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NEW STRUCTURAL INSIGHTS INTO ANOMERIC CARBOHYDRATE RECOGNITION BY FRUTALIN: AN α-D-GALACTOSE-BINDING LECTIN FROM BREADFRUIT SEEDS

FORTALEZA
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Área de concentração: Bioquímica Vegetal.

Orientador: Prof. Dr. Renato de Azevedo Moreira.

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A Deus, em sua Santíssima Trindade,
Ao meu pai, Vicente.

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RESUMO

As lectinas são proteínas que se ligam a carboidratos ou glicoconjugados, de forma reversível, sem alterar a estrutura dos mesmos. Desta forma, podem interagir com a superfície celular e promover as mais diversas funções biológicas. A Frutalina (FTL) é a lectina mais abundante das sementes do fruta-pão (*Artocarpus incisa*), pertencendo à família de lectinas relacionadas à Jacalina (JRL). FTL reconhece e se liga especificamente a α-D-galactose e tem sido usada com sucesso em pesquisas imunobiológicas para o reconhecimento de oligossacarídeos associados ao câncer. Neste trabalho, nós relatamos toda a estrutura 3D da FTL, conforme determinada por cristalografia de raios-X. Os cristais obtidos foram difratados a 1,81 Å (Apo-frutalin) e 1,65 Å (FTL–D-Gal) de resolução. A lectina exibe clivagem pós-traducional produzindo uma cadeia α- (133 aminoácidos) e uma cadeia β (20 aminoácidos), apresentando um homotetrâmero quando em solução, com um típico β-prisma presente nas JRL. O β-prisma da FTL é composto de três folhas-β formando três motivos chave grega antiparalelos. O sítio de ligação à carboidratos (CBS) envolve o N-terminal da cadeia α e é formado por quatro resíduos chave: Gly25, Tyr146, Trp147 e Asp149. Estes resultados foram usados em simulações de dinâmica molecular em soluções aquosas para esclarecer as bases molecular da ligação de glicanos à FTL. As simulações sugerem que a excisão do peptídeo Thr-Ser-Ser-Asn (TSSN) reduz a rigidez do CBS da FTL, aumentando o número de interacões com ligantes e resultando em locais de ligação múltipla e reconhecimento anomérico das moléculas de açúcar. Nossos achados fornecem uma nova perspectiva para elucidar ainda mais a versatilidade do FTL em muitas atividades biológicas, incluindo o reconhecimento de glicoproteínas do soro sanguíneo que provocam neoplasias através de sua super-expressão. A interação da FTL com glicoproteína Complement-C3 foi investigada por simulações de docking molecular e as bases moleculares deste reconhecimento foram observadas, comprovando o envolvimento do CBS no reconhecimento do ligante.

Palavras-chave: Fruta-pão. Lectinas. Frutalina. Complement-C3.

ABSTRACT

Lectins are proteins that bind to carbohydrates or glycoconjugates, reversibly, without changing the structure of them. They can interact with the cell surface and promote the most diverse biological activities. Frutalin (FTL) is the most abundant lectin in breadfruit seeds (*Artocarpus incisa*), belonging to the jacalin-related lectin family (JRL). FTL specifically recognizes α-D-galactose and has been successfully used in immunobiological research for the recognition of cancer-associated oligosaccharides. Here, we report the full 3D structure of the FTL, as determined by X-ray crystallography. The crystals obtained were diffracted at 1.81 Å (Apo-frutalin) and 1.65 Å (FTL-D-Gal) resolution. The lectin exhibits post-translational cleavage producing an α- (133 amino acids) chain and a β chain (20 amino acids), exhibiting a homotetramer when in solution, with a typical β-prism present in the JRLs. The β-prism of the FTL is composed of three β-sheets forming three antiparallel Greek key motifs. The carbohydrate binding site (CBS) involves the N-terminus of the α-chain and is formed by four key residues: Gly25, Tyr146, Trp147 and Asp149. These results were used in simulations of molecular dynamics in aqueous solutions to clarify the molecular bases of glycan binding to CBS. The simulations suggest that the excision of the Thr-Ser-Ser-Asn peptide (TSSN) reduces the CBS rigidity of the FTL, increasing the number of interactions with ligands and resulting in multiple binding sites and anomeric recognition of the sugar molecules. Our findings provide a new perspective to further elucidate the FTL's versatility in many biological activities, including the recognition of blood serum glycoproteins that elicit neoplasms through their overexpression. The interaction of FTL with Complement-C3 glycoprotein was investigated by molecular docking simulations and the molecular bases of this recognition were observed, proving the involvement of CBS in ligand recognition.

Keywords: Breadfruit. Lectins. Frutalin. Complement-C3.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

C1	Carbono 1
C4	Carbono 4
CBS	“Carbohydrate binding site”
FTL	Frutalina
JRL	“Jacalin-related lectin”
MCA	“Minimal concentration for agglutination”
MD	Dinâmica Molecular (“Molecular dynamics”)
NO	Óxido nítrico
PDB	“Protein Databank”
RMSD	“Root-mean square deviation”
SDS-PAGE	Eletroforese em gel de poliacrilamida na presença de SDS

SUMÁRIO

1 INTRODUÇÃO

As lectinas vegetais, por serem proteínas capazes de se ligar reversivelmente a glicanos sem alterar a estrutura dos mesmos, possuem uma grande versatilidade em aplicações biotecnológicas. Desta forma, o trabalho a seguir busca investigar detalhes da estrutura da Frutalina (FTL), uma lectina abundante nas sementes de *Artocarpus incisa*, conhecida popularmente como fruta-pão. Nas sementes de fruta-pão é possível encontrar três lectinas com reconhecimento distinto a carboidratos: frutalina, frutapina e frutaquina [1]. A Frutalina possui alta homologia estrutural com outra lectina amplamente estudada, a Jacalina, presente nas sementes de Jaca (*Artocarpus integrifólia*), porém as lectinas são capazes de promover diferentes aplicações biológicas [2].

As sementes de fruta-pão são compostas por um alto teor de água (até 60%) e moderado teor de proteína (12,25% do peso seco). A FTL é a lectina mais abundante desta espécie, apresentando propriedades de ligação múltipla em que o mesmo CBS reconhece uma gama de diferentes ligantes, embora tenha maiores afinidades para os monossacarídeos de α-D-galactose e carboidratos complexos que contêm glicanas Gα-1-3. [17]. O padrão da FTL em SDS-PAGE é semelhante a Jacalina: duas bandas entre 20 e 14 KDa que correspondem às frações glicosilada e não glicosilada, respectivamente, além de apresentar espectro de massa deconvoluído com diferentes massas dentro de 16,5 kDa, consistente com a presença de glico-isoformas de monômeros idênticos. Em solução, a frutalina pode formar homotetrâmeros nos quais cada monômero contém uma cadeia α (16 kDa) e uma cadeia β (2 kDa) [3,4]. Semelhante às lectinas de Moraceae, o CBS da frutalina está localizado em um domínio próximo ao terminal N da cadeia α, consistindo de quatro resíduos chave Gly25, Tyr146, Trp147 e Asp149. No reconhecimento ao ligante, ocorrem cerca de dez interações envolvendo hidroxilas: hidroxila C1 na Tyr146, hidroxila C3 na Gly25, hidroxila C4 nos resíduos Gly25 e Asp149 e hidroxila C6 em Tyr146, Trp147 e Asp149 [5], estas interações também foram evidenciadas no capítulo I deste trabalho, nas simulações in silico. Além disso, há evidências que sugerem que a frutalina possui estereoespecificidade, capaz de ligar especificamente α-D-galactose, uma vez que foi previamente isolada em uma coluna galactomanana reticulada, mas não em matrizes imobilizadas com β-galactosil [3, 6], o que deu origem à modelagem dos fragmentos ligantes destas matrizes para as simulações computacionais a seguir.

A Frutalina, por ser uma proteína bioativa, é uma lectina de interesse mundial, tendo sido investigada por muitos anos, com pesquisas multidisciplinares, dentre elas, destacam-se: levantamento preliminar de lectinas em extratos de fruta-pão [7], isolamento e caracterização

parcial da frutalina [3], desdobramento e redobragem da estrutura da frutalina [8], modulação das funções dos neutrófilos pela frutalina [9], mecanismos envolvidos no efeito mitogênico da frutalina nos linfócitos humanos [10], expressão de frutalina na levedura Pichia pastoris [11], expressão funcional da frutalina em Escherichia coli [12], estudo comparativo da ligação de frutalina recombinante e nativa aos tecidos da próstata humana [13], especificidade da frutalina usando modelos de biomembranas [14], efeitos citotóxicos da frutalina nativa e recombinante em células de câncer cervical Hela [15], potencial gastroprotetor da frutalina contra lesões gástricas induzidas pelo etanol [16], produção aumentada de frutalina heteróloga em Pichia pastoris sob pressão de ar aumentada [17], cristalização e estudos preliminares de difração de raio-X da frutalina [18], redução dos comportamentos nociceptivos agudos e neuropáticos em modelos de roedores da dor orofacial usando frutalina [19], painel de glicoproteínas como biomarcadores candidatos para o diagnóstico precoce e avaliação do tratamento da leucemia linfoblástica aguda de células-β [20], análise proteômica livre de marcadores de plasma de pacientes com câncer de mama: expressão de proteínas específicas do estágio [21], caracterização neurofarmacológica da frutalina em camundongos: Evidência de um efeito semelhante ao antidepressivo mediado pela via do receptor NMDA / NO / cGMP [22], reconhecimento da proteína Complement-C3 super-expressa em neoplasias pela frutalina [23].

Com base no que foi dito acima, pode-se sugerir que as pequenas diferenças e nuances conformacionais da Frutalina são fatores diferenciais nas suas funções biológicas e aplicações, o que impulsionou os objetivos deste trabalho, divididos aqui em dois capítulos. No capítulo I, o objetivo é a elucidação da estrutura tridimensional e a descrição dos detalhes estruturais da Frutalina, que apesar de homólogos à uma grande parte da Jacalina, apresenta detalhes que fazem da frutalina uma lectina versátil e relevante. Já o capítulo II traz como objetivo a promoção da interação in silico da Frutalina com uma glicoproteína presente no sangue, que se apresenta super-expressa em pacientes com neoplasias, buscando elucidar as interações específicas e inespecíficas que fazem da Frutalina uma ferramenta de detecção aplicável e valiosa para as ciências biomédicas.

2 CAPÍTULO I:

Running title: New structural insights into anomeric carbohydrate recognition by frutalin.

New structural insights into anomeric carbohydrate recognition by frutalin: an α-D-galactose-binding lectin from breadfruit seeds

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ABSTRACT

Frutalin (FTL) is a multiple-binding lectin belonging to the jacalin-related lectin (JRL) family and derived from *Artocarpus incisa* (breadfruit) seeds. This lectin specifically recognizes and binds α-D-galactose. FTL has been successfully used in immunobiological research for the recognition of cancer-associated oligosaccharides. However, the molecular bases by which FTL promotes these specific activities remain poorly understood. Here, we

report the whole 3D structure of FTL for the first time, as determined by X-ray crystallography. The obtained crystals diffracted to 1.81 Å (Apo-frutalin) and 1.65 Å (frutalin–D-Gal complex) of resolution. The lectin exhibits post-translational cleavage yielding an α- (133 amino acids) and β-chain (20 amino acids), presenting a homotetramer when in solution, with a typical JRL β-prism. The β-prism was composed of three 4stranded β-sheets forming three antiparallel Greek key motifs. The carbohydrate-binding site (CBS) involved the N-terminus of the α-chain and was formed by four key residues: Gly25, Tyr146, Trp147 and Asp149. Together, these results were used in molecular dynamics simulations in aqueous solutions to shed light on the molecular basis of FTL ligand binding. The simulations suggest that Thr-Ser-Ser-Asn (TSSN) peptide excision reduces the rigidity of the FTL CBS, increasing the number of interactions with ligands and resulting in multiple-binding sites and anomeric recognition of α-D-galactose sugar moieties. Our findings provide a new perspective to further elucidate the versatility of FTL in many biological activities.

2.1 INTRODUCTION

Plant lectins are proteins that are capable of interacting specifically and reversibly with glycans without altering their covalent structure. These proteins can mediate a variety of biological processes when in contact with glycoconjugates on the cell surface. This carbohydrate recognition ability also confers inflammatory and anti-inflammatory properties to plant lectins, as well as immunostimulatory activities [1]. Indeed, plant lectins are generally considered to be a very heterogeneous group of proteins, given that comparative biochemical studies indicate distinct biochemical/physicochemical properties, molecular structure, carbohydrate-binding specificity and biological activities, even among homologous molecules [2].

The genus Artocarpus (Moraceae) is a group among forest plants comprising more than 1400 species widely used in traditional medicines. Thus, prompting scientific interest in the secondary metabolites produced by this genus, which possess useful biological activities. Among these species, jackfruit (*Artocarpus integrifolia*) and breadfruit (*Artocarpus altilis*, also known as *Artocarpus incisa*) are well-known sources of lectins [3]. Jacalin is the predominant protein in jackfruit crude seed extract [4,5]. Since 1998, our group has surveyed A. incisa seeds and found lectins with similar characteristics to those in jackfruit seeds. The most abundant lectin in breadfruit is frutalin (FTL), first identified by Moreira et al. [6].

FTL belongs to the jacalin-related lectins (JRLs) with a preference for α-D-galactose moieties. At pH >8.0, FTL appears to be in a tetramer form composed of four protomers and with an apparent molecular mass of 60 kDa in native electrophoresis. FTL presents bands of 12 and 15 kDa upon SDS–PAGE, which correspond to a glycosylated fraction and a slightly or non-glycosylated fraction [7]. FTL is expressed in different isoforms, which mainly reflect differences in its post-translational glycosylation pattern [8]. Monteiro-Moreira et al. observed several masses ∼16 kDa in the deconvoluted spectra, which is consistent with the presence of these isoforms.

FTL has become an attractive and versatile biotechnological tool based on its ability to specifically recognize glycoconjugates. FTL has a multitude of activities, such as in the identification of prostate cancer tissues [9], as a tool for pivotal cancer biomarkers [10], neutrophil activation [11], gastroprotection in ethanol-induced lesions [12], and as an inhibitor of orofacial nociception in acute and chronic pain [13]. Therefore, based on such attractive properties, we aimed to elucidate the structure–activity relationship of FTL, to provide an understanding of its protein–carbohydrate interactions, which could help to identify and/or improve its therapeutic value.

2.2 EXPERIMENTAL

2.2.1 Isolation and purification of FTL from *Artocarpus incisa* seeds

A. incisa seeds were collected in Maranguape, Ceará, Brazil, and FTL extraction was performed according to previous studies [6]. Purity was confirmed by SDS–PAGE, and functional activity was assessed by a routine hemagglutination assay to measure the minimal concentration for agglutination (MCA). FTL-induced hemagglutination was determined as described previously [14]. After 1 h incubation at 37°C, duplicate wells were assessed to determine the MCA, i.e. the lowest lectin concentration that gave visible agglutination. PBS blanks were used as control [15]. After tryptic digestion, FTL was also submitted to mass spectrometry using a SYNAPT HDMS mass spectrometry (Waters, Manchester, U.K.) spectrometer, coupled to a nanoUPLC-ESI system. The sample was diluted to 1 mg/ml in 0.1% (v/v) formic acid. One microliter of the sample was used to perform reverse-phase chromatography using a gradient from 3% to 70% (v/v) acetonitrile for 30 min with 0.1% formic acid (v/v) at a flow rate of 300 nl/min in a BEH C4 column. For intact mass analysis, an aliquot was diluted in 0.1% (v/v) formic acid and applied directly to the spectrometer without

being submitted to reverse-phase chromatography. The acquired MS data were processed using a technique that prioritized the maximal entropy (MaxEnt) to obtain a deconvoluted spectrum [16].

2.2.2 Crystallization, data collection, and processing and refinement of FTL structure

Crystallization and data collection of Apo-frutalin has been previously described [8]. The molecular replacement was performed with the use of PHASER [17], using jacalin co-ordinates (PDB ID: 3P8S) as the initial model (98% identity with FTL). Co-crystallization experiments were performed using FTL crystals incubated with 5 mM D-galactose (Sigma–Aldrich®, purchased as a mixture of α/β-D-galactose) and 10% PEG 20 000, 50% (v/v) PEG MME 550, 0.1 M bicine/Trizma base (pH 8.5). Data collection was performed at 100 K up to 1.6 5 Å using a Rigaku MicroMax 007 HF equipped with RAXIS IV++ IP, to 1.81 Å (Apo-frutalin) and 1.65 Å resolution (FTL–D-galactose complex). The diffraction data were indexed, integrated and scaled using the XDS package [18]. We used jacalin co-ordinates for molecular replacement, which shares 98% sequence identity with FTL (PDB ID: 3P8S); both structures were refined using Phenix [19] and Coot [20]. R and Rfree were monitored to evaluate the validity of the refinement protocol, and the stereochemistry of the model was assessed using Molprobity [21]. The co-ordinates and structure factors have been deposited in the RCSB Protein Data Bank with the accession codes 4wog and 5bn6 for the Apo and FTL–D-galactose complex, respectively. The representative figures for both models were generated, and the two structures were validated through the validation server of the Protein Data Bank.

2.2.3 Molecular docking studies

AutoDock 4.2 was used to perform the molecular docking analysis [22]. The grid maps of 40 Å x 40 Å x 40 Å centered on the CBS of FTL (PDB ID: 4WOG) and α-D-Gal-(1 → 6)-D-Man) and β-D-Gal-(1 → 2)-α-D-Xyl-(1 → 6)-D-Glu residues mimicking, respectively, D-galactose found in galactomannan and xyloglucan polysaccharides, were calculated with AutoGrid. These molecules were modeled with Chem3D software and had their energies minimized to maintain the most stable structure. The saccharide substrates exhibited all of the torsional bonds with free rotation, while the protein was held rigid. The 10 best structures were analyzed and ranked according to the predicted binding affinity (expressed in kcal mol−1). Three-dimensional images of the interactions between ligands and proteins were depicted with the aid of Pymol [21].

2.2.4 Molecular dynamics

The structure without ligands (PDB ID: 4WOG) was submitted to the program H++ using the webserver (http://biophysics.cs.vt.edu/H++) [23] to obtain pK estimates for residues and define which groups would be ionized in the molecular dynamics (MD) simulation at pH 9.0 (tetramer). Histidine residues were protonated at the delta positions of His68 and His95 on the four β-chains. The Asp, Glu, Lys, and Arg residues were ionized and the N- and C-terminal chains of the α- and β-chains resulting in a net charge of −4.

MD were analyzed using the GROMACS-5.1.2 package [24]. The system was defined by a 10 854 nm cubic box, and the crystallographic co-ordinates of the protein and α-D-galactose were used to generate the topology and place the geometric center of the protein in the box center. The water molecules that accompanied the crystallographic structure were excluded, and 38645 SPC water molecules were added. Additionally, four Na+ ions were used to neutralize the system. The system consisted of 122 107 atoms.

The GROMOS53A6 force field was used to model proteins and ions, and a modified version of the GROMOS53A6 was used to model sugar molecules [25]. A cutoff radius (rc = 1.4 nm) was used to determine the interactions between unrelated atoms for both van der Waals and electrostatic interactions, and the PME method [26] was used to treat long-range interactions. The bond distances of hydrogen atoms were constrained with LINCS [27] in the case of proteins and SETTLE [28] for water. The integration timestep was 2 fs during MD runs, at 1 atm and 298 K.

Root-mean-square deviations (RMSDs) from the starting structure for the alpha carbon (Cα) atoms from αand β-chains were calculated during the 400 ns of the MD simulation. Contacts between the α- and β-chains were identified by the observation of percentage hydrogen bonds (HBs) between atoms of backbone (bb) and side-chain (sc) proteins formed during the MD (molecular dynamics) trajectory analysis. The geometric criterion for detecting HBs was used as proposed by IUPAC: X H^ ... Y, where the angle is >165° and the distance H……Y is <0.3 nm.

2.3 RESULTS AND DISCUSSION

In SDS–PAGE, the profile of an isolated FTL batch matched the typical jacalin-like mass pattern of two bands ∼12 and 15 kDa, corresponding to monomers with different

glycosylation levels, as previously reported [6]. Evidence based on FTL mass deconvoluted spectra suggests that the protein is naturally expressed as a mixture of isoforms [29]. Using a hemagglutination assay, we found FTL to be in a biologically active form and able to agglutinate human erythrocytes, which was preliminarily required before submitting the lectin to crystallization assays (Supplementary Figure S1).

2.3.1 X-ray diffraction

The best solution for both Apo-frutalin and FTL–D-galactose complexes was obtained in space group I2 with four monomers per asymmetric unit. The data collection statistics are summarized in Table 1. The final models were refined to an Rfactor of 0.163 and an Rfree of 0.203 for Apo-frutalin, and to an Rfactor of 0.166 and an Rfree of 0.200 for the FTL–D-galactose complex. In both cases, the asymmetric unit contains for monomers, with 153 residues in each (Figure 1A,B). The residues (96.78%) are in the most favored regions, and 1.21% are in the allowed regions of the Ramachandran plot.

Similar to jacalin, FTL is synthesized in vivo as an unusual preproprotein, which becomes two chains after co-translational and post-translational processing: an α-chain (133 amino acids) and a β-chain (20 amino acids). FTL also shows conserved consensus sequences, which suggests that three N-glycosylation sites may be present [6,8]. In contrast, mannose-specific lectins such as frutapin (also found in breadfruit seeds) consist of uncleaved protomers of ∼150 amino acid residues [15].

Table 1 - X-ray parameters for FTL structures
(Values in parentheses are for the outer resolution shell.)

	Apo-frutalin	FTL–galactose complex
PDB ID	4WOG	5BN6
X-ray source	Rigaku MicroMax 007HF	Rigaku MicroMax 007HF
Detector	Rigaku Raxis IV^{++}	Rigaku Raxis IV^{++}
Cell parameters (Å) a, b, c	76.17, 74.36, 118.98	75.93, 74.60, 119.04
Space group	I2	I2
Resolution (Å)	26.74–1.81 (1.85–1.81)	19.65–1.65 (1.65–1.68)
X-ray source	Rigaku MicroMax 007 HF	Rigaku MicroMax 007 HF

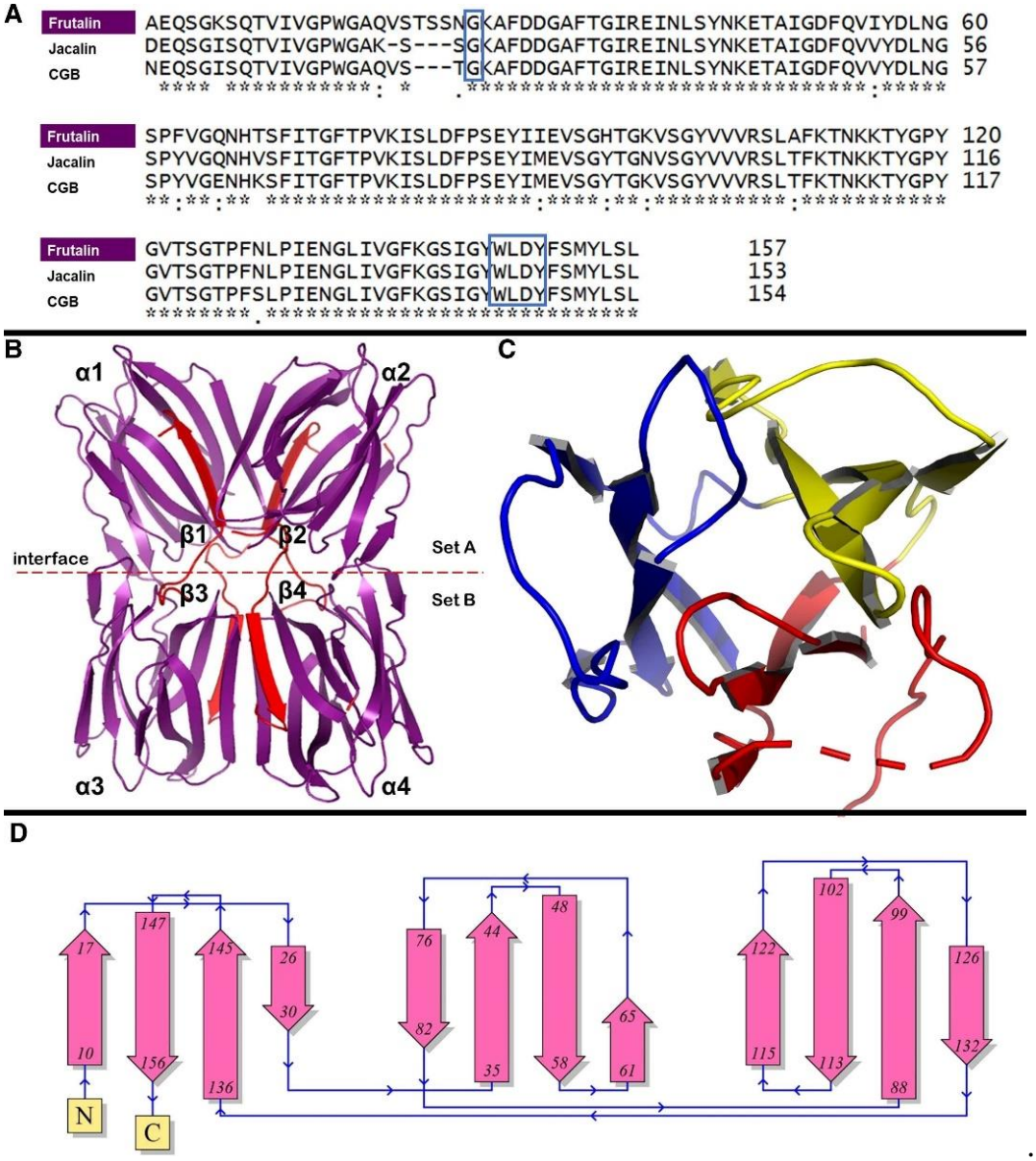
λ (Å)	1.5418	1.5418
Multiplicity	4.0 (3.7)	2.3 (1.5)
R_{merge} (%)	10.0 (53.3)	4.9 (44.6)
R_{pim} (%)	4.9 (27.1)	3.9 (39.5)
CC(1/2)	0. 996 (0.784)	0.998 (0.736)
Completeness (%)	99.8 (97.0)	97.3 (75.6)
Reflections	240 259 (12 493)	174 200 (4489)
Unique reflections	59 769 (3386)	77 162 (2939)
I/σ	10.3 (2.6)	13.7 (1.9)
Reflections used for refinement	59 762	77 151
Rfactor	0.163	0.166
R_{free}	0.203	0.200
No. of protein atoms	4629	4626
No. of ligand atoms	0	48
B ($Å^2$)	14.19	13.83
Co-ordinate error (ML based) (Å)	0.21	0.15
Phase error (°)	19.33	19.15
Ramachandran plot		
Favored (%)	96.59	96.59
Allowed (%)	3.41	3.41
Outliers (%)	0.00	0
All-atom clashscore	1.86	2.38
RMSD from ideal geometry		
r.m.s. bond lengths (Å)	0.007	0.006
r.m.s. bond angles (°)	1.017	1.038

The FTL structure showed a typical symmetric β-prism fold, which is found in jacalin and other lectins [30]. This β-prism is composed of three 4-stranded β-sheets forming three antiparallel Greek key motifs, generating an approximate 3-fold symmetry (Figure 1C,D). FTL shares high structural similarity with jacalin, CGB, and frutapin, which superposed well and

gave RMSDs ranging from 0.31 to 0.43 Å for the superposition of Cα atoms in these homologous structures (Supplementary Figure S2) [15,30,31].

Another major difference between the mannose-specific JRLs and galactose-specific JRLs is their biosynthesis, processing, and topogenesis. For example, jacalin is synthesized as a preproprotein, which undergoes a complex series of processing steps and is presumed to be located in the vacuolar compartment [32]. In contrast, the mature polypeptides of the mannose-specific JRLs correspond to the entire open reading frame of the respective lectin genes, presenting both α- and β-chains linked by a loop, and therefore are synthesized and located in the cytoplasm [32]. This alteration is believed to expose amino acids that are involved in carbohydrate recognition by FTL.

Figure 1**:** Predicted structural features of FTL



(A) Sequence alignment of FTL (PDB ID: 5BN6) with jacalin (PDB ID: 1UGW) and CGB (PDB ID: 4AKB). Alignment was performed using Clustal Omega. The expanded carbohydrate-binding site for each protein is boxed in blue. (B) Tetrameric structure of FTL with the α- (magenta) and β-chains (red). Interactions between α- and β-chains can be observed in α1–β1–α2– β2 chains (set A) and α3–β3–α4–β4 chains (set B). (C) Ribbon diagram of β-prism, each part of the β-prism, is colored differently to facilitate the understanding of this structure (red, blue, and yellow). Topology diagram of FTL generated by PDBSUM. (D) The FTL structure is composed of three Greek keys and could be classified as N- (N- and C-termini) and C-types.

Indeed, leguminous lectins have considerable conservation in their primary, secondary, and tertiary structures. Comparisons of these sequences and structures demonstrate that differences in carbohydrate specificity appear to occur due to changes in carbohydrate-binding site-adjacent amino acids. The conformation of these loops is determined by the presence of calcium or transition metal ions in the protein structure, which helps CBS orientation and affinity for ligands. Although structurally analogous, with some reaching up to 90% similarity, these lectins present several distinct biological activities [33].

2.3.2 Carbohydrate-binding site and molecular docking studies

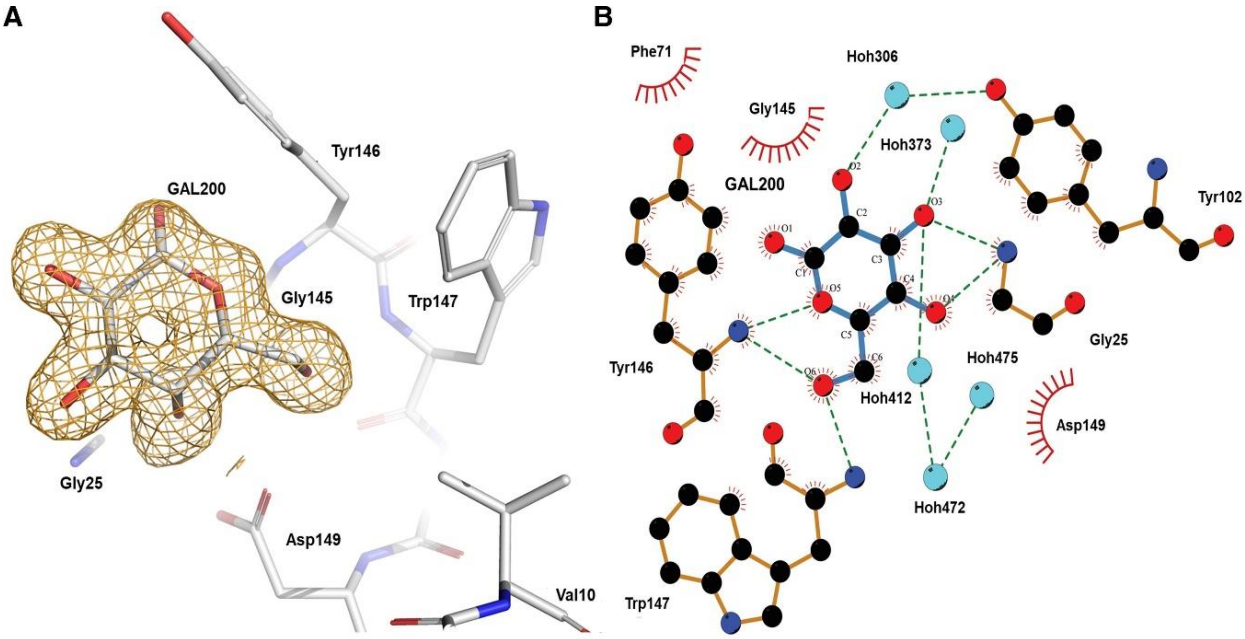
Although FTL recognizes a range of ligands, it has great affinity for α-D-galactose monosaccharides and complex carbohydrates that contain Galα1–3 glycans [6,9]. Galactose binding is dominated by hydrogen bonding with the sugar hydroxyl groups O1, O3, O5, and O6 (Figure 2A). Hydrogen bonding is the most dominant interaction in recognition of sugar molecules by the lectin CBS via carbonyl and hydroxyl groups (Figure 2B) of the backbone and side chains [34]. The crystal structures of the FTL–D-galactose complex also showed this pattern. The FTL–D-galactose complex does not present significant structural differences when compared with Apo-frutalin.

Overall, the FTL-binding site is similar to those in Moraceae lectins and consists of a domain close to the N-terminus of the α-chain consisting of four key residues: Gly25, Tyr146, Trp147, and Asp149 [35–37]. In the three-dimensional structure of the jacalin–α-D-galactose complex (PDB ID: 1KU8), eight hydrogen bonds form directly between CBS amino acids and the hydroxyls, especially those of C3 and C6 positions in D-galactose. Similarly, in the galactose-binding lectin from champedak fruit (CGB)–Gal complex, FTL–galactose binding occurs via many hydrogen bonds between the O atoms on the sugar ring and with side-chain and main-chain N and O atoms on the α-chain (O3 and Gly1 N, O4 and Gly1 N and Asp125 OD1, O6 and Trp123 O, Trp123 N and Tyr122 N, O5 and Tyr122 N) through O3, O4, and O5 [31]. In contrast, the FTL–D-galactose complex displays 10 interactions through the C1 hydroxyl to residue Tyr146, C3 hydroxyl to residue Gly25, C4 hydroxyl to residues Gly25 and Asp149, and C6 hydroxyl to residues Tyr146, Trp147, and Asp149 (Figure 3A).

Jacalin is among the most thoroughly studied lectins. Jeyaprakash et al. [38] identified three components of the jacalin sugar-binding site, a primary binding site and two secondary sites, named A and B. In this postulation, the primary site is responsible for binding galactose. The secondary site can bind any α-linked sugar moiety, but cannot tolerate any β-substitutions

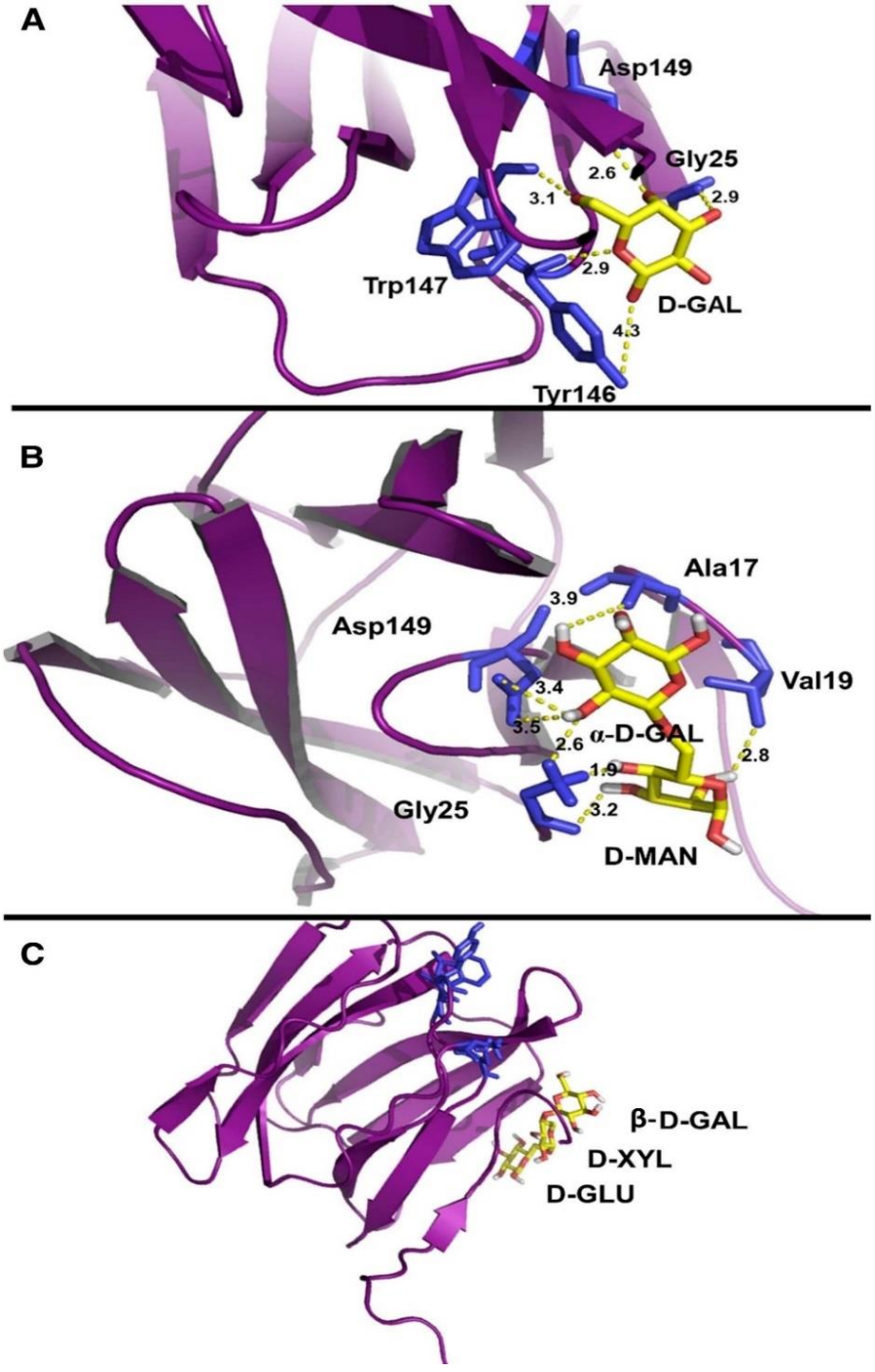
[39]. In addition, jacalin and CGB bind carbohydrates at one primary and two secondary binding sites [31,40]. The large number of interactions is consistent with FTL's affinity for galactose. The FTL agglutination of erythrocytes is due to its multi-subunit proteins, which contain multiple carbohydrate-binding sites that enable them to agglutinate cells [41]. These carbohydrate-binding domains are identical or very similar and bind a wide range of ligands. A comparison of the FTL CBS to other Moraceae lectins suggests that the site closely resembles that of jacalin. There are equivalent residues in both lectins (such as Asp149 in FTL and Asp125 in jacalin), and both form two hydrogen bonds: one with the C3 hydroxyl and another with the C4 hydroxyl. Based on these interactions, we suggest that FTL has an affinity for D-galactose epimers such as D-mannose, as the C4 hydroxyl in this sugar is equatorial instead of axial, and the interaction would also be allowed by Asp149.

Figure 2. FTL carbohytdrate-binding site with D-galactose.



(A) Composite omit map contoured at 1σ for a galactose molecule in the FTL structure. (B) Galactose-neighboring residues are labeled. Ligplus interaction drawing of galactose interactions in the FTL structure.

Figure 3. FTL CBS anchoring specific sugars.



(A) Close-up view of the FTL sugar-binding site in the D-galactose complex. Protein and carbohydrate molecules are depicted as ribbon and stick models, respectively. Amino acid residues, which interact with carbohydrates, are highlighted in blue. (B) Molecular docking experiments suggest that the FTL CBS can accommodate the α-D-Gal–D-Man disaccharide present in galactomannans, but not the β-D-Gal–Xyl–Glu trisaccharide present in xyloglucans (C), as evidenced through affinity chromatography in those cross-linked polysaccharide matrices.

Interactions between derivatives of Gal β-(1,3) Gal-α-OMe and jacalin have been structurally and thermodynamically characterized [39]. It is now known that distortion of the ligand occurs as a strategy for modulating affinity. β-Substituted methyl derivatives of disaccharides can also bind to jacalin without changing the pattern of interactions in the complexes involving the corresponding α-substituted derivatives. This is achieved through distortions of the ligand molecule at the anomeric carbon and the glycosidic linkage in addition to a small lateral shift. The higher internal energy caused by the distortion reduces the affinity of β-substituted b-(1,3)-linked disaccharides to jacalin when compared with the α-substituted variants [36,39,42]. Gal β-(1,3) Gal and its derivatives preferentially bind to jacalin with the reducing sugar at the primary binding site, although binding with the non-reducing Gal at the primary site is possible. α-Methyl substitution further strengthens binding in the first arrangement. In contrast, β-substitution weakens the binding due to ligand distortion. The β-substituted disaccharides continue to bind with the reducing Gal at the primary binding site, thus, indicating that the affinity reduction is not strong enough to overcome the intrinsic propensity of Gal β-(1,3) Gal to bind to jacalin with the reducing Gal at the primary site. This propensity is believed to be an important determinant in the biologically relevant interactions between jacalin and oligosaccharides [39].

Interestingly, native FTL was previously isolated in an affinity chromatography step using an *Adenanthera pavonina* cross-linked galactomannan [6]. In this type of polysaccharide, galactose is naturally α-linked (1-6-D-galactopyranose) to the β-1-4-D-mannopyranose backbone. On the other hand, in xyloglucan from Tamarindus indica seeds, these galactose branches are bound by β-(1-2) to xylosyl residues attached to the main glucan backbone. When using xyloglucan cross-linked matrices, we found FTL to always be easily removable from the column with PBS buffer during the washing steps. This result suggests that FTL has a lower or no affinity for β-galactose residues, implying an anomeric recognition. In looking for further evidence of this anomeric recognition, docking experiments were performed to check whether the FTL carbohydrate-binding site could accommodate the α-D-Gal-(1 → 6)-D-Man disaccharide and β-D-Gal-(1 → 2)-α-D-Xyl-(1 → 6)-D-Glu trisaccharide, which mimic the FTL interaction in galactomannan and xyloglucan cross-linked matrices, respectively. FTL interactions with those saccharides yielded 10 energetic clusters. Figure 3B,C shows the pivotal interface between the FTL–galactomannan residue (−4.9 kcal mol−1) and FTL–xyloglucan residue (−6.5 kcal mol−1), respectively. These results are consistent with affinity chromatography data in which xyloglucan matrices were inefficient at retaining FTL. In

contrast, accommodation of the α-D-Gal-(1 → 6)-D-Man disaccharide with Ala17 and Val19 residues was observed in FTL–galactomannan docking, in addition to those involved in the CBS. Thus, FTL has anomeric carbohydrate recognition.

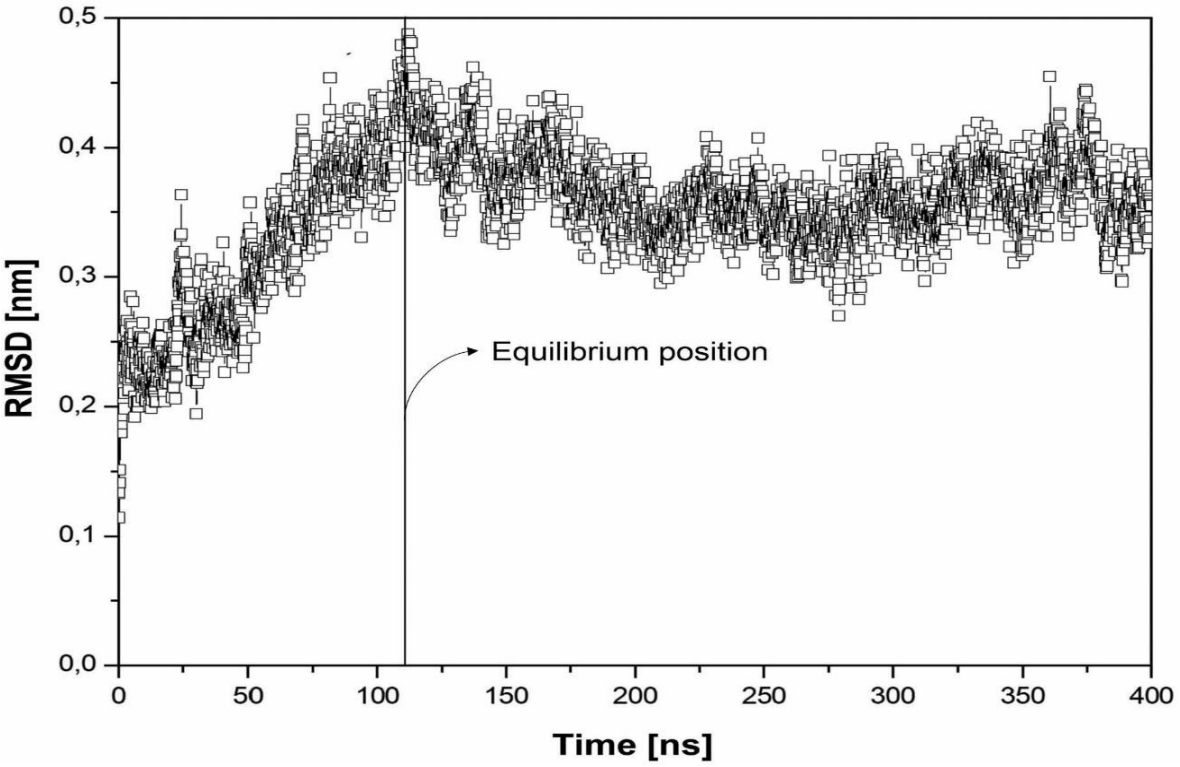
In addition, the tetrapeptide-linker 'T-S-N-N' is not a structural component of mature FTL, as it is excised during lectin processing to separate FTL β- and α-chains, giving rise to new N- and C-terminal sequences and reducing CBS rigidity, which results in the multiple-binding abilities of FTL, jacalin, and CGB [37]. The specificity of KM+ (Artocarpin), the mannose-binding lectin in jackfruit seeds, is attributed to the increased structural rigidity caused by the presence of the binding peptide GGPGGNGW. This peptide linker is not eliminated by post-translational processes, and is rich in glycine residues, thereby yielding extremely strong bonds between the two chains [43]. Therefore, we investigated MD to determine how this peptide linker may affect carbohydrate recognition by FTL.

2.3.3 Molecular dynamics

Simulations of the FTL structure with four α- and β-chains revealed an increasing RMSD with a remarkable peak ~110 ns and 0.5 nm (Figure 4). This observed behavior indicates structural relaxation, which might occur through hydration after 110 ns and therefore reach equilibrium. Structural properties were then analyzed considering the trajectory at the same time point (equilibrium position) for all α- and β-chains (as shown in RMSD profiles by MD time in Supplementary Figure S3A,B). Table 2 shows the mean RMSD for the interaction of tertiary FTL structures (α- and β-chains), β-chain moieties (RMSD-M and RMSD-E), and the sum of the four α- and β-chains. Higher averages were observed for single β-chains when compared with single α-chains, implying that β-chains are more flexible.

The RMSDs for α-chains varied between 0.14 and 0.16 nm, whereas β-chain RMSDs varied between 0.16 and 0.41 nm, suggesting that the positions of the α-chains have shorter distanced than the beginning of the β-chains until reaching the equilibrium position of the quaternary structure.

Figure 4. RMSD FTL during MD simulation.



RMSD from the α-carbon positions after finding maximal overlap between α-carbons of the initial structure and structures collected during the MD simulation in all four FTL α- and β-chains, with its peak within 110 ns and 0.5 nm.

Table 2 - The mean RMSD ± SD obtained in the last 300 ns of the MD simulation

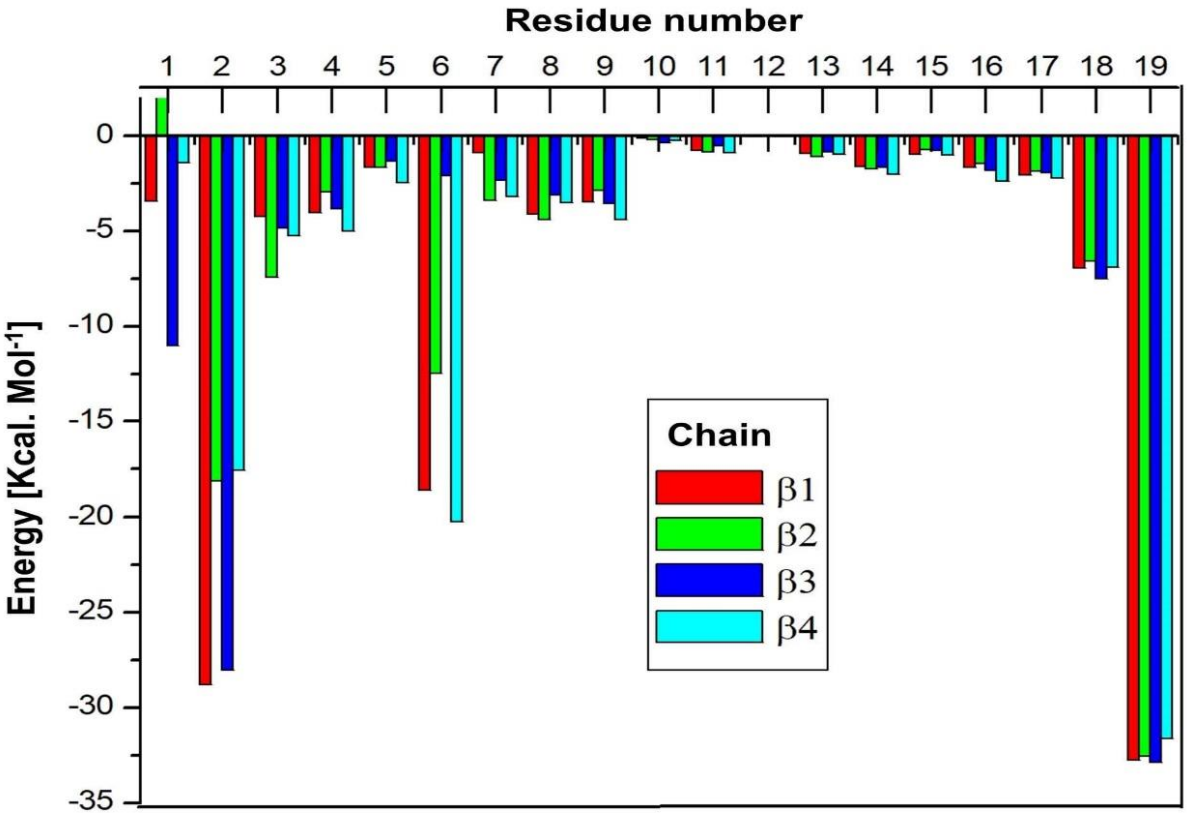
ID	RMSD (nm)	RMSD-M	RMSD-E
β1	0.26 ± 0.05	0.07 ± 0.02	0.32 ± 0.11
β2	0.16 ± 0.05	0.08 ± 0.02	0.39 ± 0.08
β3	0.41 ± 0.04	0.07 ± 0.02	0.38 ± 0.14
β4	0.28 ± 0.04	0.09 ± 0.02	0.60 ± 0.09
β1+ β2 + β3 + β4	0.37 ± 0.02		
α1	0.15 ± 0.01		
α2	0.16 ± 0.01		
α3	0.16 ± 0.03		
α4	0.14 ± 0.02		
α1 + α2 + α3 + α4	0,36 ± 0.03		
All β- and α-chains	0.35 ± 0.05		

The RMSD-M includes the superimposing step and calculating the RMSD between the Cα of residues in the 7–17 range. In the RMSD-E, Cα atoms between residues 7 and 17 are used for the superimposing step, and the deviation calculation is between the atoms of residues near the N- and C-termini of the β-chain from 1 to 6 and 18 to 19.

Similarly, the RMSDs were higher for the β-chains (range 0.04–0.05 nm), suggesting that after reaching the equilibrium position, β-chain positions vary more than α (range 0.01–0.03). These variations can be viewed and compared between the RMSD profiles in Supplementary Figure S3A,B. Therefore, even though β-chains move more than α, they are maintained in the structure, and the chain set stabilizes the octameric structure of FTL. Taking into account the relative positions of the four α- and β-chains in the quaternary structure, the RMSDs of (α1 + α2 + α3 + α4) 0.36 ± 0.03 nm and (β1 + β2 + β3 + β4) 0.37 ± 0.02 nm presented similar variations in the means and SD, suggesting that positions of the eight chains supporting the quaternary structure are preserved in equilibrium (Table 2). The RMSD-E and standard deviation were much larger than for the overall side-chain (RMSD). The high RMSD-E values suggest that the residues placed in these regions do not establish effective interchain contacts

with the α-chains, which remain free to move. However, the RMSD-M for the 7–17 region happens to be smaller than the RMSD and RMSD-E, which indicates increased rigidity in this regions.

Figure 5. PII between FTL residues and water molecules.



The distribution of IIP between the residues of the four β-chains and the water molecules within a 0.5 nm cutoff radius.

Table 3 - Percentage of HBs observed (% OBS) between amino backbone (N–H) and carbonyl groups (C=O) for α- and β-chain residues

Chain	α1		α2		α3		α4	
Residue	N–H	C=O	N–H	C=O	N–H	C=O	N–H	C=O
Gly5				Ser156* β3 (47)				
Lys6					Thr10 β2 (45)			
Ser7		Thr34 β4 (95)					Ser156* β1 (23)	
Gln8		Leu157^{CT} β4 (39)	Leu157^{CT} β3 (96)				Leu157^{CT} β1 (31)	
Val10	Leu155 β1 (59)	Leu155 β1 (100)	Leu155 β2 (60)	Leu155 β2 (100)		Leu155 β3 (99)	Leu155 β4 (96)	Leu155 β4 (99)
Ile11	Asn134 β2 (89)	Glu133 β2 (55) Asn134 β2 (93)	Asn134 β1 (85)	Glu133 β1 (74) Asn134 β1 (86)	Asn134 β4 (98)	Glu133 β4 (52) Asn134 β4 (89)	Asn134 β3 (96)	Glu133 β3 (78) Asn134 β3 (86)
Val12	Met153 β1 (99)	Met153 β1 (99)	Met153 β2 (99)	Met153 β2 (99)	Met153 β3 (99)	Met153 β3 (99)	Met153 β4 (99)	Met153 β4 (99)
Gly13	Pro131 β2 (87)		Pro 131 β1 (86)		Pro131 β4 (89)		Pro131 β3 (83)	Phe 151 β4 (96)
Trp15	Phe151 β1 (98)	Phe151 β1 (99)	Phe151 β2 (98)	Phe151 β2 (99)	Phe151 β3 (98)	Phe151 β3 (99)	Phe151 β4 (98)	
Ala17	Asp149 β1 (73)		Asp149 β2 (88)		Asp149 β3 (90)		Asp149 β4 (67)	

*HB is formed between the backbone and α-chain side-chain groups.

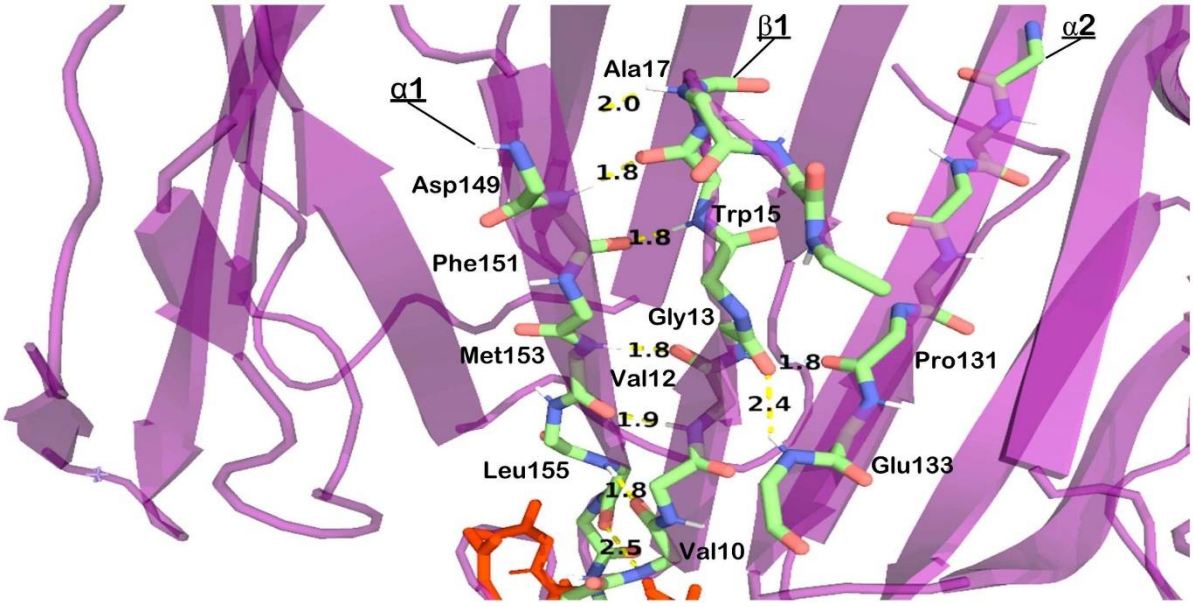
Blank locations represent the absence of HB.

The residue name and position is represented by Res in the α1–4 chains, which make intermolecular HBs with residues of the β1–4 chains. Only the HBs between the amino backbone (NH) and carbonyl (C=O) groups with % OBS > 25 are shown.

Figure 5 shows the distribution of the intermolecular interaction potential (IIP) between the residues, building up the four β-chains and water molecules within a 0.5 nm cutoff radius. It is evident that regions from 1 to 6 and 18 to 19 are hydrated (energy less than −5 kcal mol−1), which is in contrast with those from 7 to 17. The IIP results combined with the RMSD-E values found in the simulations allow us to conclude that the β-chain region from 7 to 17 residues increases the rigidity of the referred chain by interacting with the α- and β-chain residues. These interactions reflect the intermolecular HBs between groups of α- and β-chains, as can be seen in Table 3. We expected to find interactions among these α- and β-chains by the breakdown in FTL structure, which are mostly by α1–α2 and α3–α4 pairs. However, the majority of pivotal HBs were negligible. β-Chains appear to be useful in maintaining the structure, and they occur close to the amino acids from 10 to 18 in α-chains and interact through HB between the backbone amino and carbonyl groups (C=O……H–N) of both α-chains (Table 3). Figure 6 shows a representation of these connections formed by HBs between pairs of αand β-chains. The β1 and β2 chains connect the α1 and α2 chains, while the β3 and β4 connect the α1 and α2 chains via intermolecular HBs.

The amino and carbonyl groups from 10, 12, 15, and 17 β-chain residues form HBs with amino and carbonyl groups of one α-chain, while residues 11 and 13 form HBs with groups of another.

Figure 6. Interface between 03B1; and 03B2; chains of FTL connected by hydrogen bonds.



Carbonyl and amine group HBs (C=O……H–N) between α1, β1-chains from monomer 1 and the α2-chain of monomer 2 are depicted in the tetrameric FTL structure (magenta). The interactions occur on two faces of the lectin and involve both α- and β-chains from monomers 1 and 2, resulting in an α2–β2–α1 cluster. Likewise, the connections are prone to occur in α3–β3–α4 and α4–β4–α3 interchain clusters.

Thus, the HBs establish β1–α1, β2–α2, β3–α3, and β4–α4 pairs; while for residues 11 and 13, the pairs are β1–α2, β2–α1, β3– α4, and β4–α3. Indeed, the α-chain segment from 10 to 19 residues (VIVGPWGAQ), with hydrophobic side chains, helps to maintain these HBs by keeping the water molecules away from the amino and carbonyl groups.

The stability of the entire FTL structure can be understood when we consider interaction sets between αand β-chains, which occur as α1–β1–α2–β2 (set A) and α3–β3–α4–β4 chains (set B) (Figure 1B). Sets A and B form an interface maintained through interactions between the C-terminal regions of the β-chains (residues 1– 8) and complementary regions of the α-chains in set B. These interactions result in the formation of HBs between α1–β4, α2–β3, α3–β2, and α4–β1 (mainly by the carboxylic acid of the C-terminal Leu157 in α-chains and amine of Gln8 in β-chains) (Table 3).

Additionally, Asp149 in the α-chain is stabilized in the carbohydrate-binding site by HBs between the amine and carbonyl groups of Ala17 in the β-chain (Figure 1D and Table 3). The occurrence of HB is greater than 67% in all α–β pair interactions. Furthermore, Ala17 and Val19 promote a hydrophobic environment by enhancing interchain HBs and preventing the α-D-Gal-(1 → 6)-D-Man disaccharide hydration, which helps its accommodation into the FTL CBS. Meanwhile, Ala17 appears to play a structural role, helping to stabilize Asp149, with Val19 oscillating in its position, as shown by the RMSD-E (Table 2). The MD results validate the functional importance of the β-chains in maintaining the FTL structure. In addition, Ala17 in the β-chains helps to stabilize the Asp149 position in the α-chains through HB formation, which corroborates Asp149 functioning as the key residue for interactions and carbohydrate-binding. Indeed, Asp149 allows entry of α-D-Gal-(1 → 6)-D-Man disaccharide to the CBS site and its subsequent stabilization.

In regard to this carbohydrate recognition, FTL has been evaluated in several biomedical applications and found to establish hydrogen bonds with the glycosylated fraction of the TRPV1 ion channel, inhibit the orofacial pain mechanism [13], and specifically recognize the NMDA receptor in its glycosylated fraction, interfering in a cellular mechanism and producing antidepressant-like NMDA receptor-mediated activity [44].

2.4 CONCLUSION

Over the years, the therapeutic relevance of FTL has been demonstrated in many biomedical mechanisms. However, little was known about FTL's structure. Taken together, the results in this work provide a new perspective for further elucidation of the functional properties of this lectin. While FTL is conserved across species, which explains the high similarity to other lectins in the Moraceae family, some plants have developed unique and specialized mechanisms to deal with their complex intracellular signaling pathways, mostly triggered by the intricate relationships between the carbohydrate-binding specificity of lectins. Thus, a better understanding of FTL binding to sugar moieties provides insights into its functionality and sheds light on the biological activities of this lectin.

ABBREVIATIONS

CBS, carbohydrate-binding site; CGB, champedak galactose-binding lectin; FTL, frutalin; HB, hydrogen bond; IIP, intermolecular interaction potential; JRL, jacalin-related lectins; KM+, Artocarpin; MCA, minimal concentration for agglutination; MD, molecular dynamics; PME, particle mesh ewald; RMSD, root-mean-square deviation; UPLC-ESI, ultra-performance liquid chromatography with electrospray ionization.

AUTHOR CONTRIBUTION

A.E.V.N., T.B.G., and F.D.S. were involved in all aspects of purifying the protein, while H.M.P. and F.B.M.B.M determined the X-ray structure. M.R.L. performed the MD experiments. A.C.O.M.M. and R.A.M. obtained funding for the study and provided overall supervision. A.E.V.N and F.D.S. wrote the initial drafts of the paper and all authors contributed to the final manuscript.

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COMPETING INTERESTS

The Authors declare that there are no competing interests associated with the manuscript

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3 CAPÍTULO II

Running title: Frutalin Recognizes Complement C3 Over-Expressed by Neoplasms

Frutalin Recognizes Complement C3 Over-Expressed by Neoplasms

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ABSTRACT

Frutalin (FTL) is an α-D-galactose-binding lectin obtained from the seeds of *Artocarpus incisa* L. It has presented several biomedical activities based on the recognition of the carbohydrate binding site. Bioinformatics techniques have sought to elucidate the molecular bases of interaction with biological receptors. Previous studies have demonstrated that Frutalin, in addition to promoting biological activities, is able to recognize differentially expressed glycoproteins in the blood in patients with neoplasms such as breast cancer and acute lymphoblastic leukemia (ALL). The aim of this work was to elucidate the molecular bases of interaction of Frutalin with Complement C3 Protein, a glycoprotein over-expressed in serum of patients with ALL and breast cancer. The possible interaction between FTL with the surface of the complement protein C3 was analyzed using molecular docking, with the Hex 8.0.0 platform. The 10 most energetic clusters were investigated for analysis of interaction energy, binding specificity, CRS-glycoprotein affinity, the amino acids involved and the attraction/repulsion forces. Molecular docking demonstrated high affinity to between FTL and C3 glycans, with high specificity and complexation energy, with 08 hydrogen bonds measuring up to 0.8 angstroms, with high reproducibility. It can be concluded that FTL is a lectin capable of promoting biological and biomedical activities due to the flexibility of its CRS, which allows

the recognition of complex glycans present in glycoprotein's involved in biological phenomena, which makes it a relevant biotechnological tool.

3.1 INTRODUCTION

3.1.1 Biomarkers in cancer research

Biomarkers are increasingly useful tools for cancer research. The search for new, specific and sensitive biomarkers continually aims to discover improvements in the diagnosis and confirmation of the neoplasms or that may have practical value in the evaluation of the treatment and in the behavior of the disease [1].

Acute lymphoblastic leukemia of Precursor Cells B (ALL-B) and Breast Cancer are examples of diseases that occur in worrying proportions and have to be more studied. Precursor Cell Lymphoblastic Leukemia-Lymphoma is the most common malignancy in childhood and breast cancer and the most common cancer among women worldwide after non-melanoma skin cancer. Featuring clinically and biologically heterogeneous diseases, early detection, reliable characterization, and accurate diagnosis are critical for reducing mortality [2].

3.1.2 Frutalin as biomedical tool

This work investigates the interaction of Complement C3, which is a differentially-expressed glycoprotein with changes in its glycosylation patterns in both diseases, with Frutalin (FTL), that is a plant lectin with affinity for α-D-galactose and α-D-mannose, obtained from the seeds of *Artocarpus incisa* L. FTL is a glycoprotein with approximately 66 kDa, which has a CBS in each monomer of its homotetramer. It is able to agglutinate erythrocytes since it is oligomerized under physiological conditions and promotes glycans recognition in two or more CBS simultaneously, agglutinating the red blood cells [3]. The three-dimensional structure of Frutalin has been elucidated by crystallographic methods [4] and has already shown to be compatible with other biological components [5,6]. FTL proved to be efficient in detecting the overexpression of Complement C3, which suggests an association with the development and characterization of cancer in LLA and breast cancer situations [2,7]. The aim of this study was elucidating the molecular bases of interaction of FTL with the C3 protein, in the recognition observed in previous studies.

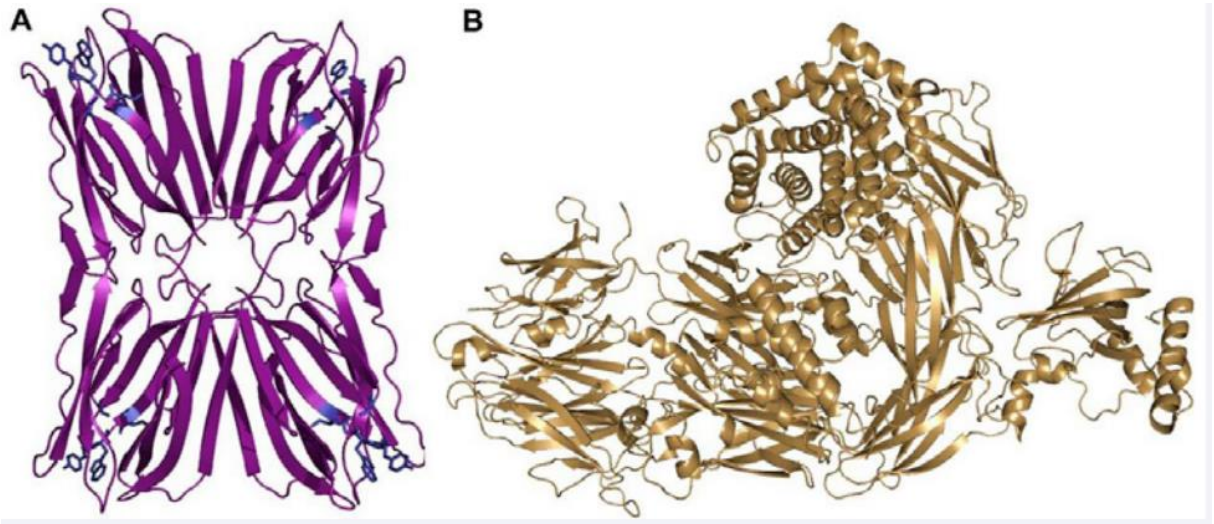
3.2 MATERIALS AND METHODS

3.2.1 Molecular docking

The interaction between Complement C3 and Frutalin (FTL) was analyzed using molecular docking. FTL and Complement C3 structures were available in the Protein Data Bank (codes 4WOG and 2A73, respectively).

Frutalin and C3 structures (Figure 1) were solved by crystallographic methods [4,8] and have high resolution (1.6 and 3.3 angstroms, respectively). The C3 structure had its ligands removed manually, hydrogens and charges were added in both molecules, prior to perform molecular docking, and all chains were maintained in order to simulate the real interaction in physiological conditions (Figure 1).

Figure 1: Frutalin structure (purple) with one carbohydrate recognition site (blue) in each monomer (A); Complement C3 structure (B).



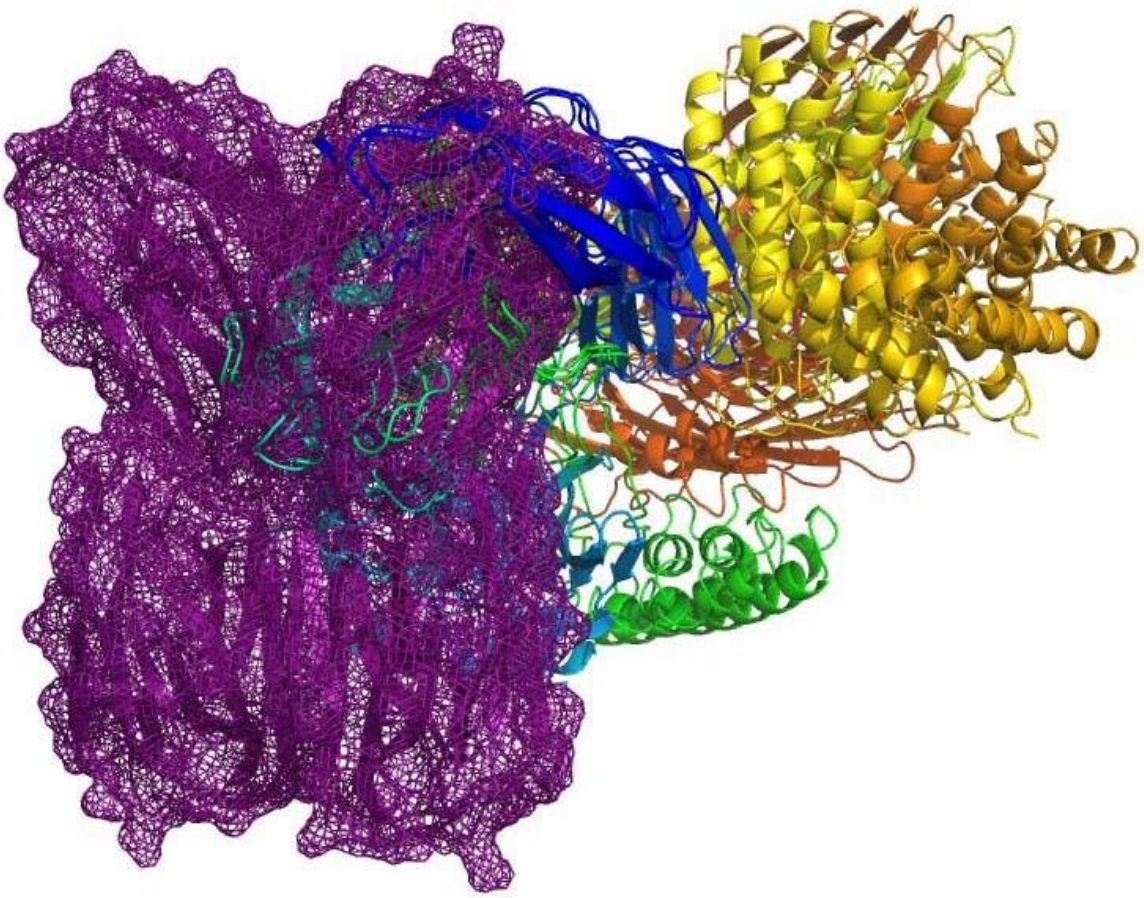
The HEX software was used for molecular docking calculations [9], as it is able to predict interactions large molecules in intermediate processors (academic), besides having a platform compatible with didactic purposes. The parameters applied for the docking process were: Correlation type - Shape only; Calculation Device – GPU (graphic process units); FFT Mode (fast Fourier transform) - 3D fast life; Grid Dimension - 0.6; Receptor range - 180; Ligand Range - 180; Twist range - 360 and Distance range – 40; Solutions - 50000. Three-dimensional images of the interactions between ligands and the proteins were depicted using the PyMol software [10].

3.3 RESULTS AND DISCUSSION

Molecular docking showed the interaction of FTL (receptor molecule) with Complement C3, after 50,000 possible fittings. In in vitro proteomic investigations serum samples from patients with cancer were applied in chromatography column to interact with the FTL immobilized matrix [2,7].

These results showed that the C3 molecule interacts with matrix, being considered a ligand while the FTL was considered receptor. The molecular bases of this interaction were elucidated through computational simulation. The 10 most stable clusters of this interaction were analyzed and initially presented high specificity, since they were overlapped in the same place, which indicates good energy stabilization of the formed complex, in that position (Figure 2).

Figure 2: Overlapping of the 3 most energetic clusters (colored) of C3, which suggests specific affinity for the FTL surface (purple) surrounding the CBS.



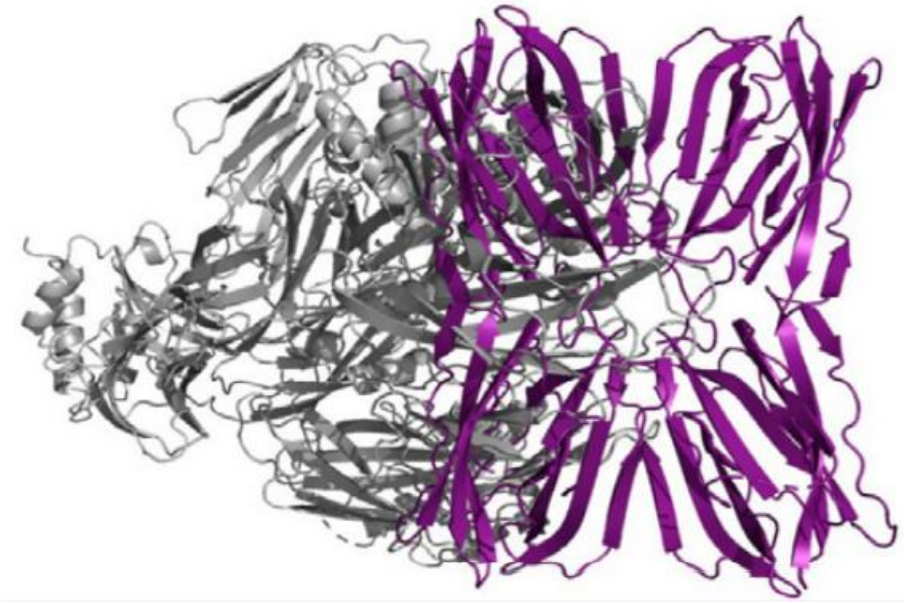
A protein has an intrinsic vibratory energy and when it binds with another molecule; its energy is stabilized by the complexation, which was also evidenced with the connection to Complement C3. The fit provided an energy stabilization of up to -26767.43 kcal/mol and proved to be efficient in all 10 most energetic clusters (Table 1). A similar result has been as have been found in other studies concerning the molecular interactions of the FTL in biomedical activities [5,6] (Table 1).

Table 1: Total energy of the interactions between FTL (receptor) and Complement C3 (ligand), after molecular docking simulation.

Complex obtained by FTL-C3 interaction	**Total energy obtained (kcal/mol)**
Cluster 01:	-26767.43
Cluster 02:	-26556.11
Cluster 03:	-25255.56
Cluster 04:	-24777.30
Cluster 05:	-24430.97
Cluster 06:	-23427.78
Cluster 07:	-22620.34
Cluster 08:	-22402.28
Cluster 09:	-22321.76
Cluster 10:	-21322.84

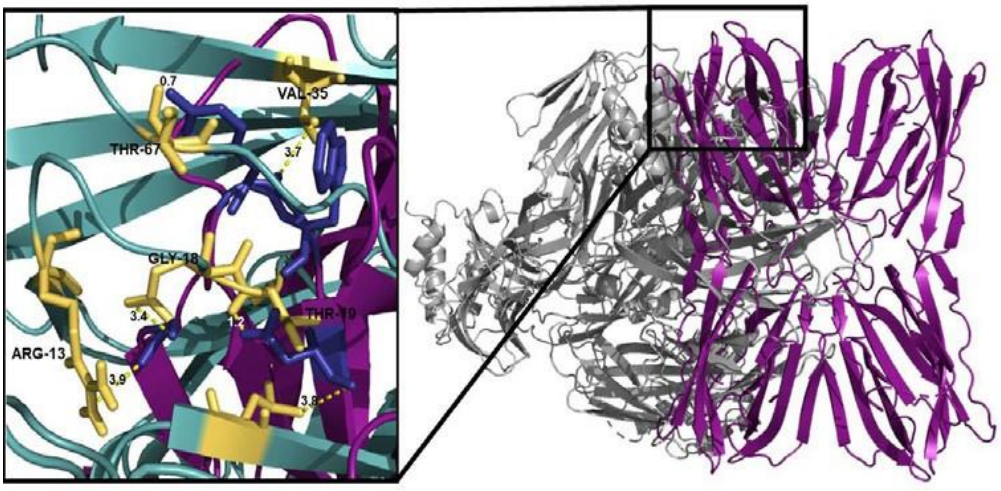
The high complexing energies observed in the 10 analyzed clusters indicate that there is high affinity of CBS for the ligand, and regarding the role of CBS in this recognition, it was important to investigate the surface of FTL and the location of its CBS in each monomer. In fact, C3 is a large glycoprotein and interact with FTL with the involvement of two monomers. In general, plant lectins have their lectin activity measured by the ability to agglutinate erythrocytes, due to their ability to recognize glycans, agglutinating them. This agglutination occurs when the lectin binds to two or more erythrocytes, which recruits more than one CBS [11]. Based on this, FTL recognizes complement C3 with two monomers simultaneously. This interaction increases the stabilization and neutralizes the repulsions and the hydrophobic effects of the region that does not belong to the lectin activity, in Frutalin (Figure 3).

Figure 3: Interaction of Complement C3 (gray) with FTL (purple), evidencing lectin recognition activity, interacting with two tetramer subunits simultaneously.



According to previously information about FTL amino acid residues [6] that promote lectin activity (Gly25, Tyr146, Trp147 and Asp149), the interaction of these residues with the amino acids of Complement C3 was measured. As a result, was observed the action of 6 amino acid residues (please specify these 6 residues), which made bonds of up to 0.8 angstroms and could stabilize the region and promote the binding between the two molecules (Figure 4). The results corroborate with in vitro findings [2,7] (Figure 4).

Figure 4: Carbohydrate recognition site of FTL (blue) showing hydrogen bonds with 6 amino acid residues (yellow) of Complement C3 (0.8 – 3.9 angstroms).



In order to assess the affinity of the FTL for glycoprotein's, oligosaccharides and glycans with higher molecular size, it can be suggested that the size and flexibility of the CBS promotes greater recognition by stabilizing in the middle of so many bonds. Therefore, the binding energy against a monosaccharide is much lower than that observed in biological activities already reported [12], in which FTL showed a high recognition activity in silico. It is known that plant lectins have been studied because they have specificity and affinity to carbohydrates in several biological processes [13]. Based on this, this work strengthens the suggestion of investigating plant lectins with high specificity to glycans characteristic of specific diseases, seeking biotechnological potential.

3.4 CONCLUSIONS

The molecular docking results reflect a specific interaction between Frutalin and Complement C3 protein over expressed in some neoplasms added to its specificity, stability in physiological conditions and its affinity to glycans make it a useful tool in research concerning cancer and biomarkers. FTL is a plant lectin capable of promoting biomedical activities due to the flexibility of its CBS, which allows the recognition of glycoproteins involved in ALL and breast cancer.

ABBREVIATIONS

ALL: Acute Lymphoblastic Leukemia; BC: Breast Cancer; CBS: Carbohydrate Binding Site; FFT: Fast Fourier Transform; FTL: Frutalin

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4. CONSIDERAÇÕES FINAIS

O aprofundamento no estudo estrutural da Frutalina em associação ao uso dos recursos de simulação computacional foi fundamental para a determinação dos detalhes e "insights" estruturais deste trabalho. No capítulo I os dados foram esclarecedores em relação às interações que promovem estabilidade à estrutura, estabilizando-a, além da ação do CBS no reconhecimento anomérico específico e na flexibilidade do sítio, permitindo as ligações químicas observadas no docking molecular. As simulações foram eficientes na elucidação das bases moleculares de interação da Frutalina com os ligantes, responsáveis pelas suas inúmeras atividade biotecnológicas e isto também se deu devido à resolução da estrutura. Os cristais de Frutalina foram de excelente qualidade, alcançando padrões de difração com alta resolução (~1,6 Å), originando dois depósitos de coordenadas moleculares no Protein Data Bank (PDB): 4WOG e 5BN6, referentes a Apo-FTL e o complexo FTL–D-Galactose, respectivamente. Esta melhor compreensão das ligações entre FTL e ligantes específicos fornece insights sobre sua funcionalidade e lança um melhor esclarecimento sobre as atividades biológicas desta lectina.

No Capítulo II o conhecimento prévio da estrutura permitiu a aplicação da FTL em uma interação in silico com base num fenômeno observado por outros autores: a capacidade da FTL se ligar a glicoproteínas específicas (contendo glicanos de α-D-galactose) presentes no soro sanguíneo em pacientes com neoplasias (leucemia linfoide aguda e câncer-de-mama). Usando a proteína Complement-C3 como objeto de estudo, devido sua incidência e super-expressão em mais de uma doença, foi evidenciada a afinidade da FTL por ligantes maiores, que promovem algumas interações inespecíficas e mesmo assim se ligam ao CBS da Frutalina, recrutando todos os aminoácidos do sítio com ligações químicas eficientes.

Os dados aqui coletados também fomentaram outras pesquisas no âmbito da farmacologia, com abordagem semelhante ao capítulo II, porém com viés farmacológico. Destas pesquisas, foram gerados dois artigos complementares:

- Frutalin reduces acute and neuropathic nociceptive behaviours in rodent models of orofacial pain. Chemico-Biological Interactions (DAMASCENO, et al., 2016).

Neste trabalho, nosso grupo conseguiu promover e elucidar a interação da Frutalina com o canal proteico TRPV1 (PDB ID: 3J5P), responsável pela passagem de íons pela membrana celular, uma proteína que faz parte do mecanismo bioquímico associado à dor. Nestes ensaios in vivo e in silico, a FTL bloqueou a passagem de íons, ao se associar à glicanos do canal

TRPV1, e essa interação se deu, principalmente, pela ação dos resíduos de aminoácidos do CBS da FTL.

- Neuropharmacological characterization of frutalin in mice: Evidence of an antidepressant-like effect mediated by the NMDA receptor/NO/cGMP pathway. International Journal of Biological Macromolecules (ARAÚJO, et al., 2018).

Neste segundo artigo, o grupo conseguiu mostrar o envolvimento da FTL com duas glicoproteínas presentes no efeito depressivo em roedores: o receptor proteico NMDA (PDB ID: 5I57) e a enzima NOS (1ZVI). Ao observar o encaixe in silico, foi demonstrado que a ação dos resíduos de aminoácido do CBS da FTL foi fundamental na estabilização estrutural destas duas proteínas, promovendo efeito antidepressivo nos animais.

Os artigos complementares a este trabalho e que foram publicados, foram anexados ao material suplementar desta Tese, a fim de promover ao leitor uma visão complementar e multidisciplinar dos dados bioquímicos gerados neste trabalho, inserindo a molécula vegetal (Frutalina) ao contexto farmacológico, o que amplia ainda mais o potencial biotecnológico da frutalina.

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ANEXO A – ARTIGO COMPLEMENTAR EM EXPERIMENTAÇÃO ANIMAL

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Frutalin reduces acute and neuropathic nociceptive behaviours in rodent models of orofacial pain

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ABSTRACT

Orofacial pain is a highly prevalent clinical condition, yet difficult to control effectively with available drugs. Much attention is currently focused on the anti-inflammatory and antinociceptive properties of lectins. The purpose of this study was to evaluate the antinociceptive effect of frutalin (FTL) using rodent models of inflammatory and neuropathic orofacial pain. Acute pain was induced by formalin, glutamate or capsaicin (orofacial model) and hypertonic saline (corneal model). In one experiment, animals were pretreated with L-NAME and naloxone to investigate the mechanism of antinociception. The involvement of the lectin domain in the antinociceptive effect of FTL was verified by allowing the lectin to bind to its specific ligand. In another experiment, animals pretreated with FTL or saline were submitted to the temporomandibular joint formalin test. In yet another, animals were submitted to infraorbital nerve transection to induce chronic pain, followed by induction of thermal hypersensitivity using acetone. Motor activity was evaluated with the rotarod test. A molecular docking was performed using the TRPV1 channel. Pretreatment with FTL significantly reduced nociceptive behaviour associated with acute and neuropathic pain, especially at 0.5 mg/kg. Antinociception was effectively inhibited by L-NAME and D-galactose. In line with *in vivo* experiments, docking studies indicated that FTL may interact with TRPV1. Our results confirm the potential pharmacological relevance of FTL as an inhibitor of orofacial nociception in acute and chronic pain mediated by TRPA1, TRPV1 and TRPM8 receptor.

1. Introduction

Orofacial pain is any pain associated with the soft or mineralized tissues (skin, vessels, bone, teeth, glands, muscles) of the oral cavity and the face [11,39]. In some cases, it can cause intense distress and compromise occupational performance, thereby producing negative impacts on the economy and on public health spending [7,38,45]. More research is necessary to understand the mechanisms involved and to develop new forms of treatment for patients with this condition [21].

Orofacial tissues are innervated by the three main branches (mandibular, maxillary and ophthalmic) of the trigeminal system. As with other tissues in the body, Aδ and C fibers transmit nociceptive signals from the orofacial region to the CNS [40].

Orofacial pain may be controlled with a range of drugs, including NSAIDs, muscle relaxants, opioids and antidepressants, but prolonged treatment is associated with considerable toxicity and adverse effects [20]. However, significant advances have been made over the past few years, spurred by the urgent need for safer and more efficient drugs [10].

Lectins are a heterogeneous group of proteins classified according to their ability to bind reversibly to simple sugars or complex carbohydrates. Due to their role as information mediators in biological systems, interacting with glycoproteins, glycolipids and oligosaccharides, lectins are widely used in research models evaluating protein/carbohydrate interactions and as tools in carbohydrate analysis [44].

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ANEXO B – ARTIGO COMPLEMENTAR EM FARMACOLOGIA

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Neuropharmacological characterization of frutalin in mice: Evidence of an antidepressant-like effect mediated by the NMDA receptor/NO/cGMP pathway

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ABSTRACT

In this study we evaluated the effect of frutalin (FTL) on mouse behavior. Mice (n = 6/group) were treated (i.p.) with FTL (0.25; 0.5 or 1 mg/kg) or vehicle and submitted to several tests (hole-board/HBT, elevated plus maze/PMT, open field/OFT, tail suspension/TST, or forced swimming/FST). Yohimbine, ketamine, L-NAME, aminoguanidine, 7-NI, methylene blue, L-arginine or DL-serine was administered 30 min before FTL (0.5 mg/kg). To evaluate the subchronic effect, animals were injected with FTL or vehicle for 7 days and submitted to the FST. Molecular docking was simulated using FTL against NOS and the NMDA receptor. No changes were observed in the HBT or the OFT. FTL (0.25 mg/kg) increased the number of entries into enclosed arms in the PMT. FTL reduced immobility in the TST (0.25 and 0.5 mg/kg) and the FST (0.25 mg/kg; 0.5 mg/kg). The effect of FTL was dependent on carbohydrate interaction and protein structure integrity and was reduced by ketamine, L-NAME, aminoguanidine, 7-NI and methylene blue, but not by L-arginine, yohimbine or DL-serine. The antidepressant-like effect remained after subchronic treatment. The molecular docking study revealed a strong interaction between FTL and NOS and NMDA. FTL was found to have an antidepressant-like effect mediated by the NMDA receptor/NO/cGMP pathway.

1. Introduction

Mental disorders affect around 700 million people worldwide, representing 13% of all disease [1]. Depression and anxiety are among the most prevalent forms. The former is characterized by changes in appetite/weight, sleep, psychomotor activity and by feelings of guilt, lack of concentration, recurrent thoughts of death, suicide ideation and suicide attempts [2]. The latter is characterized by feelings of tension or fear and by increased nervous system activity associated with tremors, shortness of breath, accelerated heart beats, sweating, gastrointestinal changes, dizziness, fainting and other symptoms [3].

Despite the existence of several classes of antidepressants and anxiolytics on the market, pharmacological treatment is often unsatisfactory [4,5]. For example, many antidepressants require 2–4 weeks (or even more) to produce clinically significant improvement of symptoms. This lag can be a serious problem for patients with suicide ideation (15% of depressed individuals commit suicide) [6,7].

Much effort is therefore made to develop antidepressants and anxiolytics with faster onset of action and greater efficacy [8]. In addition, more studies are needed on the role of the glutamate system and the L-arginine-NO pathway in the pathogenesis of mental disorders [7,9–11].

A number of active substances, including proteins isolated from plant sources, are currently being evaluated as tools for the study of neurotransmission. Some have revealed biological properties that may be useful in the treatment of mental disease [11,12].

Lectins are proteins with at least one non-catalytic domain which allows selective recognition of and reversible binding to specific glycans, whether free or as part of glycoproteins and glycolipids [13]. Plant lectins have proven capable of modulating molecular targets in the CNS that may be involved in behavior regulation and neuroplasticity

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