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New biochemical and physicochemical insights on a muskmelon [*Cucumis melo* (L.)] chitinase

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Abstract: Chitinases (EC 3.2.1.14) are enzymes that catalyse the degradation of chitin, a vastly abundant polymer of N-acetylglucosamine. Chtin is a structural component of insects, nematodes, fungi and other plant pests and pathogens. A chitinase (*Cm*chi1) was previously identified in *Cucumis melo* (L.). The enzyme was identified as not being active against an analog of chitin. However, the authors did not made any inference on the possible reasons to this absence of activity. In an attempt to better characterize *Cm*chi1 the present study was developed to explore several physicochemical, biochemical and structural parameters regarding to this enzyme through computational analysis. New and relevant insights on the mechanism of action of *Cm*chi1 over chitin are discussed.

Keywords *- Computational biology, Physicochemical property analysis, Amino acid sequences*

I. INTRODUCTION

Plants are constantly being challenged by biotic and abiotic stress. Despite their inability to move and the absence of an elaborated immunologic system like that found in animals plants present a diversified arsenal of chemical and structural or physical barriers that can confer resistance to both biotic and abiotic stress [1,2].
Pathogenesis related proteins (PR proteins) are a specific group of plant proteins (chemical barrier) whose level and activity are known to be severely increased or even *de novo* induced when a number of stress conditions including the infection by fungi [3] and bacteria [4], or the attack by phytophagous insects [5] and phytonematodes [6], as well as, extremes of heat, cold, drought etc are posed to plants.

One of the most important group of PR proteins are chitinases (EC 3.2.1.14). Chitinases are a class of hydrolytic enzymes that breakdown the glycosidic bonds that compose the polymer chitin [7]. The importance of chitinases as a part of the chemical defense mechanism of plants relies on the fact that chitin is a major and very important structural component of several phytopathogens and plant predators [8]. The disruption of chitin by endogenous chitinases is an elegant strategy plants have developed to defend themselves against its biological enemies in addition to other defense mechanisms.

Several plant cultures of great economic importance such as *Glycine max* [9], *Vigna unguiculata* [10], *Zea mays* [11], *Cucumis sativus* [12], and *C. melo* [13] have been characterized as source of PR-proteins including chitinases. Two genes encoding for chitinases were previously isolated and characterized from developing seeds of *C. melo* [13]. The enzymes derived from these genes were termed *Cm*chi1 and *Cm*chi2. *Cm*chi1 was expressed in bacteria and the recombinant product purified. When tested on an *in vitro* assay against glycol chitin (substrate) the enzyme did not presented activity [13]. The authors mentioned this fact as a justification to prevent the direct investigation of the potential defensive role of *Cm*chi1 in fungal inhibition assays.

In this work we have conducted a systematic analysis by using a bioinformatic approach with the aim to better characterize *C*mchi1 according to biochemical and physicochemical properties. Finally we have got a more complete understanding of the structural and molecular aspects surrounding the interaction between *Cm*chi1 and N-acetyl-D-glucosamine (the monomer of chitin) and evaluation of its interactions through molecular docking studies. New and relevant insights on the mechanism of action of *Cm*chi1 over chitin are discussed.

II. METHODS

2.1. Sequence retrieval and analysis

The amino acid sequence of the *Cm*chi1 enzyme (Accession GenBank nº AAF64474.1) was downloaded in FASTA format from National Center for Biotechnology Information (NCBI) database

(http://www.ncbi.nlm.nih.gov/). The algorithm employed by SignalP 4.1 Server [14] was used to identify the presence of signal peptide within the obtained sequence. The mature protein sequence obtained after SignalP evaluation was used for additional analysis.

2.3. Physicochemical characterization

For physicochemical characterization of mature *Cm*chi1, theoretical molecular weight (MW), isoelectric point (pI), instability index (II) and grand average of hydropathicity index (GRAVY) were computed using the Expasy's ProtParam server [15]. DISULFIND Server [16] was used to assess the presence of disulphide bonds.

2.4. Prediction of secondary structure

Secondary structure of *Cm*chi1 chitinase was predicted using the following web servers: CFSSP – Chou & Fasman Secondary Structure Prediction Server [17], GOR V – Protein Secondary Structure Prediction Server [18], HNN - Hierarchical Neural Network [19], PCI-SS - PCI-Based Protein Secondary Structure Site Prediction Server [20], SopMa - Self Optimized Prediction Method with Alignment [21] and YASPIN Secondary Structure Prediction [22].

2.5. Domain analysis and linker prediction

Sequence of *Cm*chi1 in FASTA format was submitted to the following servers: Conserved Domains Database (CDD) and Resources [23], Simple Modular Architecture Research Tool (SMART) [24] and SBASE Domain Prediction (http://pongor.itk.ppke.hu/protein/sbase.html#/sbase_form) in order to identify possible boundary domains as well as to predict linker sequences.

2.6. 3D structure prediction and analysis

2.6.1. *Template identification*

In order to find suitable templates for the prediction of the three dimensional structure of *Cm*chi1 protein the RCSB Protein Data Bank Server [25] was used. The following parameters were used to find protein models with the highest levels of structural relationships to *Cm*Chil: (I) - Advanced Search; (II) - Query type - Sequence (BLAST/FASTA/PSI-BLAST), and (III) - E-value cutoff - 0.001. All templates were selected based on sequence identity and maximum query coverage.

2.6.2. *Model construction and refinement*

The software Modeller [26] was used to construct the theoretical model of *Cm*chi1. The obtained theoretical structure was refined using WinCoot 0.8.1 [27] and its quality was improved by energy minimization using the method of GalaxyRefine server [28,29].

2.6.3. *Model evaluation*

The quality of the refined model was evaluated using Molprobity [30], ERRAT [31] and VERIFY_3D [32] servers. The overall model quality for the final structure was determined using Protein Structure Analysis (ProSA) server [33].

2.6.4. *Docking study*

The final refined model of *Cm*chi1 was submitted to binding analysis to chitin ligand (here we termed "chitin" an oligomer of 4 units of N-acetyl-D-glucosamine joined by β-1,4-glycosidic linkage). The binding pocket was identified using CASTp server [34]. The structure of the ligand was obtained from ChemSpider database (http://www.chemspider.com/). The interactions between *Cm*chi1 and chitin were simulated by the AutoDock Vina software v. 1.1.2 [35].

III. RESULTS AND DISCUSSION

3.1. *Analysis of primary sequence*

The analysis of *Cm*chi1 primary structure using SignalP 4.1 Server [14], showed that the 292 amino acids full length protein contains a 25 amino acid long *N*-terminal signal peptide which is cleaved off to form the mature polypeptide (267 amino acid residues). The data suggests the presence of a cleavage site between Ala^{25} and Ala^{26} residues with a mean S score of 0.856 and a discrimination score of 0.857 (Figure 1).

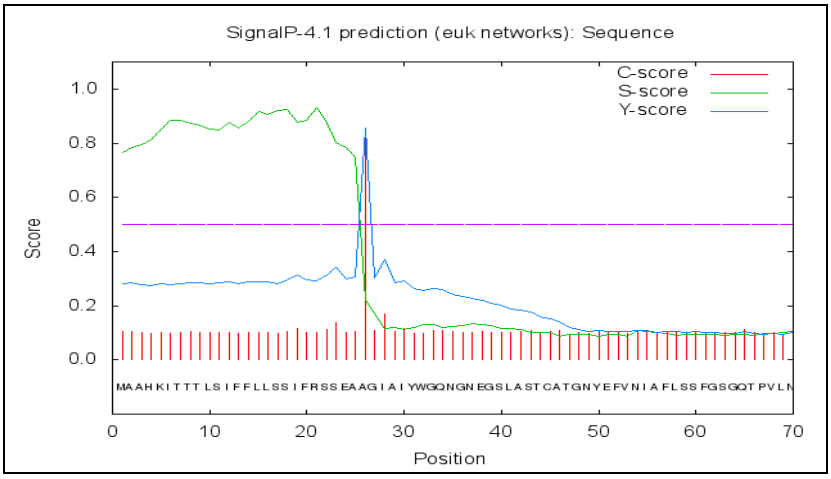


Figure 1. Signal peptide prediction. Signal peptide sequence was predicted using SignalP online tool (http://www.cbs.dtu.dk/services/SignalP/). The analysis revealed the presence of a cleavage site between Ala[25] and Ala[26] residues.

3.2. *Physicochemical properties*

The characterization of a polypeptide amino acid sequence may provide important insights over the structure and properties of the protein which can contribute to a better understanding of its biochemical and cellular functions. Chitinase family comprises a diverse group of enzymes that differ in enzymatic activities, amino acid primary sequence, isoelectric point (pI) and subcellular localization [36], as well as other structural features such as the chitin-binding domain and a carboxyl-terminal extension sequence flanking the main catalytic domain [37]. After an extensive bibliographic review it was noted that no significant data about the physicochemical properties or biological activities of *Cm*chi1 chitinase is available.

Therefore, physicochemical properties such as: molecular weight, theoretical pI, instability, aliphatic and Grand average of hydropathicity indexes of *Cm*chi1 were predicted using ProtParam server (http://web.expasy.org/protparam/).

Computational analysis revealed that the whole *Cm*chi1 protein has a molecular mass of 30.78 kDa [13], while the mature protein (with no signal peptide) has a predicted molecular mass of 28.03 kDa which is almost identical in comparison to other members of 18 class of plant chitinases, such as *C. sativus* (28 kDa) [38] and *Ficus microcarpa* (27 kDa) [39]. In addition *Cm*chi1 is an acidic protein (pI 4.17). This pI value is in accordance to the pI obtained in the work of Witmer and colleagues [13]. Acidic and basic chitinases are present in a number of plant cultures including: *Arabidopsis thaliana* [40], *Capsicum annuum* [41] and *Sporisorium scitamineum* [42].

The instability index provides an indirect estimative of the metabolic stability of a protein [43]. This index is based on a scale of stability in which values lower than 40 can be considered to classified a protein as stable while values higher than 40 predict that the protein may be unstable [43]. Based on this analysis *Cm*chi1 could be considered a stable protein as its instability index showed a value of 33.63 (< 40) (Table 1). The GRAVY value for a peptide or protein is calculated as the sum of hydropathy values of all the amino acids divided by the total number of residues in the sequence. The GRAVY value of *Cm*chi1 was found to be 0.052, which suggests low possibility of interactions with water molecules.

In terms of thermal stability *Cm*chi1 can be considered as a stable protein. Two hypothesis are discussed here to clarify this question. *Cm*chi1 shares with members of class 18 plant chitinases the occurrence of two signature sequences which lie along barrel strands 3 and 4. These structures are known to help to form the active site cleft on the carboxyl end of the *β*-barrel and appear to be important both for stability of the fold and for catalytic activity [44]. Furthermore, it is possible that the thermal stability of *Cm*chi1 chitinase is in part due to the presence of disulfide bonds. Indeed the search for possible disulfide bonds in *Cm*chi1 structure using the DISULFIND server [16] revealed the existence of 3 putative Cystine (CyS-SCy) interactions among the residues (Cys^{20}- Cys^{67}); (Cys^{50} - Cys^{155}) and (Cys^{57} - Cys^{184}) residues.

3.3 Domain Analysis

According to the analysis performed using CDD [23], SMART [24] and SBASE servers, *Cm*chi1 belongs to the family 18 of Glycosyl Hydrolases (GH_{18}), with an E-value of 2.51e-23 (CDD), E-value of 3.16e-01 (SMART) and Score of 100% (SBASE), respectively.

3.3. *Secondary structure analysis*

The predicted secondary structures of *Cm*chi1, using CFSSP, GOR 5, HNN, PCI-SS, SopMA and YASPIN servers (Table 1), showed the predominance of random coils (34.83 – 57.68%) followed by helices (19.10 – 31.08%) and strands (16.85 – 34.46%). Although these Servers use different algorithms and approaches to predict secondary structure pattern from primary amino acid sequences, in all the cases the presence of random coils as secondary structure elements was found to be dominant. Similarly, the class III GH_{18} family rice chitinase (DIP3) when analyzed for possible secondary structure elements, i.e., helices, strands and random coils showed the prevalence of random coils as the main secondary structure element [45], which also supports the present findings. Random coils are known to be very important to protein folding, translocation and stability [46].

Table 1: Analysis of secondary structures of *Cm*chi1 enzyme (Accession GenBank nº AAF64474.1) using different servers

Cmchi1	Server name	α-Helix		Extended-strand		Random coil	
		Nº of residues	Percentage (%)	Nº of residues	Percentage (%)	Nº of residues	Percentage (%)
	CFSSP	82	30,71	92	34,46	93	34,83
	GOR 5	51	19,10	62	23,22	154	57,68
	HNN	81	30,34	45	16,85	141	52,81
	PCI-SS	66	24,72	57	21,35	144	53,93
	SopMa	68	25,47	78	29,21	121	45,32
	YASPIN	83	31,08	62	23,22	122	45,7

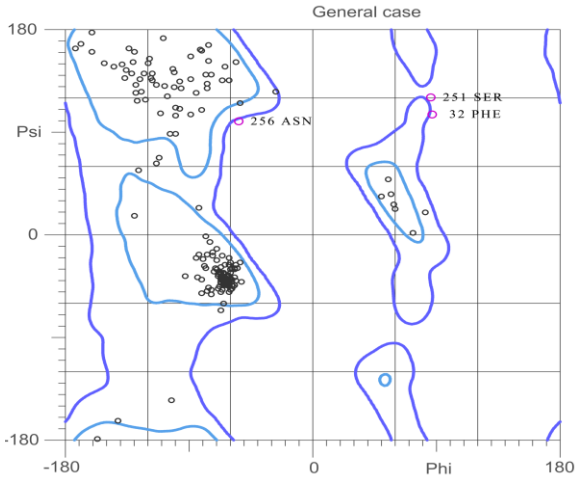
3.4. *Model construction, refinement and stereo-chemical evaluation*

Computational modeling of protein has been considered more reliable when there is a clear evolutionary relationship (homology) between a target sequence of the protein on analysis and protein structures already stored and available in protein structure data banks. The first step in predicting a 3D model for *Cm*chi1 through comparative molecular modeling consisted in an extensive search for suitable models in the RSCB-PDB Protein Data Bank. As a result the structures termed: 1HVQ, 1LLO and 2GSJ, all belonging to family GH_{18} of plant chitinases, were selected according to parameters like query coverage, E-value, sequence identity and structural resolution.

The *Cm*chi1 3D model was constructed using the software Modeller [26] and the quality of the predicted structure was checked in the MolProbity server [30]. It was observed that the vast majority of $\phi - \psi$ dihedral angle pairs were distributed in the most favored and allowed regions of the Ramachandran's plot (Figure 2A). On the other hand, an additional optimization step was made using the WinCoot tool [27] in order to rearrange the Phe^{32}, Ser^{251} and Asn^{256} amino acids in the allowed regions of the Ramachandran's plot (Figure 2B). The overall structure comprises an 8 β-strains and 10 α-helices, which together form a TIM-barrel protein fold. The eight parallel β-strains form the core of the enzyme (Figure 3).

The final model of *Cmchi1* was subjected to energy minimization using GalaxyRefine server [28,29]. GalaxyRefine performs the refinement in the overall structure [44]. The method takes into account the general pattern of the initial protein model and then it performs the refinement based on the backbone structure using a high-accuracy global distance test (GDT_{-HA}) [47]followed by a side-chain global distance test GDC_{-SC} [48] and finally, for physical correctness, the software makes use of MolProbity score [30].

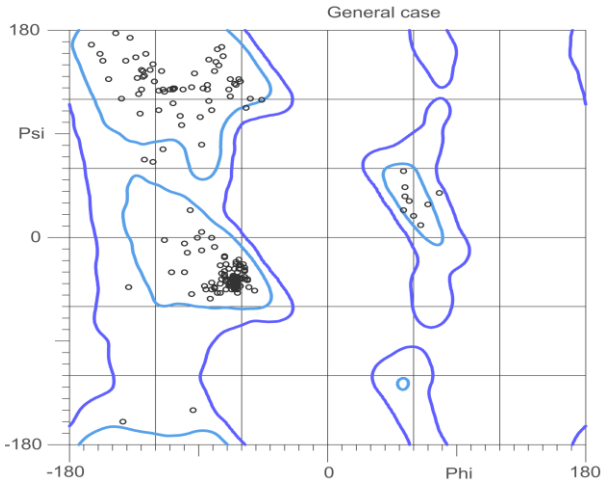
The overall model quality (Z-score), overall quality factor and 3D-1D Averaged Score of the *Cm*chi1 model were measured using ERRAT, VERIFY_3D and ProSA-web, respectively. Using the Protein Structure Assessment Server (ProSA-web) [33], it was obtained a value of - 9.69 (black dot – Figure 4A). Figure 4B shows the quality of the model based on the global energy of its structure. According to this result *Cm*chi1 model can be classified as a good quality model and it is placed on a range of scores typically found for native conformations of proteins with similar sizes (200 - 300 amino acid residues) in which the three dimensional structures were solved by X-Ray diffraction [33].



(2A). Ramachandran's plot of *Cm*chi1 protein.

95.1% (252/265) of all residues were in favored (98%) regions.
98.9% (262/265) of all residues were in allowed (>99.8%) regions.

There were 3 outliers (phi, psi):
32 PHE (87.4, 106.9)
251 SER (87.0, 121.3)
256 ASN (-54.4, 100,2)



(2B). Ramachandran's plot of *Cm*chi1 protein after being optimized by WinCoot software and energy-minimized by GalaxyRefine.

95.5% (253/265) of all residues were in favored (98%) regions.
100.0% (265/265) of all residues were in allowed (>99.8%) regions.

There were no outliers.

Figure 2. Ramachandran's plot (MolProbity) showing the dihedral angles ϕ and ψ of amino acid residues from *Cm*chi1. The residues located in the most favored regions (95.1%) are shown in light blue curves and the residues located in the additional allowed regions (98.9%) are in dark blue curves.

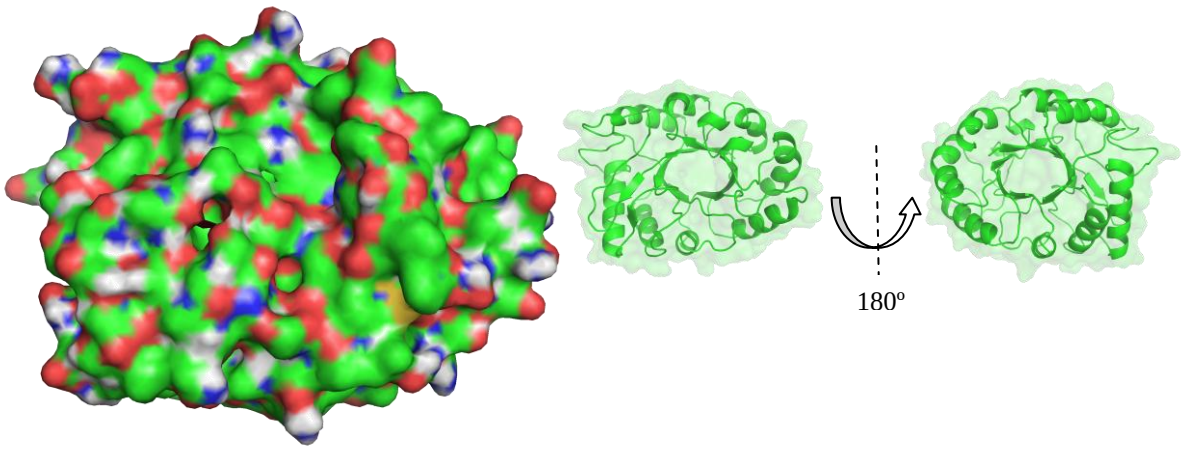


Figure 3. Electrostatic potential map shows differences in the distribution of superficial charge along *Cm*chi1 structure **(A)**. Specular image of *Cm*chi1: 180º rotation around their longitudinal axis **(B)**.

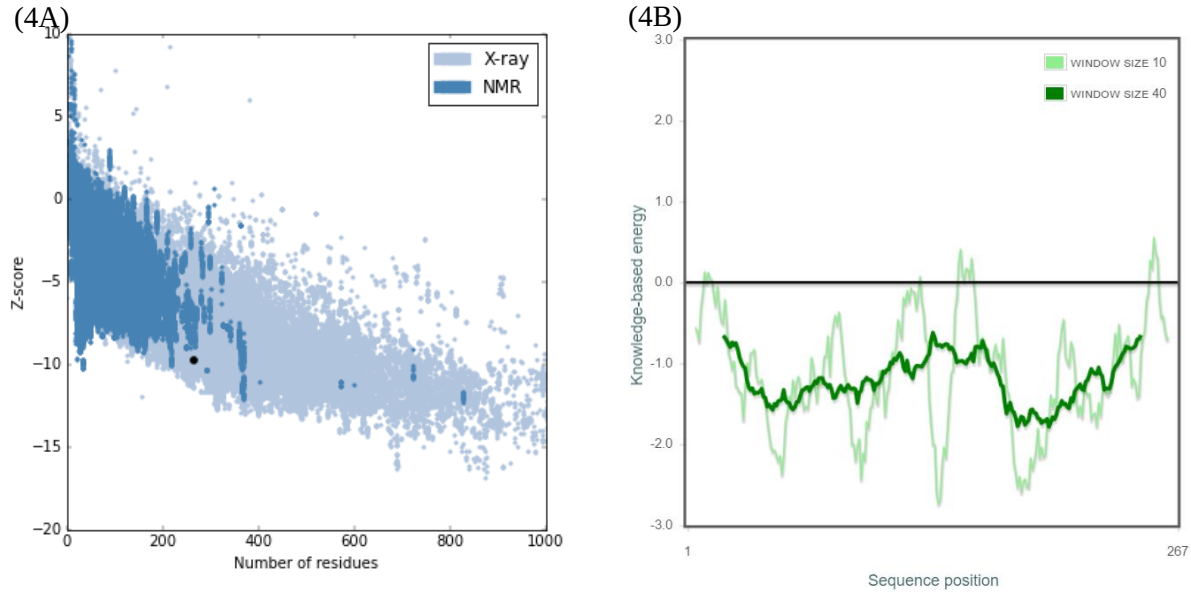


Figure 4. Protein Structure Assessment server (ProSA-web) analysis of *Cm*chi1 model. The Z-score (-9.69) of *Cm*chi1 falls in the range of values commonly found for PDB proteins whose structures were determined by NMR (dark blue region) and X-ray crystallography (light blue region). Note the black dot on the plot which represents the Z-score of *Cm*chi1 **(A).** ProSA-web analysis shows that all the amino acid residues of *Cm*chi1 are distributed within the negative region of the plot **(B)**. Taken together these results suggest that the *Cm*chi1 model proposed here is of high quality and confidence.

Non-bonded interactions among different atoms was analyzed by ERRAT [31]. The overall quality factor for the *Cm*chi1 model was 91.506% (Figure 5). This value suggests that the predicted model can be considered as being of acceptable quality, according to ERRAT confidence limit scale (< 95%). The Verify3D [32] results for the *Cm*chi1 model revealed that 100% of the amino acid residues had an averaged 3D-1D score higher than 0.2, which indicates a well built model with all residues positioned in their folded conformation (Figure 6). The final model was deposited in the PMDB Protein Model Database under the Access number PM0080774 (https://bioinformatics.cineca.it/PMDB/).

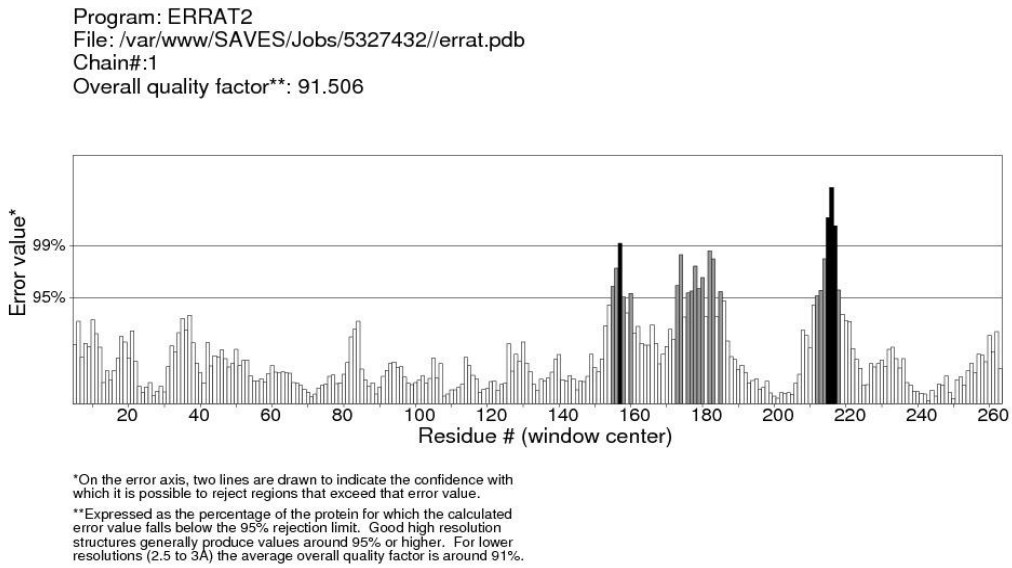


Figure 5. Overall quality analysis of *Cm*chi1 model according to ERRAT server. Less favored regions are shaded in black and the most favored regions are in white. The method of evaluation of ERRAT returned a value of 91.506% for the predicted structure of *Cm*chi1. This data suggests a model of good quality which is in accordance with the other analysis discussed here.

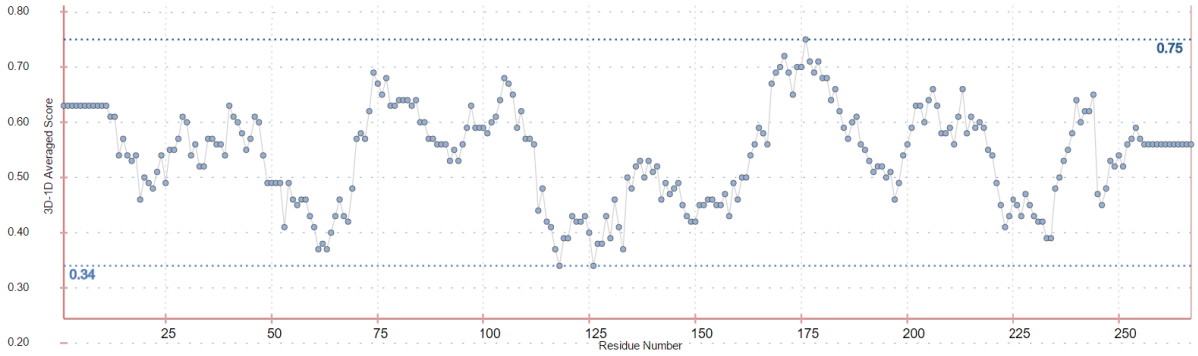


Figure 6. Verify 3D plot of *Cm*chi1 model. Vertical axis represents the averaged 3D-1D profile score for residues in a 25-residue sliding window. Horizontal axis represents the number of residues in the primary sequence of the protein. The first and the last 9 residues of the primary sequence are not subjected to analysis. According to the plot the structure of *Cm*chi1 shows no error in the distribution of amino acid residues along the three dimensional space.

3.5. *Docking study*

A chitinase from muskmelon, *Cm*chi1, was initially identified in the work of Witmer *et al*., [13]. A recombinant version of the enzyme was heterologously expressed in bacteria and purified. Besides to being a genuine chitinase the recombinant protein was unable to properly hydrolyze glycol chitin, a potential substrate for this type of enzyme. According to authors this fact (absence of activity) prevented a direct investigation of the possible protective role of *Cm*chi1 against phytopathogenic fungi [13]. Based on these information we had developed a docking study in order to elucidate the molecular properties that possibly justify the absence of activity of *Cm*chi1 using the predicted and refined 3D model of the enzyme.

The first step in the docking study consisted in the search for the amino acid residues responsible for the interaction between *Cm*chi1 and chitin. Besides the fact that *Cm*chi1 appears to be unable to properly interact with chitin or chitin-like ligands [13] there are a sequence of conserved amino acid residues located in the potential active site of *Cm*chi1 in which its presence appears to be essential to the catalysis mechanism, according to CAZypedia database (https://www.cazypedia.org/index.php/Glycoside_Hydrolase_Family_18). In classical GH_{18} family chitinases the active site of the enzyme is comprised by the motif, Asp-X-X-Asp-X-Asp-X-Glu, where "X" stands for any of the other known amino acids residues. In the case of *Cm*chi1 the conserved sequence is: Asn^{120}-X-X-ASP^{123}-X-ASP^{125}-X-GLU^{127}. Surprisingly *Cm*chi1 presents a mutation in the first conserved amino acid of the active site, in which a Asn is located in a place where a Asp should be present in the majority of commonly active chitinases. We suggest that this substitution may explain at least in part the absence of activity of the enzyme. In addition, when the same region (active site motif) was analyzed in the sequence of the chitinases used as template, PDB ID: 1HVQ, 1LLO and 2GSJ by means of a multiple sequence alignment using the software CLC Sequence Viewer v. 7.7.1 (www.clcbio.com) we observed that *Cm*chi1 was the only protein that do not conserved the first Asp. Indeed the Asn was present as discussed before (Figure 7).

In addition to the fact that *Cm*chi1 has a substitution mutation in the first amino acid residue of the conserved active site motif of chitinases, docking studies revealed that the enzyme cannot perform an optimal interaction with the substrate. A total of 10 simulations were performed in which the binding of $(GlcNac)_4$ to the active site of *Cm*chi1 was examined. The best value of interaction was E-value = – 6.2 kcal.mol-^{1}. This result demonstrated that the amino acids of the active site of *Cm*chi1 were prone to interact with $(GlcNac)_4$ molecules (Figure 8A). However it was observed a distance of ligation of around 12.1 Å from the Asp^{123} to the ligand $(GlcNac)_4$, what is completely incompatible with an ideal interaction between enzyme and substrate (Figure 8B).

Taken together these findings: 1) The mutation of Asp^{120} to Asn^{120} and 2) The distance of Asp^{123} to the ligand may give us a molecular overview that partially explain the possible reason why *Cm*chi1 is a chitinase that lacks chitinolytic activity and they are in complete agreement with the work of Witmer *et al* [13].

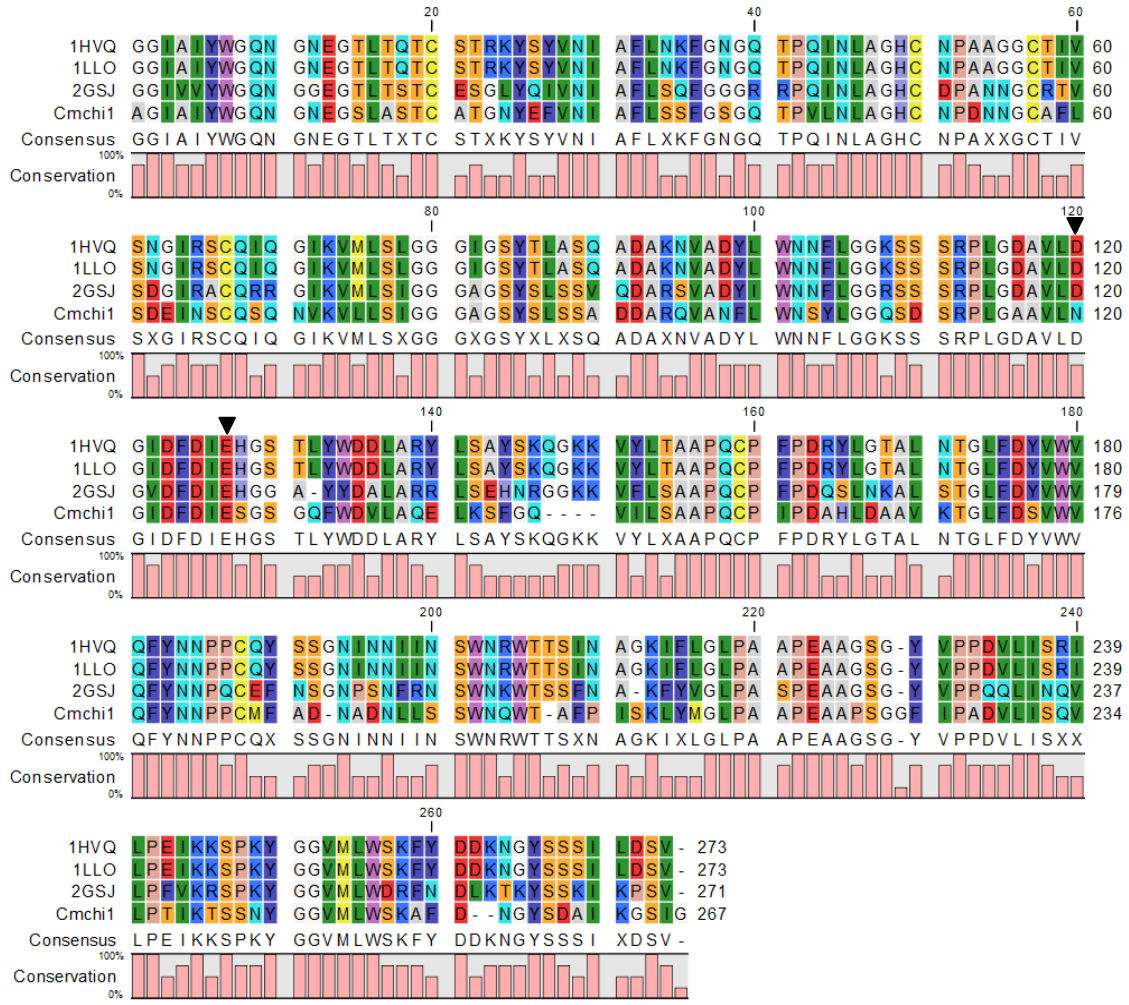


Figure 7. Multiple amino acid sequence alignment of four chitinases from family 18 (GH_{18}) of plant Glycosyl Hydrolases: 1HVQ, 1LLO, 2GSJ and *Cm*chi1 using the software CLC Sequence Viewer v. 7.7.1. The active site motif of the enzymes is indicated by black triangles. The number of residues relative to the N-terminal segment of the mature proteins is shown on the right side of each sequence.

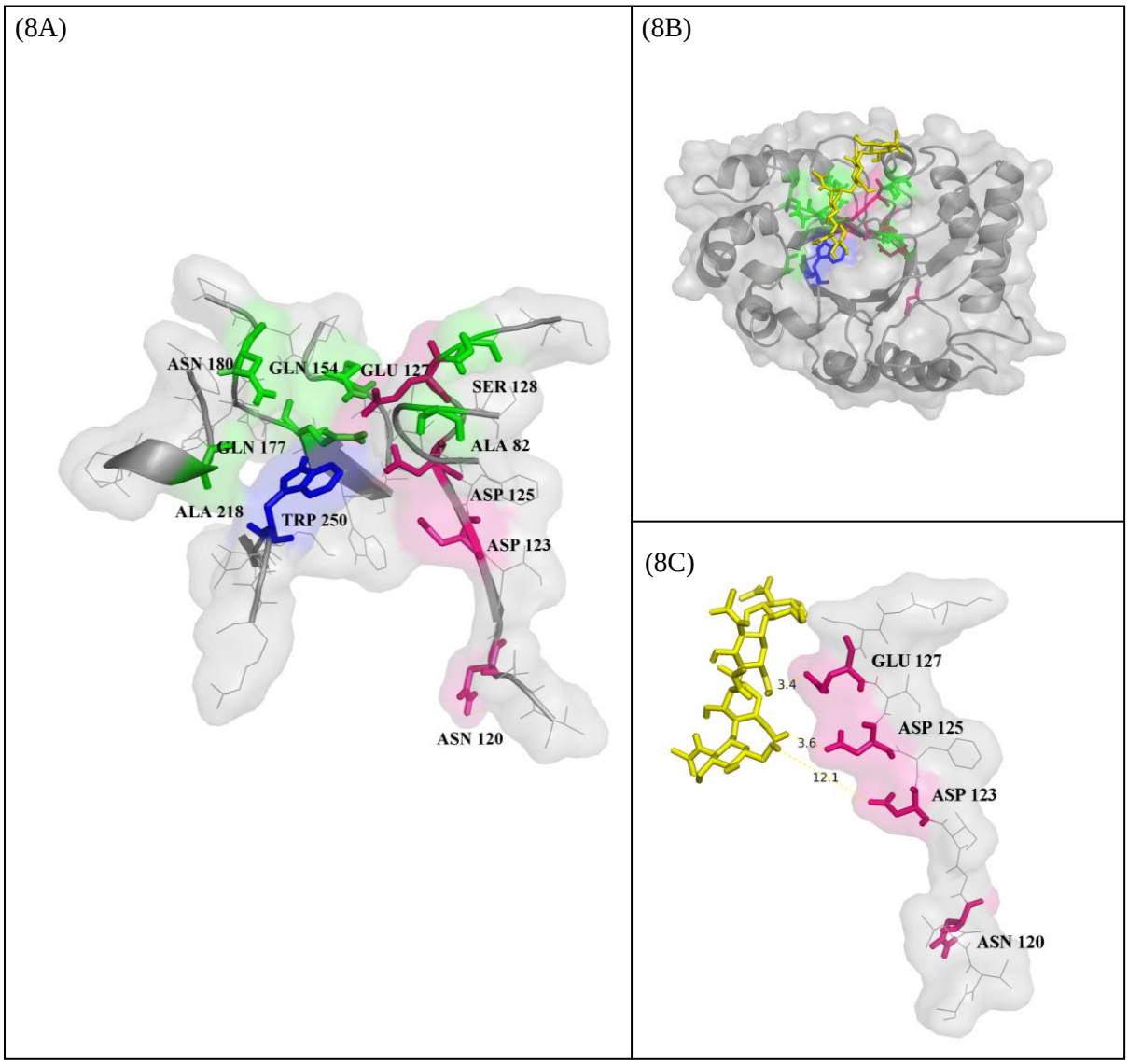


Figure 8. Interacting residues on the binding pocket of *Cm*chi1 by molecular docking analysis. The residues related to the catalytic site of *Cm*chi1 are shown (8A). An overview of the molecular interaction between *Cm*chi1 and $(GlcNac)_4$ (yellow structure). Green colored residues are interacting with hydrogen atoms of $(GlcNac)_4$ ligand; the blue residue is forming a hydrophobic interaction with the ligand while magenta residues are those related to the catalytic site (8B). A detailed overview of the interaction through active site motif residues of *Cm*chi1 and $(GlcNac)_4$. Note that conversely to the conserved residues present in the catalytic site motif of other plant chitinases *Cm*chi1 presents a substitution of Asp^{120} to an Asn^{120}. In addition the distance of Asp^{123} to the ligand may potentially hamper the occurrence of favorable interactions between *Cm*chi1 and $(GlcNac)_4$ (8C).

IV. CONCLUSION

A three-dimensional model of a chitinase from *Cucumis melo*, *Cm*chi1, was constructed in this work using the molecular modeling method based on three chitinase structures deposited on the PDB databank. Different bioinformatic tools demonstrated that *Cm*chi1 is a typical plant chitinase of the family (GH_{18}) which shares the same physicochemical properties as other known (GH_{18}) plant chitinases. However, particular alterations on the catalytic motif of *Cm*chi1 were in depth investigated through molecular docking analysis.

These studies revealed that *Cm*chi1 may be hampered to form an ideal interaction with $(GlcNac)_4$ due to a substitution mutation (Asp^{120} to Asn^{120}) and a non ideal binding distance through Asp^{123} and the ligand. Taken together these results suggest that *Cm*chi1is a classic (GH_{18}) plant chitinase that potentially may not be active against its more common substrate, the polymer of chitin.

V. ACKNOWLEDGEMENTS

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