of ammonium sulfate ($(NH_4)_2SO_4$) (6 mM stock solution) with Nessler reagent with the concentration range of 0.375 to 12 mM. A unit (U) of L-asparaginase activity is defined as the amount of enzyme necessary to produce 1 μmol of ammonium per minute at 37° C.

2.5. Effects of pH and thermal stability

The parameters used in previous reports were followed with a few adaptations (AL-HAZMI; NAGUIB, 2022; MOHAN KUMAR; MANONMANI, 2013). The optimum pH for the enzyme activity was determined by testing several buffers in different pH (0.3 M Citrate-Phosphate buffer, pH 2.6-8; 50 mM Tris-HCl buffer, pH 8-9; 50 mM Glycine buffer, pH 9-10). In transition pHs, both buffers were tested (pH 8: Citrate-Phosphate and Tris; pH 9: Tris and Glycine). For the optimum temperature, the standard assay was performed in temperatures ranging from 30 to 70° C. This experiment was performed in two different pHs, 7.5 and 9.0. On the other hand, the enzyme's thermostability was evaluated by incubating it at 50° C for 5,

10, 15, 30, 60 and 90 min. After each incubation time, the sample was cooled in ice bath and then its activity was assayed with the standard method. The experiment was performed in triplicates and analyzed using ANOVA in GraphPad Prism version 8.0.

2.6. Kinetic parameters and inactivation studies

In order to determine the kinetic parameters, two pH conditions were used: pH 7.4 in 50 mM Sodium Phosphate, 50 mM KCl (near physiological pH conditions) and pH 9 in 50 mM Tris-HCl, 50 mM KCl (optimal pH conditions). Using these conditions, the L-asparagine final concentration varied, ranging from 0.25 to 30 mM. Also, to determine the ability of Asp-P to use L-glutamine as a substrate, both L-asparagine and L-glutamine at the concentration of 10 mM were tested and results were compared. Two enzyme concentrations were used with L-glutamine: 92 and 300 $\mu g.mL^{-1}$. The obtained data were fitted to a Michaelis-Menten function using the software GraphPad Prism version 8.0.

The activity of the enzyme was also assessed in the presence of the interferents Tween 20 (1 %), Triton X-100 (1 %), SDS (2 %), β-mercaptoethanol (2 mM), Urea (2 mM), Thiourea (2 mM) and Ethylenediaminetetraacetic acid (EDTA) (2 mM) (MOHAN KUMAR; MANONMANI, 2013). For the experiment with the interferents, the enzyme was previously incubated with each reagent for 1 h at 4° C and then the enzyme activity assay was performed according to item 2.4. The experiment was performed in triplicates and also analyzed using ANOVA in GraphPad Prism version 8.0.

2.7. Mass spectrometry

All mass spectrometry experiments were performed by Plataforma de Proteômica da Fundação Oswaldo Cruz – Ceará. Firstly, partial purified Asp-P samples by affinity chromatography at 2 $mg.mL^{-1}$ were dialyzed against 0.1 % formic acid solution. After dialysis, the samples were incubated with 0.2 % RapiGest SF (Waters™) at 80° C for 15 min. Proteins were reduced in 100 mM Dithiotreitol (DTT) in 50 mM NH_4HCO_3 for 30 min at 60° C. Alkylation was performed in 300 mM iodoacetamide in 50 mM NH_4HCO_3 as well in the dark for 30 more min. Trypsin digestion was performed utilizing sequencing grade trypsin (Promega®) with a 1:100 proportion (protease:protein) at 37° C, 16 h. 5 % Trifluoroacetic acid was used to stop the reaction. The produced peptides were desalted, purified and concentrated with ZipTip® C18 Pipette Tips (Merck, Millipore) and resuspended in 0.1 % formic acid solution. All solutions were prepared in LC-MS grade water (LiChrosolv®, Merck, Millipore).

Peptides were analyzed by nanoLC-MS with Q Exactive™ Plus Biopharma (ThermoFisher Scientific™) equipment, a hybrid quadrupole-orbitrap analyzer and electrospray ionization source. Peptides were separated by reverse phase chromatography using

Dionex Ultimate™ 3000 RSLCnano System (ThermoFisher Scientific™), which was equipped with a trap column C18 PepMap P100 (C18 5 µm, 0.3 x 5mm) and an analytical column Acclaim™ PepMap RSLC (C18, 2 µm, 100A°, 75 µm x 50 cm), E803A rev2, (ThermoFisher Scientific™). 1 µL samples were injected with a constant flow of 400 $nL.min^{-1}$ and elution gradient was performed within the range 5-98 % of solvent B (ACN with 0.1 % formic acid) in solvent A (0.1 % formic acid solution).

Precursor mass was measured by an Orbitrap mass analyzer at 70,000 resolution, mass range 375-1500 m/z. Simultaneously, a data dependent acquisition analysis was performed with the 5 most abundant precursor ions selected for a high energy collision-induced dissociation (HCD) fragmentation with a resolution of 17,500.

All data acquisition used Thermo Xcalibur 2.2 software. PatternLab 5.0.0.141 software was used for protein identification using *Escherichia coli* BL21 (DE3) (Uniprot KnowledgeBase accession: UP000002032) (CARVALHO *et al.*, 2016; SANTOS *et al.*, 2022). Asp-P recombinant protein sequence was manually inserted in this database so it could be also identified. Protein abundance was classified by Normalized Spectral Abundance Factors (NSAF). Final results are a mean of two independent biological replicates and experimental triplicates.

2.8. Circular Dichroism (CD)

To determine the secondary structure content and melting temperature (T_m) of Asp-P, a Jasco® J-815 spectropolarimeter (Jasco International Co., Japan) was used. In order to do so, the enzyme was previously dialyzed against 20 mM Potassium Phosphate buffer, pH 7, and diluted to the concentration of 75 $\mu g.mL^{-1}$. Asp-P was analyzed in the far-UV (190-240 nm) with a 5 mm path length, 1 mm bandwidth and scanning speed of 50 $nm.min^{-1}$ at 20° C. The buffer was used for baseline correction. On the other hand, the T_m analysis was performed at 220 nm wavelength within a range of 20-95° C and 3° $C.min^{-1}$ scan speed. The data obtained was then analyzed with SELCON3 algorithm using dataset 4 as reference on Dichroweb web server (SREEMA; WOODY, 1993; SREERAMA; VENYAMINOV; WOODY, 2000; WHITMORE; WALLACE, 2008).

2.9. Cytotoxicity assay

Raji (Burkitt's lymphoma) and K562 (chronic myeloid leukemia) cells were cultured in RPMI medium supplemented with 10 % fetal bovine serum, 100 $U.mL^{-1}$ Penicilin, 100 $\mu g.mL^{-1}$ Streptomycin. The growth conditions were 37° C and a 5 % CO_2 atmosphere. For the assay, these cells were seeded into 96-well culture plates in their respective concentrations: $0.4 * 10^6$ $cells.mL^{-1}$ for Raji cell line; $0.3 * 10^6$ $cells.mL^{-1}$ for K562 cell line. The medium containing

Asp-P in different concentrations (seriated dilutions starting from 250 μg.mL^{-1}) was then added after plating. Plates were incubated for 72 h at 37° C, 5 % CO_2. After incubation, 20 μL MTT (0.5 mg.mL^{-1}) were added to each well and plates were incubated again foŕ 4 h in the same conditions (MOSMAN, 1983). Once finished, plates were then centrifuged, the supernatant removed and the pellet dissolved in 200 μL DMSO. Finally, plates were agitated for better dissolution of Formazan and read at 570 nm in a microplate reader (EnVision 2105 multimode plate reader - PerkinElmer, Inc). The final results were obtained from three independent experiments performed in triplicate. The mean of controls was defined as 100 % and results were analyzed and plotted using ANOVA in GraphPad Prism version 8.0.

2.10. In silico immunogenicity prediction

In order to predict the immunogenicity of Asp-P, the sequence of the recombinant protein and the available structure (PDB 4PU6) were analyzed by *in silico* methods using online tools. The tools available in the Immune Epitope Database (IEDB) were then used: NetMHCIIPan 4.1 EL (MHCII binding prediction) with alleles HLA-DRB1*07:01, HLA-DQA1*02:01 and HLA-DQB*02:02, which are associated with asparaginase hypersensitivity (FERNANDEZ *et al.*, 2014; KUTSZEGI *et al.*, 2017; REYNISSON *et al.*, 2021); CD4 T Cell immunogenicity prediction, with a focus on the allele HLA-DRB1*07:01 as well (DHANDA *et al.*, 2018); Bepipred Linear Epitope Prediction 2.0 for B cells (JESPERSEN *et al.*, 2017) and DiscoTope 2.0 for structure based antibody prediction (KRINGELUM *et al.*, 2012). All analysis were also performed with the commercial enzyme, *E. coli* L-asparaginase (PDB 3ECA), to be able to compare results. The standard thresholds of all tools were used, except for NetMHCIIPan 4.1 EL, in which the established threshold for analysis was a percentile rank ≤ 1.

3. RESULTS

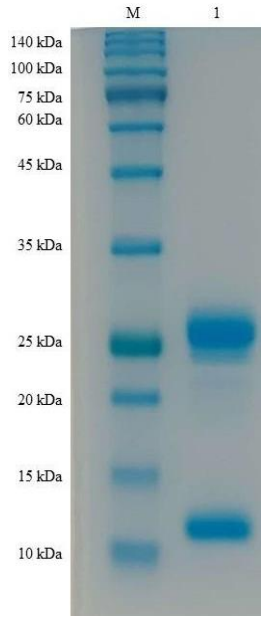
3.1 Expression and purification of Asp-P

After optimizing the expression conditions, Asp-P was produced with 0.3 mM IPTG, 20° C, 200 rpm, for 16 h. These conditions allowed maximum production of soluble protein, which was thoroughly purified after two chromatographic steps, affinity on Ni^{2+}-Sepharose and size exclusion on Superdex 200 10/300, yielding a specific activity of 905 U.mg^{-1}. The enzyme presents itself as two distinct bands on SDS-PAGE: the α subunit of theoretical 23.2 kDa and β subunit of theoretical 13.4 kDa (Figure 1). It was noted that the α subunit suffers a proteolytic degradation over time when stocked in 4° C freezers and could be seen on SDS-PAGE (Figure S1). To prevent the loss of activity caused by this degradation, samples were stocked in -80° C freezers if they were not to be used any soon.

3.2 Western blotting

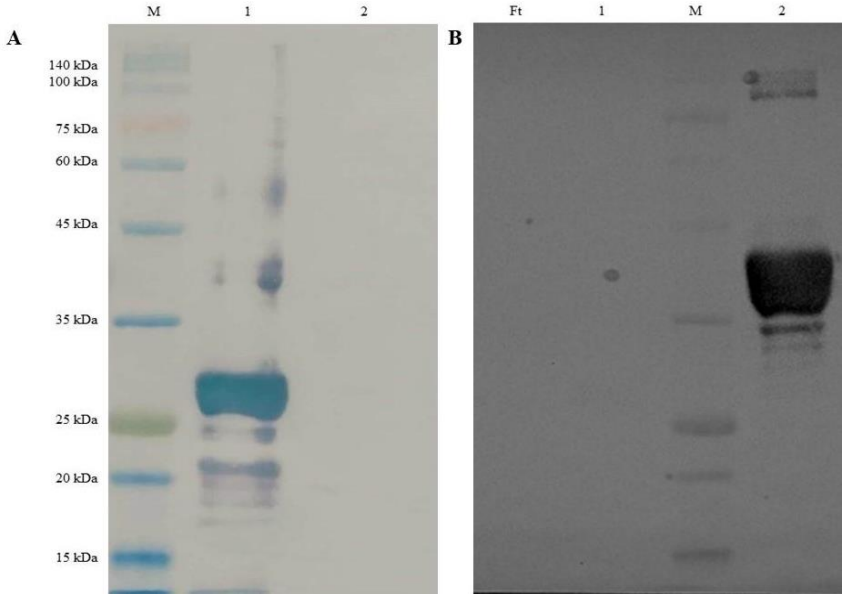
The presence of Asp-P in the samples acquired was confirmed by western blot using an anti-His tag antibody, but only its α subunit, the one that has the affinity tag on its N-terminal (Figure 2A). The same kind of experiment was done using an anti-bacterial L-asparaginase antibody in order to (I) analyze the presence of native *E. coli* asparaginase contaminants and (II) assess the ability of this antibody to recognize Asp-P. The experiment revealed that no contaminant bacterial asparaginase was present in the samples. Plus, Asp-P was not marked by this antibody (Figure 2B).

Fig. 1. SDS-PAGE (12.5%) of purified *Phaseolus vulgaris* L-asparaginase



Note: (M) molecular marker; (1) Asp-P purified after size-exclusion chromatography in Superdex 200. It is possible to see two distinct bands: α subunit (higher mass) and β subunit (lower mass).

Fig. 2. Anti-His tag and anti-bacterial L-asparaginase western blots



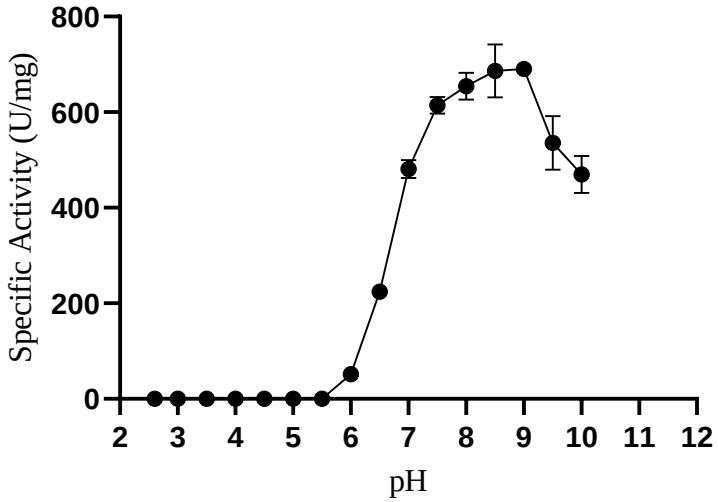
Note: (A) Western blot using anti-His tag antibody. Lane M is molecular marker; lane 1 has Asp-P after purification on affinity chromatography. The α subunit is strongly marked; lane 2 has flowthrough sample from the affinity chromatography. No protein was marked in the lane, denotating that no Asp-P was left unadsorbed to the matrix. (B) Western blot using anti-bacterial L-asparaginase antibody. The same samples used in (A) were used in figure (B), in which Ft refers to the flowthrough sample. Lane 2 is a positive control, in which *E. coli* L-asparaginase was applied.

3.3. Optimal pH and thermostability

Asp-P activity was assayed in different pHs ranging 2.6-10.0. The enzyme was active only from pH 6 and beyond. Its maximum activity was achieved in pH 9, demonstrating that the protein has a preference for a more basic environment. Still, more than 50 % of its activity was preserved in the pH 7-10 range, with impressive 68 % of remained activity in pH 10 (figure 3).

It is noteworthy that this experiment also demonstrated that the buffer composition used in the assay influences the activity of Asp-P, despite the pH. In this regard, when tested with 0.3 M Citrate-phosphate and 0.05 M Tris, both in pH 8, Asp-P showed a higher activity in the first one. Same thing happened in pH 9, in which Tris had a better performance than Glycine (data not shown). In this regard, figure 3 presents only the data of the buffer that yielded the highest specific activity *i.e.* Citrate-phosphate (pH 8.0) and Tris (pH 9.0).

Fig. 3. pH effect on Asp-P activity

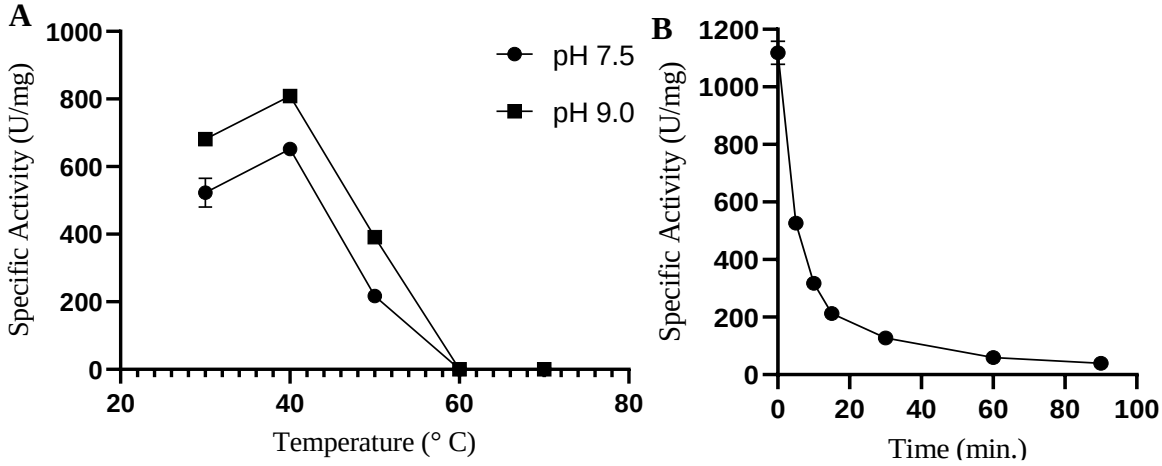


Note: optimal pH profiling for Asp-P activity. Expressed in specific activity ($U.mg^{-1}$), it is noticeable that the enzyme is active throughout the range of 6.0 to 10.0, with its peak on pH 9, reaching over 900 $U.mg^{-1}$.

It is made clear in figure 4A that, within the temperatures tested, 40° C is the optimal temperature for the asparaginase activity in both pHs tested, 7.5 and 9.0. Above that, the enzyme activity declines sharply at 50° C until no activity is measurable at 60 and 70° C. To further analyze the influence of the temperature on the enzyme and its thermal stability, Asp-P was incubated at 50° C for different times, as shown in figure 4B. Noticeably, more than half of its activity is lost after a 5 min incubation at 50° C and it goes on decreasing until almost complete

loss after 60- and 90-min incubations. This shows that Asp-P does not do great at higher temperatures.

Fig. 4. The effect of temperature on Asp-P enzymatic activity



Note: Effect of temperature parameter on enzymatic activity of Asp-P expressed in $U.mg^{-1}$. (A) Asparaginase assay performed in different temperatures and pHs 7.5 and 9.0. Note that the specific activity is higher in all temperatures on pH 9. (B) Thermal stability of Asp-P incubated at 50° C for different times. It is clear that more than half of the enzyme's activity is lost right after 5 minutes of incubation.

3.4. Kinetics parameters

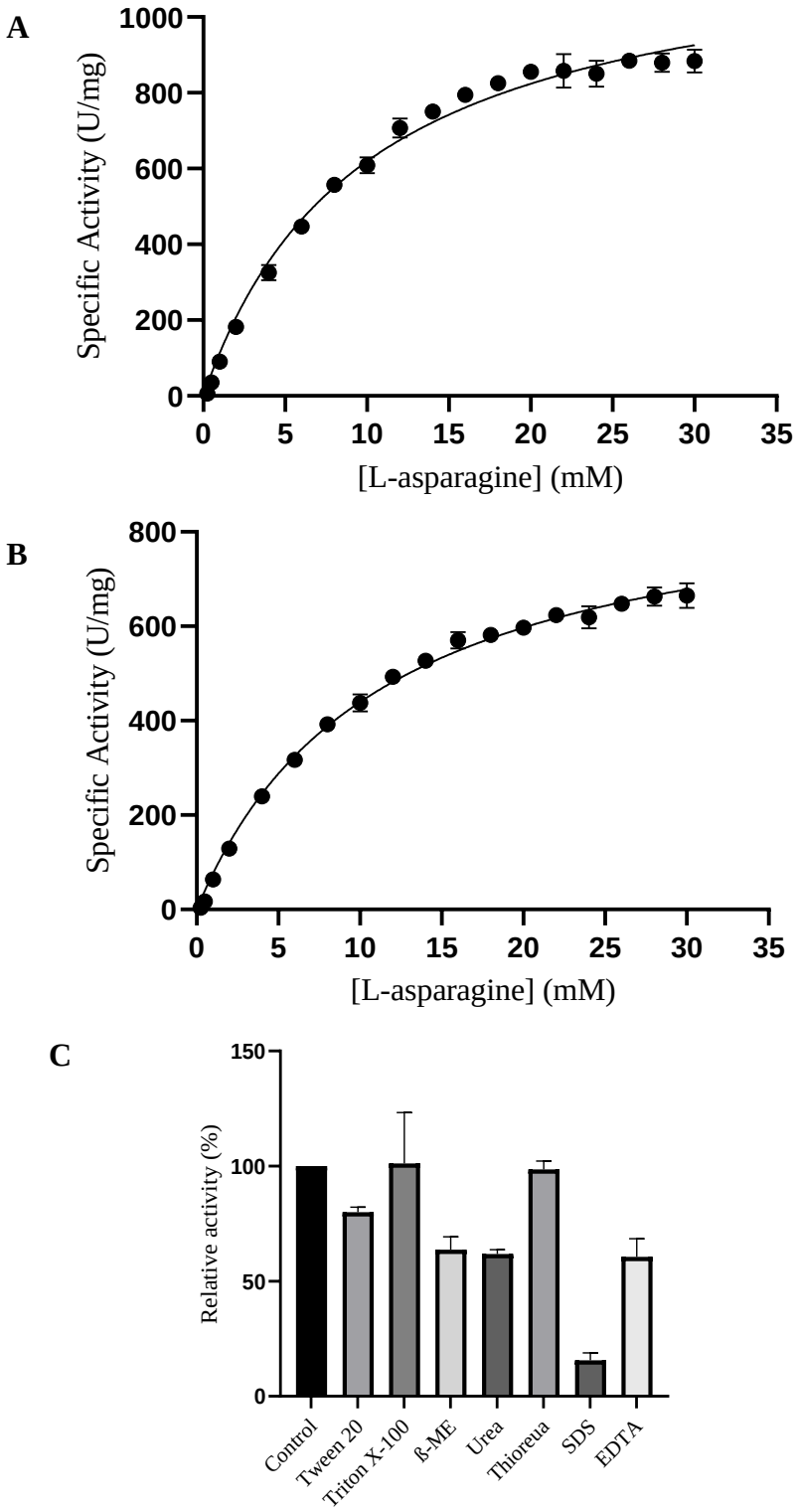
To further assess the kinetics parameters of Asp-P, 37° C (close to optimal temperature) and two pHs (7.5 and 9) were used to perform an activity assay altering L-asparagine concentrations, which ranged from 0.25 to 30 mM. The results showed a Km of 7.05 mM and Vmax of 1027 $U.mg^{-1}$ in pH 9.0 (Figure 5A). On the other hand, as expected, pH 7.5 showed higher Km and lower Vmax: 8.44 mM and 810.58 $U.mg^{-1}$, respectively (Figure 5B).

To compare Asp-P activity towards L-asparagine and L-glutamine, both substrates (10 mM) were used in an activity assay using 92 $\mu g.mL^{-1}$ of enzyme. No glutaminase activity, only asparaginase, was detected for Asp-P. Even with a higher enzyme concentration (300 $\mu g.mL^{-1}$), Asp-P showed no activity towards L-glutamine.

Interferents were also used to determine whether the enzyme was influenced by their presence or not. In figure 5C, data shows that Asp-P retained more than 50 % of its activity in the presence of Urea, β-mercaptoethanol and EDTA. Thiourea did not interfere significantly with Asp-P, which retained 98.44 % of its activity. On the other hand, only 15.57 % of activity was shown in the presence of SDS. Actually, this substance reacted with the assay buffer right after its addition, forming a white mass. Assay was performed anyway, and centrifugation after step one removed the white particles. Tween 20 and Triton X-100 were two other reagents that interfered with the assay. In this case, it was during the addition of the Nessler's reagent. The sample, despite developing color, showed a strong turbidity that could not be halted even with

centrifugation. This turbidity influenced on the absorbance measurement, making the activity values higher than they should be.

Fig. 5. Asp-P kinetic parameters



Note: Graphic representation of Asp-P kinetic parameters towards L-asparagine. The concentration of substrate ranged from 0.25 to 30 mM. (A) experiment done in the optimal pH 9.0. (B) experiment done in pH close to human physiological conditions, pH 7.4. In pH 9.0, Asp-P yields a higher Vmax (1027 $U.mg^{-1}$) and a lower Km (7.05 mM) if compared to pH 7.4 results (810.58 $U.mg^{-1}$ and 8.44 mM, respectively). Still, the difference in Km is not huge. (C) Effect of interferents on Asp-P activity. Control refers to Asp-P without any treatment with interferents.

Most of the substances showed capacity of partially inhibit the asparaginase activity of Asp-P. The results obtained for Tween 20 and Triton X-100 are biased by the fact that, by addition of Nessler reagent, the solution turned quite turbid, what interfered with the absorbance measurements.

3.5. Mass spectrometry

The samples of Asp-P after partial purification on Ni^{2+}-Sepharose were analyzed by mass spectrometry. The experiment was able to identify 195 proteins, including Asp-P, whom represented 75 % of the proteins present in the sample solution. Other than the plant asparaginase, host proteins were also identified, including chaperones (DnaK and GroEL), a nucleotide exchange factor – GrpE, as well as a transcriptional regulator (LacI) and ribosomal subunits (table S1). Also, the peptide mapping performed and the alignment of those found with Asp-P primary sequence resulted in the identification of 324 of 346 residues, a 93 % coverage. It was not possible, though, to identify the N-terminal His-tag region, along the thrombin recognition site and a minor peptide, "GTLAR". No bacterial L-asparaginase could be significantly detected (Figure 6).

Fig. 6. Asp-P primary sequence identified by mass spectrometry

```
1   MGSSHHHHHHSSGLVPRGSH MGGWAIAVHGGAGVDPNLPL ERQEEAKQLLTRCLNLGISA  60
61  LNSNVPAIDVVELVVRELET  DPLFNSGRGSALTEKGTVEM EASIMDGPKRRCGAVSGLTT 120
121 VKNPISLARLVMDKSPHSYI  AFSGAEDFARQQGVEVVDNE YFVTPDNVGMLKLAKEANTI 180
181 LFDYRIPSSAYETCGSGVES  PLQMNGLPISVYAPETVGCV VVDREGRCAAATSTGGLMNK 240
241 MTGRIGDSPLIGAGTYACDV CGVSCTGEGEAIIRGTLARE VAAVMEYKGLKLHQAVDFVI 300
301 KHRLDEGKAGLIAVSNTGEV ACGFNCNGMFRACATEDGFM EVAIWD               346
```

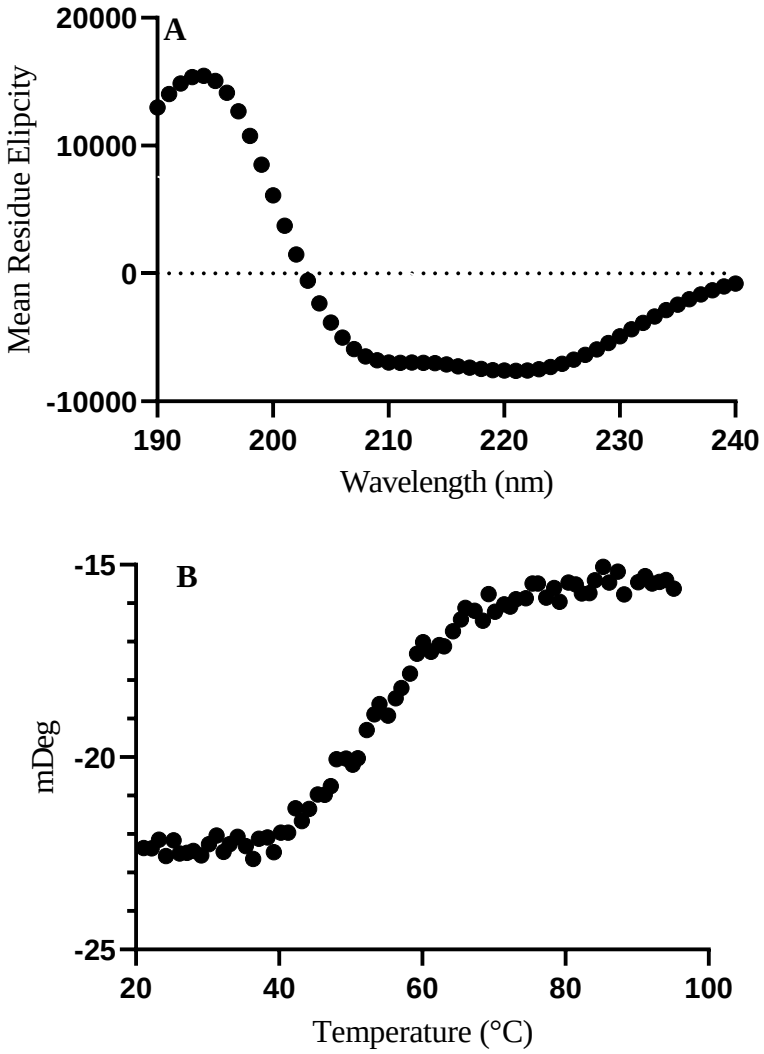
Phaseolus vulgaris L-asparaginase (Asp-P) Coverage: 324/346 = 0.936

Note: Residues marked in red were not identified by the experiment, whilst blue ones were mapped. It was possible to identify 324 out of 346 residues, a coverage of 93.6 %.

3.6. Circular dichroism

The analysis of pure Asp-P in the far-UV (190-240 nm) showed a secondary structure content of 24 % of α-helix, 49.1 % of β-strands and 26.9 % of disordered regions (Figure 7A). The T_m of the enzyme was assessed by submitting it to a range of 20-95° C and a wavelength of 220 nm. The assay demonstrated that half of Asp-P molecules are denatured at 52.78° C (Figure 7B).

Fig. 7. Circular dichroism analysis of Asp-P

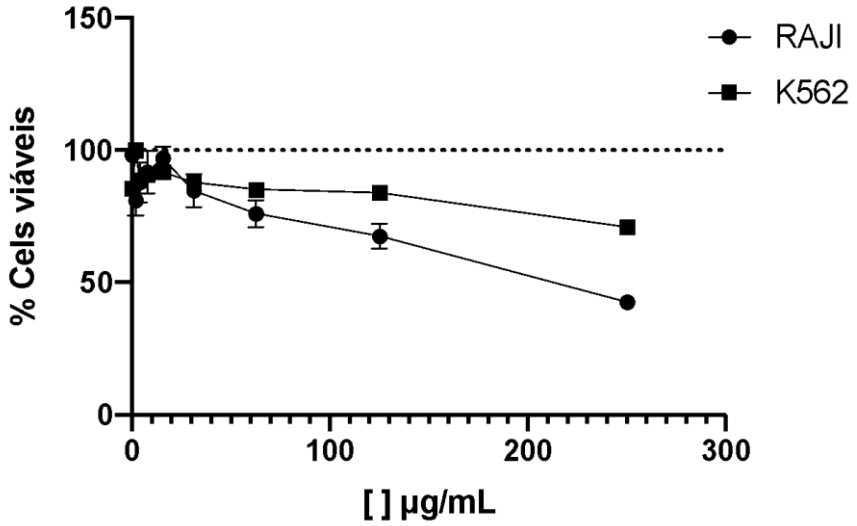


Note: (A) analysis of mean residue elipcity os Asp-P in the far-UV, 190-240 nm. (B) analysis of changes in Asp-P secondary structure in response to temperature changes within the range of 20-95° C. Both analyses were carried out in 5 mm pathlength and protein concentration of 75 μg.mL^{-1} in 50 mM potassium phosphate buffer, pH 7.0.

3.7. Cytotoxicity assay

The cytotoxicity assay of Asp-P against Raji and K562 cells showed that a high amount of enzyme is needed to induce apoptosis in both cell lines. Figure 8 shows that, in order to reduce the viable cells below 50 %, the highest concentration tested, 250 μg.mL^{-1}, is required, despite the 72 h incubation. Although this is not the case for K562, which viable cells remained up to ~70 %. Because of these data, no IC_{50} could be calculated for both cell lines.

Fig. 8. Cell viability of Raji and K562 cell lines after 72 h incubation with Asp-P



Note: The graphic shows the percentage of viable cells after incubation with different concentrations of Asp-P. Raji is more sensitive to Asp-P than K562, but only the highest concentration tested induced apoptosis in more than 50 % of the cells. More resistant, more than 70 % of the K562 cells resisted to the 72 h incubation with Asp-P. Since it was not possible to achieve 50 % of death in K562 and it was reached only in the concentration of 250 $\mu g.mL^{-1}$ in Raji, IC_{50} could not be calculated for both cell lines. The presented data are a mean of three independent experiments performed in triplicates.

3.8. Immunogenicity prediction

The online immunogenicity prediction results for Asp-P in comparison with *E. coli* asparaginase showed that, using the established threshold of ≤ 1, the plant enzyme has only one core sequence that would bind to MHCII with a percentage rank of 0.52 for the HLA-DRB1*07:01, "IRGTLAREV". The bacterial enzyme, however, has 6 predicted peptides ranging from 0.14 to 0.45 (table S2). Not only there are more predicted peptides for the commercial protein for this allele, they also have a lower percentage rank than the plant enzyme, what means higher affinity. In regards to allele HLA-DQA1*02:01/DQB1*02:02, no peptide is predicted for Asp-P sequence, while a single peptide is predicted for *E. coli* asparaginase with a percentile rank of 0.06.

The CD4 immunogenicity prediction analysis was focused on allele HLA-DBR01*07:01. While Asp-P had 4 predicted peptides with scores ranging from 7.8 to 37, the bacterial enzyme had 5 peptides with a score range of 2.9 to 65 (table S3). Meanwhile, the B cell linear epitope prediction showed that Asp-P has less predicted epitopes than *E. coli* asparaginase (Table 1). In contrast, the structural based prediction made by DiscoTope showed that Asp-P has 3 regions that may induce immunogenic response: positions 15-25, 150-160 and 215-225. Commercial enzyme, conversely, has only 2 regions: 10-20 and 160-165 (figure S2).

Table 1. B cell linear predicted epitopes for Asp-P and *E. coli* L-asparaginase

B cell linear epitopes	
Asp-P	***E. coli* L-asparaginase**
36 PNLPLERQEEAK 47	74 QVVNIGSQDMNDN 86
238 MNKMTGRI 245	140 STSMSAD 146
287 YKGLKLHQ 294	184 KTNTTDVATFKS 195
-	299 ATTQDAEVDDA 309
-	336 KDPQQIQQI 344

Note: The sequence of Asp-P containing His-tag was used. Only peptides with 7-15 residues were included.

4. DISCUSSION

L-asparaginases are essential medicines for the chemotherapeutic treatment of ALL. To date, only bacterial asparaginases, specifically *Escherichia coli* and *Dickeya sp.*, are employed in this kind of treatment due to their high affinity for the substrate, L-asparagine. In contrast, the severe adverse reactions caused by their use has been a problem still to be solved (BURKE; ZALEWSKA-SZEWCZYK, 2022; LEE *et al.*, 2023; SILVA *et al.*, 2020; TOSTA PÉREZ *et al.*, 2023). The search for novel asparaginases from different organisms that show no glutaminase activity and less immunogenic responses has been an effort made throughout the years without much of a success (HATAMZADEH *et al.*, 2020; IZADPANAH *et al.*, 2018; LAILAJA *et al.*, 2022; MOUBASHER *et al.*, 2022; VAN KERCKHOVEN *et al.*, 2017). For this purpose, among different *taxa*, plants have shown unique characteristics that still need studies, even though potential has been brought to light (AL-HAZMI; NAGUIB, 2022; DA SILVA *et al.*, 2018; MICHALSKA; HERNANDEZ-SANTOYO; JASKOLSKI, 2008; MOHAMED *et al.*, 2016).

In this context, *Phaseolus vulgaris* is a species that has been studied before obtained from the native source or by recombinant production (MOHAMED *et al.*, 2015, 2016; PAJAK *et al.*, 2023; YAZDI *et al.*, 2020). Despite the available data, the studies that work with the recombinant enzyme have a more structural bias, analyzing specifically the interaction between the metal ion and the enzyme itself, focusing on the effects of this metal coordination on protein structure and activity (AJEWOLE *et al.*, 2018; BEJGER *et al.*, 2014). It is known that recombinant proteins may have different properties when compared to native ones due to the expression systems' differences (BEYGMORADI *et al.*, 2023; BRITO E CUNHA *et al.*, 2019; ZHANG *et al.*, 2022). Since a thorough characterization of the recombinant *P. vulgaris*' asparaginase was not portrayed before, the present work focus on making such characterization.

In this work, *P. vulgaris*' L-asparaginase was successfully expressed in bacterial system and purified to homogeneity through two chromatographic steps. The enzyme has a theoretical molecular mass of 73.28 kDa in its tetramer form. However, the subunits α and β dissociate when denatured in SDS-PAGE, allowing us to see two different bands. This proves that, even in a different expression system, the enzyme was able to perform its maturation by autoproteolytic cleavage. Also, the presence of the two bands agrees with previous results for the recombinant protein and other plant and class 2 asparaginases (AJEWOLE *et al.*, 2018; BEJGER *et al.*, 2014; BRUNEAU; CHAPMAN; MARSOLAIS, 2006; MICHALSKA; HERNANDEZ-SANTOYO; JASKOLSKI, 2008; SODEK; LEA; MIFLIN, 1980).

Upon complete purification, Asp-P showed specific activity of 905 $U.mg^{-1}$, which is slightly higher than the reported value for the purified native enzyme (MOHAMED *et al.*, 2015). The western blot marked only the α subunit of Asp-P, confirming that the band corresponds to the recombinant protein. Also, since no bands were marked by anti-bacterial asparaginase antibody, it is possible to affirm that no native bacterial asparaginase contaminant was in the samples tested, hence the asparaginase activity could be attributed solely to Asp-P. Also, since there is no cross-reactivity between the anti-bacterial antibody and Asp-P, it is hypothesized that patients that already had adverse reactions caused by the bacterial asparaginase could be treated with the plant enzyme because there is lower probability of cross-reactivity and immunogenic responses (SILVA *et al.*, 2020; ZALEWSKA-SZEWCZYK *et al.*, 2009).

After a wide pH screening, Asp-P showed a preference for more basic mediums, demonstrating measurable activity only between pHs 6.0 and 10.0, having an optimal pH of 9.0. In this case, Asp-P can be addressed as an alkaline enzyme. The higher catalytic activity in pH 9.0 may be due to the ionization of sidechains of Asp-P residues, what could alter the enzyme conformation, thus allowing a better catalytic efficiency. Also, L-asparagine, the substrate, is in a transitioning ionic state, since its amine group pKa is 8.8. This way, more asparagine molecules now have a negative charge that interacts with the active site in a slightly different way. Most active site residues, whether the nucleophilic residue (Thr196) or the residues that assist on the substrate anchorage, are still neutral in this pH, except Arg224 and Asp227. The repulsion of the two negative charges from substrate and Asp227 may lead the first closer to the nucleophilic residue, Thr196, meanwhile the positive charged Arg224 has a stronger interaction with it, thus improving catalysis. However, structural studies are required to prove this.

An optimal pH of 9.0 does not agree with previous data reported for this protein, whether it was the native or recombinant one. All data from previous works report an optimal pH of 8.0 for *P. vulgaris* L-asparaginase (AJEWOLE *et al.*, 2018; MOHAMED *et al.*, 2015). Meanwhile, here, the optimal pH of 9.0 shows that recombinant enzymes may have different properties when compared to native or, in this case, recombinant ones (BRITO E CUNHA *et al.*, 2019; ESPEJO-MOJICA *et al.*, 2015; MÉNDEZ-LÍTER *et al.*, 2017). This value differs even when compared to other plant species, like *Asparagus racemosus*, *Glycine max* and the bryophyte *Sphagnum fallax*, all presenting optimal pH around 8.0 (AL-HAZMI; NAGUIB, 2022; BEULAH; HEMALATHA, 2019; HEESCHEN *et al.*, 1996). This difference may be due to alterations on post-translational modifications, whether it is the addition or the lack of them. Also, the cytosol environment and the protein-protein interactions with chaperones that assist on protein folding may cause these differences as well (BEYGMORADI *et al.*, 2023; POURESMAEIL; AZIZI-DARGAHLOU, 2023; ZHANG *et al.*, 2022). This preference for more basic medium is not a favorable feature for therapeutical L-asparaginases (BECKETT; GERVAIS, 2019; CECCONELLO *et al.*, 2020).

Regarding the effect of temperature on Asp-P, 40° C was the best temperature for the enzyme activity among the temperatures tested in both pHs (7.5 and 9.0). Above that, activity is completely lost from 60 to 70° C. This result aligns well with the previous results for *P. vulgaris*, as well as other asparaginases from different organisms, including the commercial ones, but divergent from *Pinus pinaster* PpASPG1, which showed optimal temperature for enzymatic activity at 50° C. Such optimal temperature for asparaginase activity (40° C) is a great feature, since it is close to physiological temperature, 37° C (BANO; SIVARAMAKRISHNAN, 1980; BECKETT; GERVAIS, 2019; BEULAH; HEMALATHA, 2019; JIA *et al.*, 2013; LOPES *et al.*, 2017; MOHAMED *et al.*, 2015; MOHAN KUMAR; MANONMANI, 2013; VAN KERCKHOVEN *et al.*, 2017).

Furthermore, when incubated for different times at 50° C, Asp-P specific activity sharply declined over time. In 5 minutes, more than 50 % of its activity is lost and the decrease continues until almost complete loss after 60 and 90 minutes. Despite having a minimal thermotolerance at 50° C, this does not disqualify Asp-P as a potential substitute for the commercial enzymes, since it is not expected to be active and work under such extreme temperature conditions inside the human body. This loss of activity at 50° C and beyond is not uncommon for L-asparaginases, agreeing with results reported for plant asparaginases, like *G. max* and *Lupinus luteus* (AL-HAZMI; NAGUIB, 2022; BOREK *et al.*, 2004), but also showed

by bacteria and fungi (DIAS; SANTOS AGUILAR; SATO, 2019; EL-NAGGAR *et al.*, 2018; JIA *et al.*, 2013; MOHAN KUMAR; MANONMANI, 2013).

Knowing these parameters, the kinetics of Asp-P were assayed in pHs 7.4 and 9.0 to better understand its behavior in a pH that is similar to the physiological one and its optimal pH conditions. Having said that, just as shown in the pH profiling, both pHs did not have a significant divergence in terms of values. Still, it is noticeable that pH 9.0 is optimal for Asp-P enzyme activity for it showed a greater affinity for the substrate than in pH 7.4. However, both pHs result in Km values that are in the range reported for plant asparaginases, most set in the millimolar range (AL-HAZMI; NAGUIB, 2022; BRUNEAU; CHAPMAN; MARSOLAIS, 2006; CREDALI *et al.*, 2011; HEESCHEN *et al.*, 1996; SODEK; LEA; MIFLIN, 1980; VAN KERCKHOVEN *et al.*, 2017). Comparing the Km value obtained in pH 9.0 (7.05 mM) with the reported Km for the native Asp-P (6.72 mM), it is clear that they are similar to each other (MOHAMED *et al.*, 2015). In contrast, a bigger difference is found when Asp-P Km value is compared to the one reported by Bejger *et al.* (2014) with the recombinant enzyme, 3.71 mM. This divergence may be due to the different methods used to quantify asparaginase activity, since the authors used a NADH-coupled enzymatic assay, which requires a lot of expensive reagents, but is said to be more sensitive than Nesslerization, thus being able to measure small amounts of L-aspartate produced by asparaginase activity (BRUNEAU; CHAPMAN; MARSOLAIS, 2006; MAGRI *et al.*, 2018).

Taking into account that glutaminase activity is a disadvantage for a L-asparaginase, enzymatic activity of Asp-P towards L-glutamine was tested in comparison with L-asparagine. In both protein concentrations tested, 92 and 300 $\mu g.mL^{-1}$, glutaminase activity was not measured for Asp-P. This lack of glutaminase activity is a favorable feature for Asp-P, since it is a major drawback presented by the bacterial enzymes (BECKETT; GERVAIS, 2019; NGUYEN; SU; LAVIE, 2016). This result also agrees with previous data related to the native protein and many other plant asparaginases, which, in turn, show enzymatic activity towards isoaspartyl-dipeptides, said to alter tridimensional protein structures and, when these proteins are degraded, these molecules require specific enzymes to hydrolyze them (BOREK *et al.*, 2004; BRUNEAU; CHAPMAN; MARSOLAIS, 2006; CLARKE, 2003; HEJAZI *et al.*, 2002; LEA *et al.*, 2007; MOHAMED *et al.*, 2015; SHIMIZU; MATSUOKA; SHIRASAWA, 2005).

The test of Asp-P activity in the presence of interferents/denaturants showed that, among the ones tested, only Thiourea did not hinder it significantly. All other reagents had a higher effect on Asp-P activity, specially SDS, a known denaturant detergent. Just like in SDS-PAGE, the presence of this substance in the sample may separate the subunits of the protein, still Asp-

P retained 15 % of its activity. The asparaginases from *Lasioplodia theobromae*, *Cladosporium sp.* and *Acinetobacter soli* were reported to completely lose their catalytic activity in the presence of SDS (JIAO *et al.*, 2020; MOHAN KUMAR; MANONMANI, 2013; MOUBASHER *et al.*, 2022), whilst it did not have the same effect in *Streptomyces brollosae* (EL-NAGGAR *et al.*, 2018).

EDTA, β-mercaptoethanol and Urea affected Asp-P in a similar proportion, hindering about 40 % of the catalytic activity of the protein. This result was expected for EDTA, since Asp-P is a K^+-dependent metalloenzyme (AJEWOLE *et al.*, 2018; BEJGER *et al.*, 2014; MOHAMED *et al.*, 2015). Hence, the chelating effect of EDTA may have disrupted the metal coordination on the protein, lowering its catalytic efficiency. EDTA had a similar effect on *Streptomyces brollosae* (EL-NAGGAR *et al.*, 2018), but not on *Bacillus subtilis* and *Cladosporium sp.* (JIA *et al.*, 2013; MOHAN KUMAR; MANONMANI, 2013). As a reductive agent, β-mercaptoethanol also diminished Asp-P activity by 40 %. This reagent is reported to have different effects on L-asparaginases, having no effect (EL-NAGGAR *et al.*, 2018), complete loss of activity (MOHAN KUMAR; MANONMANI, 2013) or even an increase in catalytic efficiency (ELSHAFEI *et al.*, 2012; JIAO *et al.*, 2020; MANNA *et al.*, 1995). In accord with, Urea also present divergent effects on L-asparaginases presenting a complete or a mild loss of activity (JIAO *et al.*, 2020; MANNA *et al.*, 1995; SINDHU; MANONMANI, 2018).

Tween 20 and Triton X-100 deserve a closer look in this analysis. Both detergents, even after centrifugation, made the final solution turbid upon addition of Nessler reagent. This turbidity may overestimate the quantification of ammonia by increasing the absorbance measurement not by color itself, but turbidity as well. Therefore, both quantifications have higher values than expected, especially Triton X-100, which had a higher activity than the untreated sample. Some previous works also report an increase of asparaginase activity in the presence of these detergents or similar ones, all using the Nesslerization method (EL-NAGGAR *et al.*, 2018; MOHAN KUMAR; MANONMANI, 2013; MOUBASHER *et al.*, 2022).

The mass spectrometry experiment, along with western blotting, confirmed that the asparaginase activity presented by the sample is attributed to Asp-P, since no bacterial L-asparaginase was identified in significant amounts. 93 % of the plant enzyme sequence could be identified, most of it belonging to the original protein sequence. Still, Asp-P represented 75 % of proteins in the samples, while the other 25 % were composed of 194 other proteins from the host organism. Among these proteins, most were chaperones involved in protein folding, like DnaK, GroEL and GrpE. Their presence in the sample indicates that they also help Asp-P

perform its correct folding and not form inclusion bodies (BANEYX; MUJACIC, 2004; MAMIPOUR; YOUSEFI; HASANZADEH, 2017). However, they are also probably interacting with the protein or the chromatographic column, thus contaminating the sample. Despite the relatively high purity (75 %), the purity ratio is a factor that impact drugs administered to patients, since these contaminants may lower the biological activity of the main compound, its quality and induce other reactions, like immunogenic responses (MICHALOWSKI *et al.*, 2021; SCHNUCHEL *et al.*, 2023). Hence, it is important that purification steps are performed to ensure thorough isolation of the protein of interest, yet several factors need to be taken into account, like flow velocity and buffer pH (ALDINGTON; BONNERJEA, 2007; CHE HUSSIAN; LEONG, 2023).

Circular dichroism analysis of Asp-P resulted in values that are in accord with previous reports. The deconvoluted data of secondary structure content confirms the results obtained by Ajewole *et al.* (2018), since all percentages are similar. However, in the present work, Turns are integrated with β-strands percentage. Even if authors affirm that values presented by them are not accurate reflects of secondary structure rather a comparative method, here it is provided a confirmation that Asp-P undergoes proper folding. In the same way, the T_m calculated for Asp-P was relatively close to previous reported values, but slightly higher (AJEWOLE *et al.*, 2018). A T_m of 52.78° C agrees with the fact that enzymatic activity of Asp-P at 50° C and above is majorly hindered after 5 min. The native enzyme is also reported to lose part of its enzymatic activity at 50° C and above, though the loss percentage is lower than Asp-P (MOHAMED *et al.*, 2015).

The cytotoxicity assay performed with Asp-P used two different neoplastic cell lines, Raji (Burkitt's lymphoma, B lymphoblast, ATCC CCL-86) and K562 (chronic myeloid leukemia, multipotent blasts, ATCC CCL-243). These cell lines were chosen based on the probability of being more susceptible (Raji) and more resistant (K562) to asparaginase treatment. It is clear that Raji is indeed more susceptible to asparaginase treatment than K562, as Asp-P could not reduce K562 viable cells to below 50 % even with 250 $\mu g.mL^{-1}$. In contrast, it was possible with Raji cells, although only with the highest concentration. Because of this, IC_{50} could not be calculated for both cell lines. Mohamed *et al.* (2016), utilizing the native protein, demonstrated that the L-asparaginase from *P. vulgaris* could induce apoptosis of Jurkatt clone E6-1 cells (acute lymphoblastic leukemia, T lymphoblast, ATCC TIB-152) with lower enzyme concentrations than the commercial enzymes tested. It is possible that the cell line used by these authors is more susceptible to asparaginase treatment than Raji, although *Yarrowia lipolytica* asparaginase could induce a higher level of apoptosis in Raji cells with lower

concentrations than the commercial enzyme (MAZLOUM-RAVASAN *et al.*, 2020). Besides, asparaginases show cytotoxic effects on tumorous cell lines other than hematologic ones. For example, the asparaginase from *G. max* showed lower IC_{50} than *Pseudomonas aeruginosa* for HepG2 (liver cancer), MCF-7 (breast cancer), CLS-145 (gastric cancer), and others (AL-HAZMI; NAGUIB, 2022). It is also reported that the use of asparaginase treatment may prevent metastasis without having an effect on primary tumour growth (KNOTT *et al.*, 2018). Hence, these results do not mean that Asp-P could not present a promising cytotoxic effect on other cell lines than Raji and K562 or prevent metastasis.

In regards to the immunogenic responses showed by the commercial enzymes, they represent a major drawback for these therapeutic drugs, since patients have severe adverse reactions that alter the dose or even halt the use of asparaginase on the treatment of ALL and other kinds of cancer (BURKE; ZALEWSKA-SZEWCZYK, 2022; HIJIYA; VAN DER SLUIS, 2016; LEE *et al.*, 2023; SILVA *et al.*, 2020). Besides these adverse reactions, there is also the induction of antibodies production (IgG, IgE and IgM) that inactivate the enzyme and mediate a subsequent resistance to the medication (SAEED *et al.*, 2022). In this context, the immunogenicity of Asp-P was assessed by *in silico* methods and the results were compared to the *E. coli* asparaginase ones.

In general, Asp-P showed a lower immunogenicity when compared to the bacterial enzyme. The most promising result was for the alleles HLA-DRB1*07:01 and HLA-DQA1*02:01/DQB1*02:02, which are strongly related to asparaginase immunogenic reactions (FERNANDEZ *et al.*, 2014; KUTSZEGI *et al.*, 2017). These alleles represent a genetic repertoire for the Main Histocompatibility Complex (MHC) II that mediate an immunogenic response by CD4 T cell recognition. Asp-P showed no sequence prediction within the threshold for the allele HLA-DQA1*02:01/DQB1*02:02, while the bacterial enzyme had one predicted peptide with a high affinity binding prediction of 0.06 percentage rank. For HLA-DBR1*07:01, Asp-P had only one core sequence predicted, whilst *E. coli* asparaginase had 6 with different binding affinities. All other prediction methods were in accord with previous works that already predicted that *P. vulgaris* asparaginase has a lower immunogenicity if compared to bacterial enzymes, having less predicted peptides and inducing lower antibody levels (MOHAMED *et al.*, 2016; YAZDI *et al.*, 2020). All these results suggest a lower probability of immunogenic responses caused by Asp-P administration in patients with ALL and are in accord with western blot experiment using anti-bacterial asparaginase antibody, that marked no bands on Asp-P sample, thus showing that the probability of cross-reactivity is rather low (ZALEWSKA-SZEWCZYK *et al.*, 2009).

6. CONCLUSIONS

This work provides a different view over *Phaseolus vulgaris* L-asparaginase. It is shown that recombinant proteins may have divergent properties when compared to native, and even, recombinant ones. Here, it is reported that Asp-P is, indeed, a class 2 L-asparaginase, with an optimal pH of 9.0 and optimal temperature of 40° C, losing its catalytic activity drastically at 50° C. The enzyme activity could be hindered by a range of denaturant and chelating agents, specially SDS. Asp-P represents 75 % of total proteins in partially purified samples, reaching a higher purity ratio with size-exclusion chromatography. The enzyme is cytotoxic against leukemic cell lines and has low immunogenicity when compared to bacterial asparaginases. Put together, these results shed a light on possible prospects for improving Asp-P activity and thermostability by protein engineering, once its features are already promising. Having no glutaminase activity, lower immunogenicity and optimal activity near physiological temperature conditions, Asp-P has great features that favor it on its road to substitute commercial enzymes in the treatment of ALL.

Acknowledgements

This study was partly funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). The research used resources from Fundação Oswaldo Cruz – Ceará, Grupo de Engenharia de Proteínas e Soluções a Saúde and Plataforma de Proteômica of the same institution. Also, resources from Laboratório de Espectrometria de Massas e Análises de Proteínas (LEMAP – UFC) were used as well. The institutions that provided resources are acknowledged for the assistance and conductance of experiments as well.

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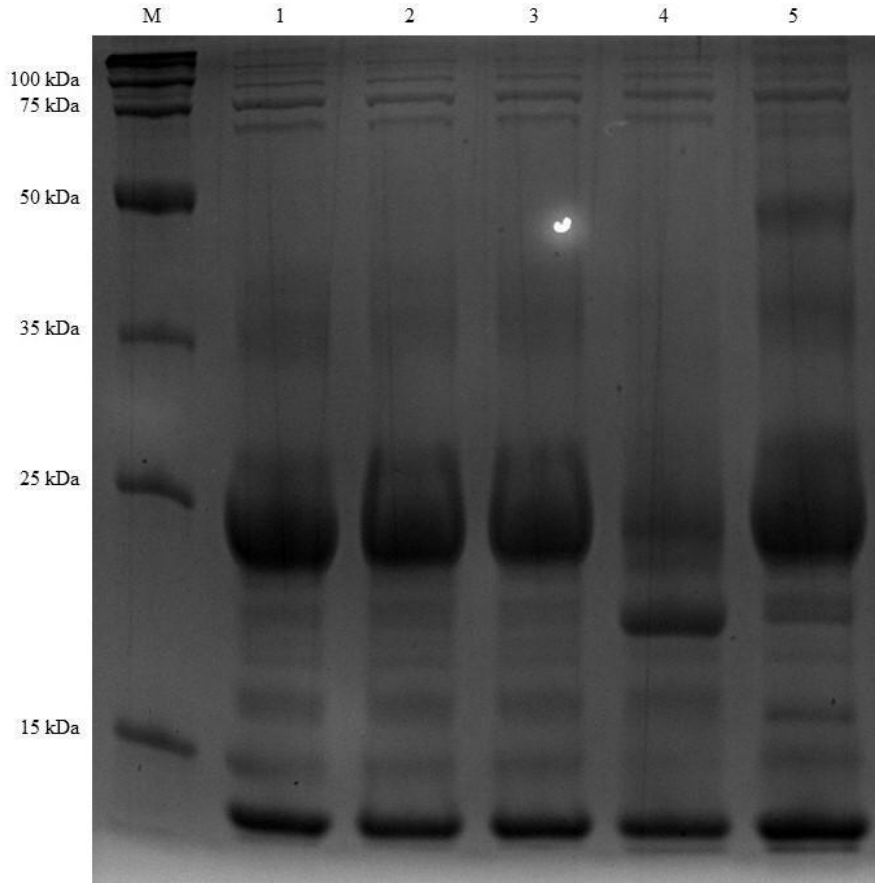
Supplementary material

Figure S1: Degradation of Asp-P α-subunit



Note: SDS-PAGE of Asp-P samples stocked in different conditions for different amounts of time. Lane M: molecular marker; lane 1: Asp-P sample stocked in -80° C freezer for 3 months. Specific activity: 149.25 $U.mg^{-1}$; lane 2: Asp-P sample containing 16 % Sucrose stocked in -80° C freezer for 3 months. Specific activity: 180 $U.mg^{-1}$; lane 3: Asp-P sample containing 16 % Sucrose stocked in -80° C freezer for 2 months. Specific activity: 184 $U.mg^{-1}$; lane 4: Asp-P sample stocked in 4° C fridge for 3 months. Specific activity: 133 $U.mg^{-1}$; lane 5: Asp-P sample stocked in 4° C fridge for a week. Specific activity: 103 $U.mg^{-1}$. Note that the α-subunit (predominant band, ~ 23 kDa) degrades over time when stocked in 4° C for long periods of time. A more recent sample stocked in the same conditions does not present a visible α-subunit degradation. Samples stocked at -80° C did not suffer proteolytic degradation over time, despite the presence or not of cryoprotectant. All samples were highly concentrated (protein concentration > 1 $mg.mL^{-1}$).

Table S1: Top 10 representative proteins identified by mass spectrometry

Protein	Length	Unique peptides	Molecular weight (Da)	NSAF (%)	Coverage (%)
Asparaginase P *Phaseolus vulgaris*	346	212	36597	75	93.6
DnaK	638	54	69054.5	3.6	57
GroEL	548	38	57274.7	1.9	49
50S ribosomal protein L28	78	4	8982.9	0.9	32
30S ribosomal protein S15	89	2	10244.5	0.7	22.4
50S ribosomal protein L3	209	6	22211.8	0.67	24.8
Transcriptional regulator LacI	363	14	38860.2	0.66	30.5
50S ribosomal protein L13	142	5	15990.5	0.63	45
GrpE	197	6	21780.2	0.62	22.3
50S ribosomal protein L14	123	3	13514.4	0.57	20.3

Table S2: Predicted peptides from NetMHCIIPan 4.1 EL

HLA-DQA1*02:01/DQB1*02:02			
Asp-P		***E. coli* L-asparaginase**	
Predicted peptide	**Percentage score**	**Predicted peptide**	**Percentage score**
-	-	295 VPTGATTQDAEVDDA 309	0.06
HLA-DBR1*07:01			
Asp-P		***E. coli* L-asparaginase**	
Predicted peptide	**Percentage score**	**Predicted peptide**	**Percentage score**
268 EGEAIIRGTLAREVA 282	0.52	308 DAKYGFVASGTLNPQ 322	0.14
-	-	237 GIVYNYANASDLPAK 251	0.21
-	-	84 NDNVWLTLAKKINTD 98	0.21
-	-	102 TDGFVITHGTDTMEE 116	0.35
-	-	255 DAGYDGIVSAGVGNG 269	0.45
-	-	63 QLKDIAANVKGEQVVN 77	0.45

Note: The percentage score threshold used was 1.0. Any non-redundant peptide with a score lower than 1.0 was included. It is noteworthy that the lower the percentage, the higher the probability of inducing immunogenic response.

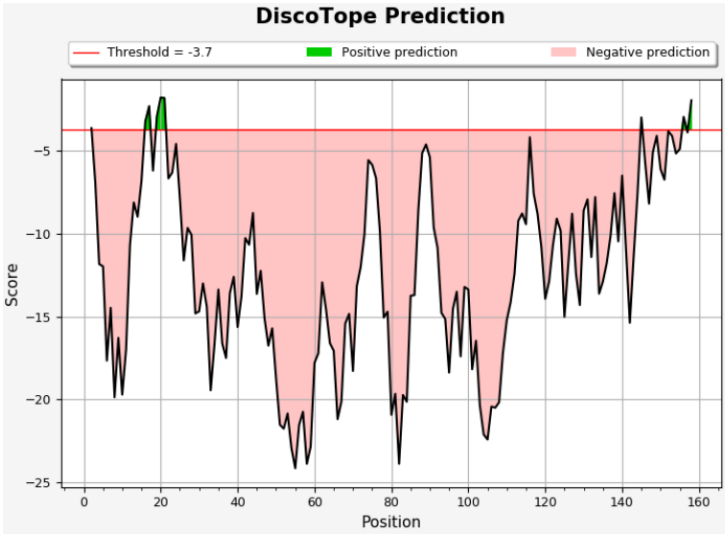
Table S3: Predicted peptides from CD4 immunogenicity predictions for HLA-DRB1*07:01

HLA-DRB1*07:01			
Asp-P		***E. coli* L-asparaginase**	
Predicted peptide	**Allele score**	**Predicted peptide**	**Allele score**
126 SLARLVMDKSPHSY 140	21.0	1 MEFFKKTALAALVMG 15	2.9
176 EANTILFDYRIPSSA 190	37.0	146 DGPFNLYNAVVTAAD 160	26.0
281 VAAVMEYKGLKLHQA 295	13.0	166 RGVLVVMNDTVLDGR 180	65.0
286 EYKGLKLHQAVDFVI 300	7.8	201 LGYIHNGKIDYQRTP 215	19.0
-	-	236 VGIVYNYANASDLPA 250	16.0

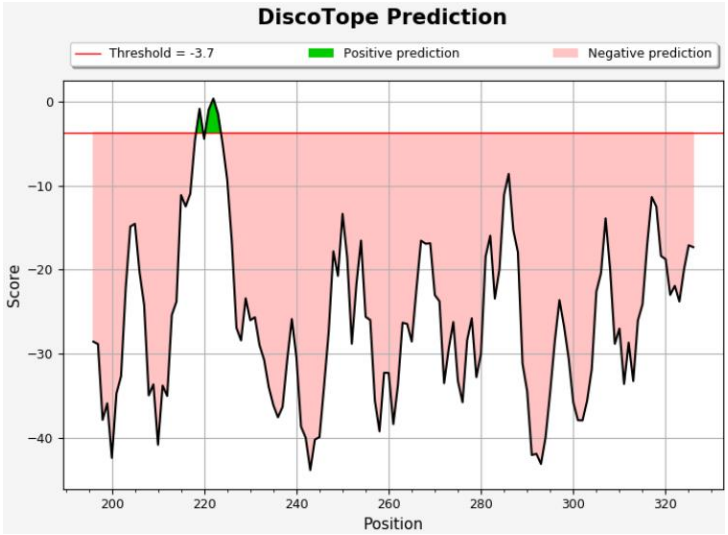
Note: The higher the allele score, the higher the probability of inducing immunogenic responses.

Figure S2: Structure antigenic antibody prediction from DiscoTope for Asp-P and commercial enzyme

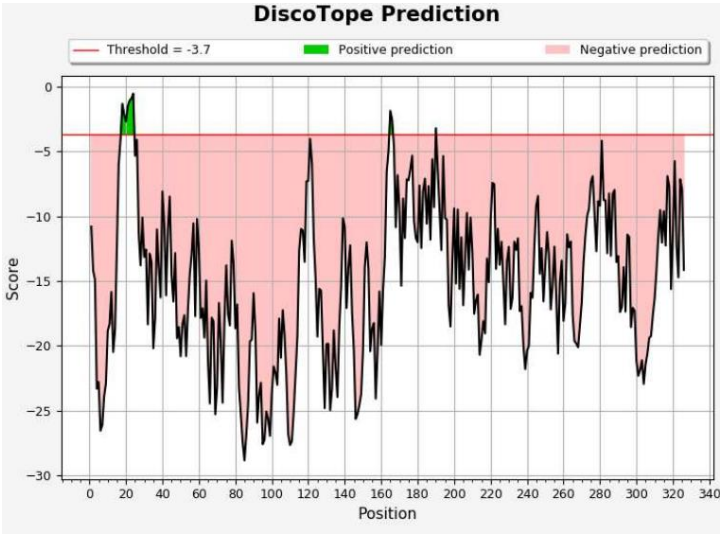
A



B



C



Note: (A) Asp-P α-subunit analysis; (B) Asp-P β-subunit analysis; (C) commercial enzyme monomer analysis. Asp-P: PDB 4PU6; *E. coli* L-asparaginase: PDB 3ECA.

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