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Molecular Modelling of Broccoli Myrosinase and its Interaction with Different Ligands

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Myrosinase (β-thioglucosidase glucohydrolase (EC 3.2.3.1)) is a glycoprotein responsible for the hydrolysis of glucosinolates, a group of sulfur-containing secondary metabolites present in *Brassicaceae* plants. Sulforaphane, a powerful anti-cancer compound, comes from the hydrolysis of glucoraphanin, the main glucosinolate found in broccoli. The aim of this work was to investigate the interaction between broccoli myrosinase and different substrates through molecular modelling. Currently the amino acid sequence and the molecular structure of broccoli myrosinase are unknown. The amino acid sequence was deduced from the nucleotide sequence, which in turn was obtained experimentally. The primary sequence of broccoli myrosinase used for modelling consisted of 548 amino acids. The three-dimensional model was generated by an *ab initio*/threading method using the crystal structure of 1MYR_A and 1DWF_M of *Sinapis alba*, 1CBG_A of *Trifolium repens* L, 3PTK_A of *Oryza sativa* and 3WQ4_A of *Camellia sinensis* as templates, employing the I-TASSER server. The interaction with glucoraphanin (GRA), sinigrin (SIN), glucoiberin (GIB) and N-acetylglucosamine (NAG) was investigated through molecular docking. The binding energy of GRA, SIN, GIB and NAG to myrosinase were -3.80; -4.71; -4.77 and -5.55 (Kcal/mol), respectively. The simulations of the interaction between GRA and myrosinase suggested that the residues of the active site responsible for substrate stabilization by hydrogen bonding were Gln207, Gly431, Ser433 and Trp 477. The residue involved in the hydrolysis of the β-D-thioglucose binding was Tyr352. NAG interacts with the active site, as inhibitor. Our results will allow further studies about molecular mechanisms of broccoli myrosinase.

1. Introduction

The use of enzymes in food and bioprocess industry has been widely studied, showing promising to produce high value-added compounds, such as bioactive compounds, functional foods or nutraceuticals. Currently, there are about 4,000 known enzymes, and among these, around 200 are used commercially (Pinotti et al., 2017). Myrosinase (thioglucoside glycohydrolase, E.C. 3.2.3.1) is found in glucosinolate-containing plants, such as *Brassicaceae*. This enzyme catalyzes the hydrolysis of the β-thioglucoside bond of secondary metabolites called glucosinolates. This reaction releases a β-D-glucose molecule, forming an unstable intermediate called thiohydroxamate-O-sulphonate, which spontaneously decomposes to yield isothiocyanates, thiocyanates, epitononitriles and nitriles. The formation of these products depends on the reaction conditions, such as, pH, temperature, presence of iron ions and cofactors (Latté et al., 2011). Among the hydrolysis products, isothiocyanates are of great interest because of their health promoting properties. For instance, sulforaphane, which comes from glucoraphanin, is an anticancer and antimicrobial compound, providing protection to cells against exogenous or endogenous carcinogenic intermediates. It also has a bactericidal effect against *Helicobacter pylori* (Moon et al., 2010).
In the *Brassicaceae* family more than 130 different glucosinolates have been reported. The glucosinolates content and profile varies between species and subspecies. In some species, more than 15 different glucosinolates have been detected. In broccoli inflorescences, the predominant glucosinolates are aliphatic,

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such as glucoraphanin, glucoadrenerin, glucocorticoids, and sinigrin (Verkerk et al., 2009). Among them, the major interest has been set on glucoraphanin, due to the health promoting properties of its hydrolysis product, sulforaphane. The glucoraphanin content in other *Brassicaceae* different from broccoli is low, even negligible in some varieties. Then the study of the broccoli myrosinase-glucosinolate system would help to elucidate the underlying enzyme mechanisms, thus contributing to exploit the benefits of sulforaphane.

In the plant, glucosinolates are physically segregated from myrosinase, but when plant cells are damaged (e.g. during processing, mastication or injury by predators such as insects) the enzyme and the substrate are released and the hydrolysis proceeds. The myrosinase - glucosinolate system constitutes a defense mechanism of the plant. Under physiological conditions of Brassicaceae, i.e. neutral pH and temperature (30-40° C in day), isothiocyanates are the main hydrolysis products (Gu et al., 2012). Myrosinase is also found in the gastrointestinal tract of mammals, coming from the gut microbiota. In stomach, given the acid pH and the presence of iron ions, the main products coming from glucosinolates are nitriles. These toxic compounds can produce ulcers or stomach cancer. In contrast, the physiological conditions in the small intestine favor the conversion of glucosinolates to isothiocyanates, since the pH of the small intestine is approximately 7.4 (Baskar et al., 2012). Then, in order to maximize the uptake of sulforaphane, we propose that an inhibitor molecule that binds to myrosinase at acid pH and is released at neutral pH would avoid the conversion of glucoraphanin into undesirable products in the stomach, allowing the production of sulforaphane in the small intestine as main hydrolysis product.

In order to address this problem, it is necessary to investigate the structural and catalytic properties of broccoli myrosinase. Accordingly, it is necessary to count on a structural model that allows the study of myrosinase-glucosinolate interaction at molecular level. The aim of this work was to elucidate molecular and structural properties of broccoli myrosinase and to investigate its interaction with different ligands.

2. Materials and methods

2.1 Template identification

The amino acid sequence of broccoli myrosinase (MYR) was obtained by directly translating the nucleotide sequence deposited in GenBank (accession number: Myr_Bro.sqn Myr_Bro MF461331) using the ORF finder of NCBI (https://www.ncbi.nlm.nih.gov/orffinder/). Subsequently, a search on the NCBI protein sequence database (http://www.ncbi.nlm.gov/protein) was performed. A local sequence alignment was made using PSI-BLAST algorithm of BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast). The database used was Protein Data Bank (PDB), and the substitution matrix was BLOSUM62. Related sequences were selected to generate the three-dimensional structure. Additionally, a multiple sequence alignment was performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Swofford et al., 1996).

2.2 Model building and refinement

The three-dimensional model of myrosinase was built using the Iterative Threading ASSEmbly Refinement (I-TASSER) server based on the ab initio/threading method (Roy et al., 2010; Zhang, 2008). The target sequence was first threaded with a representative PDB structure library, in order to find possible folds by four simple variants of PPA methods, with different combinations of hidden Markov model and PSI-BLAST profiles and the Needleman-Wunsch and Smith-Waterman alignment algorithms. A total of five three-dimensional (3D) models were generated (Figure 1). Among them, the best model was identified based on parameters such as bond angles and length, Ramachandran plot, and confidence score (C-Score).

2.3 Molecular interaction simulations

The structures of the ligands: Glucoraphanin (GRA, CID: 6602383), Glucoiberin (GIB, CID: 9548622), Sinigrin (SIN, CID: 23682211) and N-acetylglucosamine (NAD, CID: 24139) were retrieved from NCBI PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Molecular docking was performed using AutoDock Vina (Trott and Olson, 2010). The substrates structures were prepared by adding the polar hydrogens and partial charges, and defining the rotatable bonds atom types. Myrosinase was also prepared by adding polar hydrogens and merging non-polar hydrogens. Grid map dimensions were assigned to active site residues and to the surrounding surface. The best docking orientation was identified considering binding affinity score and hydrogen bond interaction to the active site based on visual inspection. The 2D graphical visualization of myrosinase and glucoraphanin interaction was generated by Discovery Studio 3.5 visualizer (DS 3.5) (Accelrys, Inc. SanDiego, CA.; http://www.accelrys.com).

3. Results and discussion

The template 3D structures selected for modeling belonged to the Glyco_hydro family, and shared an identity percentage higher than 50%. These were 1MYR_A and 1DWF_M of *Sinapis alba*, 1CBG_A of *Trifolium repens* L, 3PTK_A of *Oryza sativa* and 3WQ4_A of *Camellia sinensis.* Five 3D structural models were generated for broccoli myrosinase. The models and the respective C –scores are shown in Figure 1. C-score is typically in the range of [-5, 2]; higher C-score indicates high confidence of the model (Roy et al., 2010; Zhang, 2008). Accordingly, the structures obtained in our work are highly reliable. Among the broccoli myrosinase models, the one with the highest C-score (Figure 1A) was selected for further analysis. This model was deposited in the Protein Model Data Base (PMDB) with the access code PM0081109.

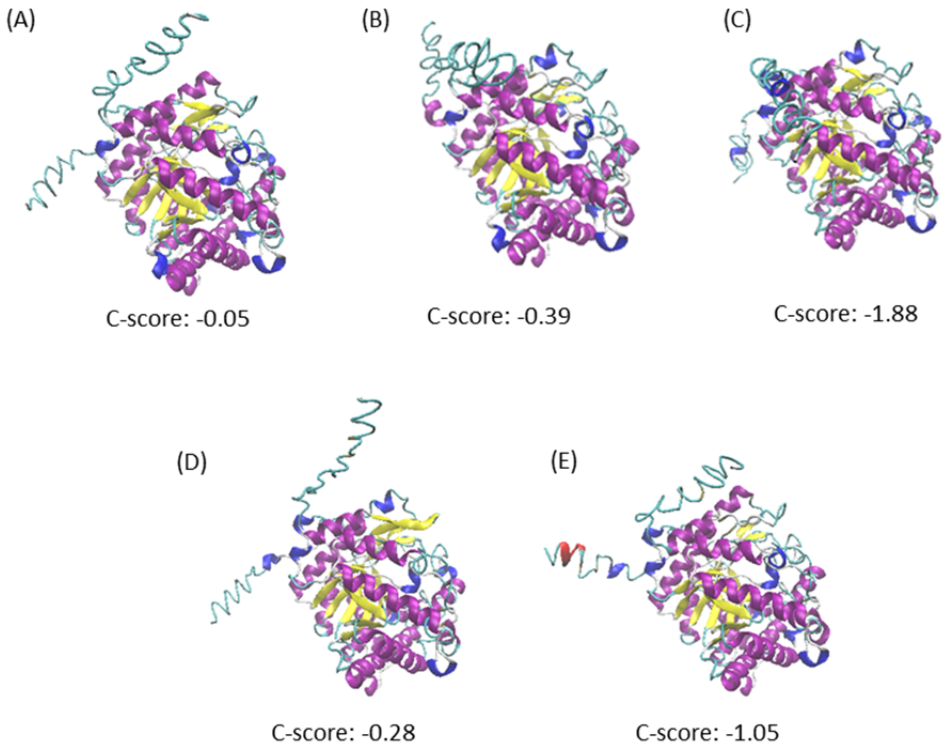

Figure 1: Myrosinase 3D models generated by I-TASSER. Colors in secondary structures represent Alpha Helix: Purple, 3-10 Helix: Blue, π-helix: Red, Beta Sheet: Yellow, Turn: Cyan and Coil: White.

Our results suggest that broccoli myrosinase presents β/α fold in its structure, it has three N-glycosylation sites and two O-glycosylation sites. Currently no structural model or molecular modeling study about broccoli myrosinase are available; however, some structural studies on others *Brassicaceae* varieties are reported. Natarajan et al. (2015) performed molecular modeling of cabbage myrosinase (*Brassica oleracea* var. *capitata*) and investigated its interaction with sinigrin. In that work, the model proposed shared features with the crystallographic structure of *Sinapis alba* myrosinase, showing β/α fold and ten conserved N-glycosylation sites. Kumar et al., (2010) reported a molecular simulations study of the myrosinase-sinigrin interaction in *Brassica juncea*. Their results indicate that the model showed 72% identity with *Sinapis alba* myrosinase, and it possesses seven N-glycosylation sites. Then, our model has similar topological properties of other myrosinases.

In our study, nine substrate conformations were generated. The criteria to select the conformation of the myrosinase – ligand complexes considered the lowest binding energy (Table 1). Figure 2 shows the model structure of the myrosinase – ligand complexes. Docking was performed in a grid set on the neighborhood of the catalytic site of myrosinase. It was observed that the residues responsible for hydrogen bonding between myrosinase and the ligand were: Gln207, Gly431, Ser433, Ser402, Arg214 and Trp477. In turn, the residue involved in the hydrolysis of the β-D-thioglucose bond was Tyr352 (Figure 3 A-C). Other authors proposed glutamic acid (GLU) and glutamine (GLN) as the nucleophilic residues responsible for cleaving the link

between sulfur and glucose (Kumar et al., (2010); Natarajan et al. (2015)). However, our results suggest that these residues form part of the catalytic site and interact with the substrate through van der Waals forces. Then, the enzymatic mechanism of broccoli myrosinase differs from that reported for other *Brassicaceae* myrosinases.

According to Iori et al. (1996), the active site of myrosinase interacts with a bidentate substrate composed by a negatively charged and a hexosidic group with beta configuration. The negatively charged group of glucosinolates is the sulfonated oxime group, and the hexosidic group is the β-D-glucoside moiety.

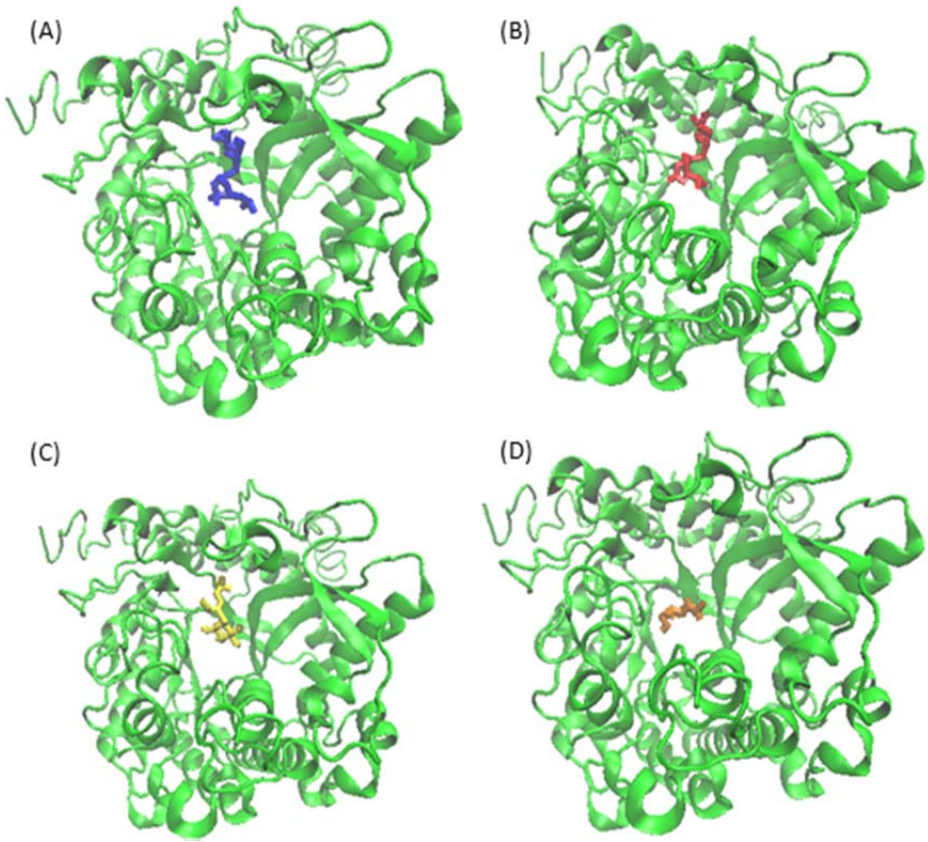

Figure 2: Myrosinase - substrate complexes. (A) Myrosinase-glucoraphanin, (B) Myrosinase-sinigrin, (C) Myrosinase-glucoiberin, (D) Myrosinase-N-acetylglucosamine.

Table 1: Binding energy, dissociation constant (K_D), and binding site of Myr_Bro with glucoraphanin (GRA), sinigrin (SIN), Glucoiberin (GIB) and N-acetylglucosamine (NAG) in Docking studies.

Myr-Ligand	Binding energy (kcal/mol)	K_D (mM)	Binding site
Myr-GRA	-3.80	1.64	Gln207, Arg214, **Tyr352**, Gly431, Ser433, Trp477
Myr-SIN	-4.71	0.35	Gln207, Ile279, **Tyr352**, Ser402, Ser433, Trp477
Myr-GIB	-4.77	0.32	Gln207, **Tyr352**, Gly431, Ser433, Trp477
Myr-NAG	-5.55	0.08	Gln59, His161, Gln207, Glu429, Glu484

Table 1 shows the binding energy, the dissociation constant and the binding sites for the myrosinase complexes. The binding energy is defined as the difference between the energy of the complex and the sum of energies of each molecule separately. It represents a measure of the affinity of the ligand and the enzyme. The dissociation constant is directly related to the bonding energy and tighter binding means more negative binding energy and smaller K_D. Lower affinity means less negative binding energy and larger K_D. The Myr-NAG complex shows the lowest binding energy, which implies that the interaction between NAG and the enzyme would result in the most stable complex. Accordingly, NAG seems to be a powerful inhibitor of broccoli myrosinase.

The active site of myrosinase may contain an acidic group and a nucleophilic anionic group, corresponding to a histidyl residue and a carboxylate group (glutamyl or aspartyl residues), respectively. The presence of a histidyl residue and a carboxylated anionic group would allow both the transfer of a proton from the enzyme to the substrate and the nucleophilic attack of the anomeric carbon of the hexoside group. In the present study, the residues involved in the hydrolysis of glucosinolates were detected to be Gln207, Gly431, Ser433, Trp 477 and Tyr352. These would be the residues responsible for the hydrolysis of the β-thioglucosidic bond the glucosinolates. Turan (2008) reported that myrosinase hydrolyzes the substrate in two steps: glycosylation and deglycosylation, and that it requires participation of the nucleophilic glutamic acid and acid/base catalyst glutamic acid residues. This agrees with the results reported by Burmeister et al. (1997), who studied the crystal structures of *Sinapis alba* myrosinase, and reported that Glu409 is the catalytic nucleophile of the enzyme. On the other hand, Husebye et al. (2005) reported Trp424 as a residue for glucose binding and Tyr180 as a catalytic residue. All these studies about the catalytic site of myrosinases agree with our results, especially Husebye et al. (2005), who reports that tyrosine is responsible for cleaving the β-thioglucosidic bond of glucosinolates.

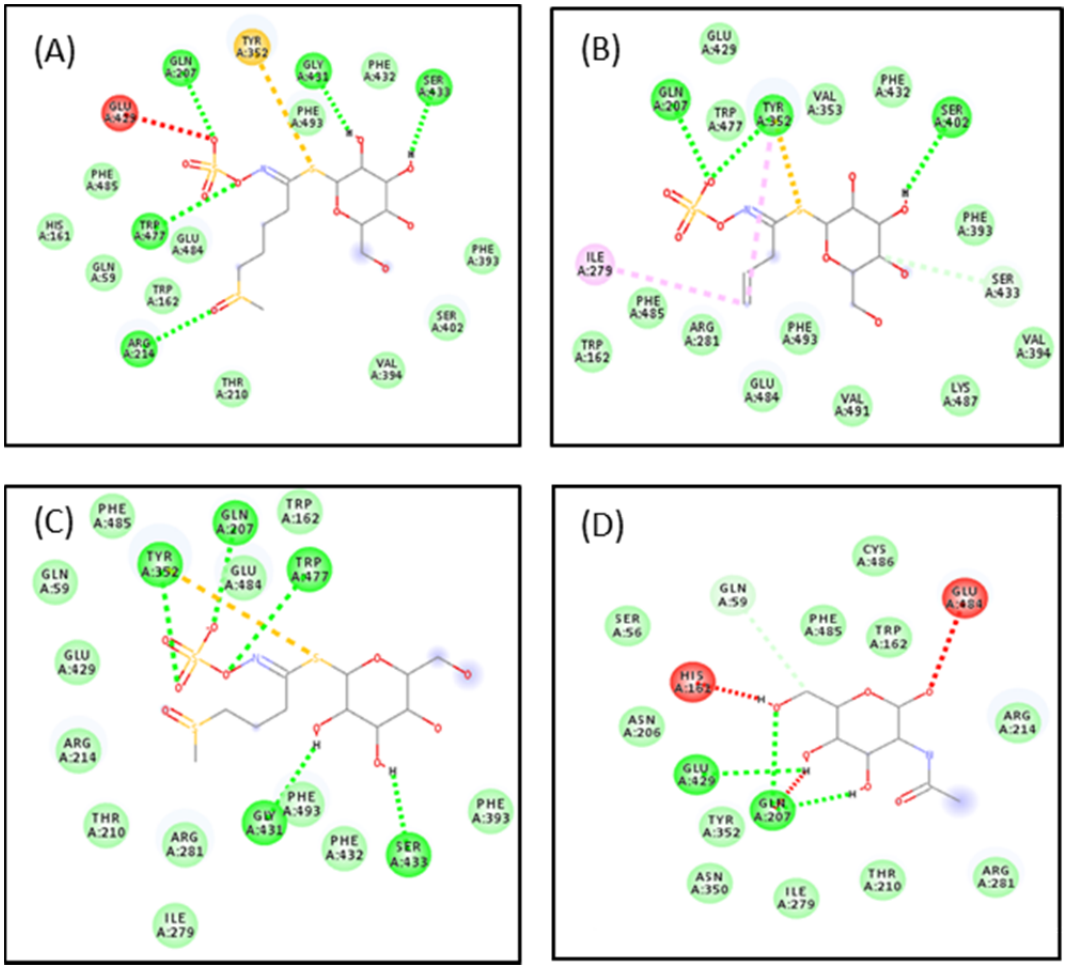

Figure 3: Interaction between myrosinase catalytic residues and (A) glucoraphanin, (B) Sinigrin, (C) glucoiberina, (D) N-acetylglucosamine. Without lines: van der Waals interactions, dotted lines; red: unfavorable acceptor, green: conventional hydrogen bond, coffee: Pi sulfur, purple: alkyl bond.

On the other hand, several potential inhibitors of myrosinase were suggested by I-TASSER, including Tetrazole (NTZ), Maltotetraoza (MAT), Cellotetraoza (CET), β-D-glucopyranose (GPS), 2,4-Dinitrophenol (DNP) and N-acetylglucosamine (NAG). NAG is a monosaccharide derived from glucose, which binds to the active site of myrosinase and interacts with the residues similarly to glucosinolates (Figure 3.D). In the present study we investigated the interaction of NAG with the active site of the enzyme as a possible inhibitor. The results shown in Figure 3 and Table 1 suggest that this substrate homologue interacts with the residues

His161, Glu429, Gln207, Glu484, and Gln59. The stability of the Myr-NAG complex at different pHs should be investigated in the future.

4. Conclusions

The molecular docking simulations of the myrosinase – ligand complexes indicated that the residues responsible for ligand stabilization by hydrogen bonding are Gln207, Gly431, Ser433 and Trp 477. The catalytic residue that responsible for the hydrolysis of the β-D-thioglucose bond would be Tyr352. Besides, the high stability of the Myr-NAG complex suggests that NAG would be a powerful inhibitor of myrosinase. The stability of the Myr-NAG complex at different pHs should be further investigated.

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