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ORIGINAL PAPER

Computational Analysis of the Effects of Mutations on Catalytic Cavity of Chitinases A

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Abstract

Computational studies are widely used to assess the effect of amino acid mutations on stability, flexibility and conformational states of proteins as well as on the interactions with different ligands. In this work the effects of mutations inchitinase A protein from Serratia marcescens and Bacillus cereus on the physicochemical properties ofactive sites and the interactions with a reaction product (N,N-diacetylchitobiose) and a fungicide (difenoconazole) were investigated using Fpocket, Chimera and SwissDock computational tools. It is shown that mutations of aromatic and acidic amino acids with aliphatic ones in both Serratia marscenses and Bacillus cereus proteins result in change of physicochemical properties of the active sites and in decrease the interaction energy with both the product and the fungicide. These effects must be considered when devising strategies for optimizing the chitinase proteins stability and/or for further research involving biotechnological applications.

Keywords: chitinase A, mutations, active site properties.

1. Introduction

There are numerous bacterial enzymes that facilitate degradation of different compounds. The glycosyl hydrolases enzymes are a class of such enzymes involved in degradation of different types of carbohydrates. An abundantcarbohydrate in natureischitin, abiotechnologically relevant sugar being hydrolyzed by chitinases, enzymes belonging to the families 18 and 19 of the glycosyl hydrolases (1). Many species of bacteria and other types of microorganisms from all studied ecosystems possess one or more chitinases from these classes, having thus the ability to hydrolyze chitin (2). Many species of bacteria produce chitinases to decompose and utilize chitin as an energy source (3). *Serratia sp.* and *Bacillus sp.*are bacteria that have been identified to produce chitinases (4, 5). The chitinolytic activity of the *Serratia marcescens* is realized by four chitinases: ChiA, ChiB, ChiC1 and ChiC2 (6) while *Bacillus cereus* produces two chitinases, ChiA and ChiB (7). Bacterial chitinases belong to the family 18 of glycosyl hydrolases with the catalytic domain containing an $(alpha\text{-}beta)_8$-barrel across of which chitooligosaccharides interact via hydrophobic forces with aromatic amino acids to result in bound complex (8, 9). Mutations in the chitinases sequences are performed either to elucidate their catalytic mechanisms, or to improve their catalytic efficiency. Directed evolution and protein-engineering approaches have been used to improve substrate specificity of chitinases toward biotechnologically relevant sugars (10, 11). The data reported in literature about directed evolution experiments ofchitinases belonging to *Bacillus sp.* illustrate that from 517 clones considered for screening, only one mutant has shown improved catalytic activity (10). The present study focuses on the evaluation of the effects of mutations on the

properties of catalytic cavities of the *Serratia marcescens* and *Bacillus cereus* chitinases A using a computational approach. The interactions of the native and mutant chitinases with the product N,N-diacetylchitobiose and the fungicide difenoconazole are also assessed.

2. Materials and Methods

To avoid redundancy of carrying calculations on similar sequences and structures, the sequences of the *Serratia marcescens* and *Bacillus cereus* chitinases A, retrieved from Uniprot database (12), were compared using ClustalW tool (13). Further, the crystal structures of the *Serratia marcescens* and *Bacillus cereus* chitinases A were obtained from the Protein Data Bank (PDB) (14). In PDB there are 14 entries of crystallographic structures of *Serratia marcescens* (15, 16) and 7 entries for *Bacillus cereus* chitinase A(17). The *Bacillus cereus* chitinase A (BcChiA) does not contain chitin binding and insertion domains, but *Serratia marcescens* chitinase A (SmChiA) contains such domains. The structures of native and mutant enzymes, both in free and bound states with substrate, product or inhibitor were considered. The structural files having the best available resolution were selected. The corresponding codes entry, resolution and a short description for the considered structural files are presented in the (table 1).

Table 1. Protein Data Bank codes entry and structures short description for *Serratia marcescens* and *Bacillus cereus* chitinases A

***Serratia marcescens* chitinase A**			***Bacillus cereus* NCTU2 chitinase A**		
PDB code entry	**Resolution (Å)**	**Observations**	**PDB code entry**	**Resolution (Å)**	**Observations**
1EDQ	1.90	region 24-563 of native protein	**3N11**	1.35	region 28-360 of the wild-type protein.
1FFR	1.80	Y390F mutant region 24-563 of the protein in complex with hexa-N-acetylchitohexaose.	**3N12**	1.20	region 28-360 of the protein in complex with zinc atoms
1EHN	1.90	E315Q mutant region 24-563 of the protein in complex with octa-N-acetylchitooctaose.	**3N13**	1.70	D143A mutant region 28-360 of the chitinase A in complex with two linked N-acetyl-D-glucosamine molecules (N,N-diacetylchitobiose)
1EIB	1.80	D313A mutant region 24-563 of the protein in complex with octa-N-acetylchitooctaose.	**3N15**	1.94	E145Q mutant region 28-360 of the chitinase A in complex with two linked N-acetyl-D-glucosamine molecules (N,N-diacetylchitobiose)
2WLZ	1.82	region 2-560 of the protein in complex with chitobio-thiazoline	**3N18**	1.20	E145G and Y227F mutant region 28-360 of the chitinase A in complex with four linked N-acetyl-D-glucosamine
1X6N	2.0	W167A mutant (entire protein 1-563) in complex with allosamidin	**3N1A**	2.0	E145G and Y227F mutant region 28-360 of the chitinase A in complex with cyclo-(L-His-L-Pro)

The comparison of the considered enzymes structures was performed using structure superposition tool under UCSF Chimera package (18) and reported as the root mean square deviation (RMSD) between the corresponding alpha carbon (CA) atoms of the superposed

structures (19). The active site cavities of enzymes were identified and characterized using the Fpocket tool (20). Fpocket detects cavities on a protein surface using exclusively geometric rules. It outputs a set of potential cavities/pockets as PDB files for predicted cavities and they may be visualized as surfaces of each pocket.

3. Results and discussion

The sequence alignment of catalytic domains of *Serratia marcescens* chitinase A (SmChiA) and *Bacillus cereus* chitinase A (BcChiA) is shown in the (figure 1). The sequence alignment reveals a low sequence similarity between SmChiA and BcChiA catalytic domains (11%), and at the same time the sequence similarity for the entire proteins sequences being as low as 16%. Like other chitinases belonging to the family 18 of glycosyl hydrolases, SmChiA and BcChiAhave a catalytic domain containing a $(beta/alpha)_8$-barrel and a signature peptide DGXDXDXE resulting in the fourth beta-strand in the barrel (1). This signature peptide is highlighted in the (figure 1), as well as the amino acids involved in the active centers of the enzymes. In the signature peptide from SmChiA, the glutamic acid 315 serves as the acid/base donor during carbohydrate hydrolysis and the aspartic acid 313 helps to properly locate the N-acetyl moiety of the glycosyl sugar (17). Correspondingly, in BcChiA, the glutamic acid 145 cleaves the glyosidic bond between the N-acetyl-D-glucosamine (NAG) subunits and the aspartic acid 143 stabilizes the conformation of the NAG subunits (15). Also, hydrophobic residues W167, Y170, W232, W245, W539 of the SmChiA [9] and F43, F66, I106, V112, Y193, Y227 and W333 of the BcChiA(17) are involved in the interaction with the chitooligosaccharides.

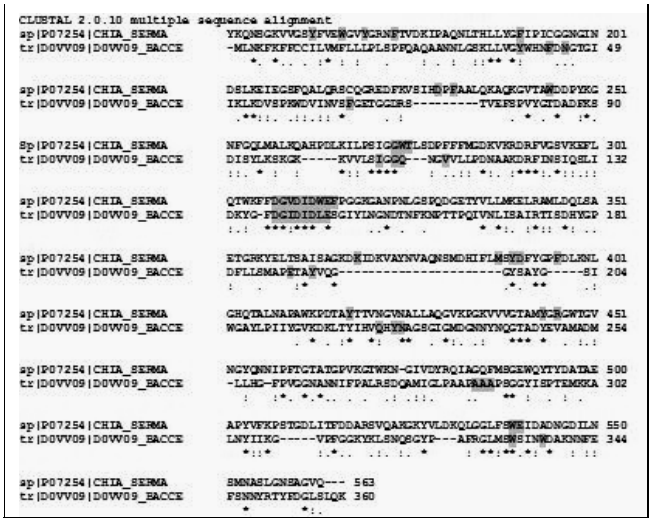
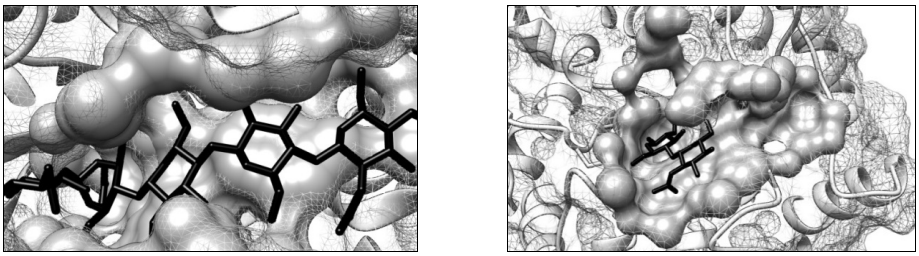


Figure 1. The alignment of the catalytic domains sequences of the *Serratia marscenses* and *Bacillus cereus* chitinases A. The signature peptide DGXDXDXE in the two sequences is highlighted in grey, as well as the other residues involved in the active center of the enzymes and in the hydrophobic interactions with the chitooligosaccharide substrate. The identical positions (*), the conserved substitutions (:) and the semi-conserved substitutions (•) are marked below the sequences

The superposition of the crystal structures of the *Serratia marcescens* (code entry 1EDQ) and *Bacillus cereus* (code entry 3N11) chitinases A results in a RMSD value of 1.163 Å for only 73 equivalent CA atoms, illustrating that the structures are not similar. Identification and characterization of the active site binding cavities of the SmChiA and BcChiA respectively were carried out using Fpocket tool (20). The identified cavities were

superposed to the crystallographic structure using the "Structure matching" facility under Chimera software in order to depict if the cavities are correctly recognized (figure 2).



A **B**

Figure 2. Active site cavity (grey surface) identified using Fpocket tool for the: (**A**) *Serratia marscenses* chitinase A (1EDQ, grey mesh surface) with the substrate hexa-N-acetylchitohexaose (black sticks) shown in the active site; (**B**) *Bacillus cereus*chitinase A (3N12, grey mesh surface) with the product N,N-diacetylchitobiose (black sticks) shown in the active site

The physicochemical characteristics of identified cavities are given in the (table 2).

Table 2. The physicochemical characteristics of the active site cavity of *Serratia marcescens* and *Bacillus cereus* chitinases A

Enzyme	Volume ($Å^3$)	Hydrophobicity score	Polarity score	Charge score	Local hydrophobic density
***Serratia marcescens* chitinase A**	1633.84	10.77	21	-6	40.89
***Serratia marcescens* chitinase A Y390F mutant**	1614.94	36.05	20	2	17.71
***Serratia marcescens* chitinase A E315Q mutant**	1660.29	22.00	19	-5	55.18
***Serratia marcescens* chitinase A D313A mutant**	2164.12	18.18	20	-5	55.41
***Serratia marcescens* chitinase A W167A mutant**	1255.18	13.45	11	3	33.08
***Bacillus cereus* chitinase A**	1284.97	30.55	15	0	37.03
***Bacillus cereus* chitinase A D143A mutant**	702.64	8	22	-1	1.00
***Bacillus cereus* chitinase AE145Q mutant**	491.88	54.00	4	1	25.40
***Bacillus cereus* chitinase AE145G and Y227F mutant**	451.33	32.29	8	0	41.69

Data presented in (table 2) reveal that BcChiAhas smaller and more hydrophobic active site cavity than SmChiA. The data also reveals that mutations strongly affect the hydrophobicity of the active site cavity.Thisis not an unexpected result taking into account that essential acidic and/or hydrophobic residues have been mutated. Another remark concerns the volumes detected for the cavities in the case of mutants. The much smaller volumes detected for the cavities of mutated *Bacillus cereus* chitinase A in compassion to that of the native protein reflect that the mutations result in local conformational changes of the protein chain affecting the pocket shape.

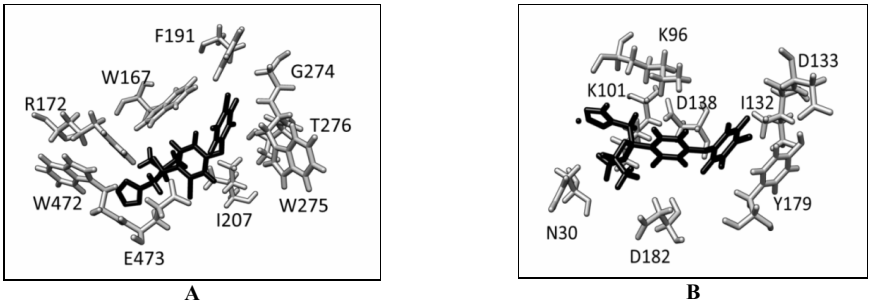
Previous molecular docking calculations revealed favorable binding of the pesticide difenoconazole on the native SmChiA and BcChiA, respectively (21). It is also true for the binding of the fungicide difenoconazole(DFC) to the mutants, but the interaction energies are

lower, as presented in the (table 3). As there are several positions where the fungicide may bind to the enzyme, the FullFitness scores and the estimated free energies of binding are given for the fungicide binding pose corresponding to the active site of the mutants. Also, the FullFitnessscores and the estimated free energies for the product N,N-diacetylchitobiose binding to the enzymes are presented.

Table 3. N,N-diacetyl chitobiose and difenoconazole interaction energies with the mutants of the *Serratia marcescens* and *Bacillus cereus* chitinases A

Enzyme	Product/ Pesticide	Structural file	FullFitness score[kcal/mol]	ΔG[kcal/ mol]
***Serratia marcescens* chitinase A**	N,N-diacetylchitobiose	region 24-563 of native protein (20)	-1912.76	-8.74
		Y390F mutant	-1898.26	-7.56
		E315Q mutant	-1869.23	-7.58
		D313A mutant	-1864.09	-8.01
		W167A mutant	-1876.08	-9.54
	difenoconazole	region 24-563 of native protein (20)	-1961.94	-7.72
		Y390F mutant	-1950.75	-7.15
		E315Q mutant	-1924.21	-7.91
		D313A mutant	-1925.46	-8.10
		W167A mutant	-1929.25	-6.86
***Bacillus cereus* chitinase A**	N,N-diacetylchitobiose	region 28-360 of the wild-type protein (20)	-1215.04	-9.12
		region 28-360 of the protein in complex with zinc atoms	-1805.27	-6.75
		D143A mutant	-1171.22	-8.01
		E145G mutant	-1172.10	-7.40
		E145G and Y227F mutant	-1128.26	-9.58
	difenoconazole	region 28-360 of the wild-type protein (20)	-1263.84	-7.13
		region 28-360 of the protein in complex with zinc atoms	-1863.48	-6.86
		D143A mutant	-1222.91	-7.63
		E145G mutant	-1235.51	-7.88
		E145G and Y227F mutant	-1180,76	-7.98

Data presented in the (table 3) illustrates favorable binding of the fungicide to the active sites of both the native enzymes and their mutants. Using the "Distance" facility under Chimera package the amino acids residues that are situated at maximum 5Å from the DFC molecule were identified. In (figure 3) there are presented only the residues of enzymes interacting with the fungicide difenoconazole. The DFC interaction with SmChiA involves abundant hydrophobic residues (F191, I207, W167, W275 and W472) in comparison to its interaction with BcChiA (I132, Y179). As a consequence, the interaction of DFC with SmChiA is stronger.



A **B**

Figure 3. Interacting residues with the fungicide difenoconazole for the *Serratia marcescens* (**A**) and *Bacillus cereus* (**B**) chitinases A

Data presented in (table 3) also reflect that the mutations of amino acids in the enzyme sequences (W167, D311, E315 and Y390 for the SmChiA and D143, E145 and Y227 for the BcChiA) negatively affect their interactions with both the product and fungicide. This result is in good agreement with experimental data providing that these mutations have a negative effect for the substrates binding to these enzymes (14-16). Except W167 of the SmChiA, the other mutated residues are not involved in the interactions of the DFC with native enzymes, suggesting that mutations affect the conformation and physicochemical properties of the binding site cavity as reflected by Fpocket outcomes (see table 2). Our data are in good agreement with several studies addressing the role of aromatic residues in the substrate converting efficiency of family 18 chitinases and on the binding surfaces of their catalytic cavities also reflecting that mutations of these residues lead to reductions of their activity (22-24).

4. Conclusions

Data presented here suggest that mutations of a few residues belonging to the active site of chitinase strongly affect the physicochemical properties of the active site cavity, especially its hydrophobicity. The mutations reduce the interacting/binding energy between the enzyme and product N,N-diacetylchitobiose or the fungicide difenoconazole. The largest consequences were observed for the W167A mutant in SmChiAand E145G/Y227F mutant in BcChiA, respectively. These effects must be considered when devising strategies for optimizing the chitinases stability and for proceeding research in the direction of their biotechnological applications. Also, our results emphasize that the computational tools are useful, their results informing about the possible effects of mutations allowing proper design of further experiments.

5. Acknowledgements

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