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Protein modeling and active site binding mode interactions of myrosinase–sinigrin in *Brassica juncea*—An *in silico* approach

Rajender Kumar[a,*], Sandeep Kumar[b], Seema Sangwan[c], Inderjit Singh Yadav[a], Rakesh Yadav[d]

[a] *Bioinformatics Section, CCSHAU, Hisar 125004, India*
[b] *Directorate of Rapeseed-Mustard Research, Sewar, Bharatpur 321303, Rajasthan, India*
[c] *Department of Biotechnology, Chaudhary Devi Lal University, Sirsa 125055, Haryana, India*
[d] *Department of Bio and Nano Technology, Guru Jambheshwar University of Science and Technology, Hisar 125001, India*

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ABSTRACT

Myrosinase, the only known S-glycosidase, occurs particularly in *Cruciferae* family. It is responsible for the hydrolysis of glucosinolates and serves as a vital element of plant defense system. The biological and chemical properties of myrosinase catalyzed products of glucosinolates are well characterized. The myrosinase-protein-sequence of *Brassica juncea* was retrieved from NCBI database and its 3-D model was generated on the basis of crystal structure of 1MYR-A, 1E4M-M and 1DWA-M chains of myrosinase from *Sinapis alba* by employing Modeller9v7 program. Homolog templates from *S. alba* exhibited 72% identity with target sequence. The model was optimized by using molecular dynamics (MD) approach together with simulated annealing (SA) methods in the same Modeller program, and eventually verified and validated on SAVES (Structure Analysis and Verification Server) and PROCHECK programs, respectively. Ramachandran plot obtained through PROCHECK program depicted that 99.8% of total residues were confined to the allowed region while only one residue (Thr92) was restrained to the disallowed region. Additionally, *B. juncea* myrosinase contains three disulphide bridges which were found to be conserved in *S. alba* homologs as well. Further, overlapping of *B. juncea* myrosinase with that of template protein 1MYR-A from *S. alba* stipulates the amino acid residues Arg115, Gln207, Thr210, Asn350, Tyr352 and Glu429 that constitute active site of the enzyme. Active site analysis also speculates the presence of a hydrophobic pocket in addition to seven N-glycosylation sites. Docking studies of enzyme and substrate illuminate the interactions of various active site residues with diverse groups of sinigrin. Therefore, the present study furnishes the first significant, *in silico* insight into the 3-D structure, active site machinery, and enzyme–substrate interactions of *B. juncea* myrosinase.

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1. Introduction

Myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1), the only known S-glycosidase, is found particularly in family *Cruciferae*, which catalyze hydrolysis of a group of the secondary metabolites glucosinolates. Native glucosinolates are biologically inactive and must be enzymatically hydrolyzed by myrosinase to produce the bioactive compounds. During the course of myrosinase mediated degradation of glucosinolates, the release of glucose is accompanied by the formation of an unstable intermediate. Depending upon the nature of side chains, plant species and reaction conditions, these unstable intermediate are spontaneously decomposes into numerous bioactive compounds such as thiocyanates, isothiocyanates, nitriles, epithionitriles or oxizolidones, etc. [1–3]. Myrosinase is generally spatially separated from the glucosinolates within plant cells [1,4,5]. Upon tissue damage due to mechanical wounding, herbivore or pathogen attack, myrosinase is released at the site of action to catalyze the hydrolysis of glucosinolates [6,7]. This arrangement is referred to as "the myrosinase–glucosinolate bomb" in cruciferous plants. Till now, myrosinase catalyzed hydrolysis of glucosinolates has largely been studied for its antinutritional effects in animal feed [8–10]. But in recent years these compounds have gained considerable attention for their unique biological and chemical properties, such as biodegradable biocides comprising antifungal, nematicidal and insecticidal activities [11–14], deterrents of generalist herbivores [15], herbicidal [16], anticarcinogenic [17–22] and allelopathic action [23]. Myrosinase is also involved in the production of some phyto-hormones from their inactive precursor glucosinolates [1], potentially in sulphur and nitrogen metabolism [24] and growth

* Corresponding author. Present address: Computer Centre, National Institute of Pharmaceutical Education and Research (NIPER), Sector - 67, S.A.S. Nagar 160062, Mohali, Punjab, India. Tel.: +91 9569022156; fax: +91 1722214692.
E-mail address: rajbioinformaticshau@gmail.com (R. Kumar).

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regulation. Further, some of the hydrolytic products could be used as an important intermediate in chemical synthesis [25].

Over 130 glucosinolates have been identified, of which more than 30 are present in *Brassica* species [26]. Earlier, the quantitative as well as qualitative variations for glucosinolate content have been reported among various species of *Brassica*, cultivars of the same species [12,27], and even in different tissues of the same plant with respect to plant age and environmental growth conditions [28,29]. In addition to this, developmental changes have also been observed in myrosinase expression patterns [30] which elucidate that the specific hydrolytic products of glucosinolates are required by the plant under certain situations or developmental stages. Further, production of designer *Brassica* crops with up- or down-regulation for synthesis of specific glucosinolates is a major concern for the breeding programs. Modification of plants is the final target to obtain the optimal combination of myrosinases and glucosinolates. An alternative approach would be to change or regulate the amount of myrosinase available for the hydrolysis of glucosinolates. For this one needs to have specific information about the various components involved in catalysis, mechanism of catalysis and overall function of the system. A structural study of myrosinase–glucosinolate system could be very useful in achieving this goal.

Additionally, bioinformatics has enormous analytical and predictive potential in the field of biochemistry, particularly in establishing correlation between structure and functions of biomolecules. Hence, it can reward a deeper insight into the structural and functional aspects of myrosinase. To date, no reports are available on the structure of myrosinase and its catalytic site in *Brassica juncea*, a major oil-seed crop in India. The current study underlines the first important *in silico* insight into the 3-D structure, active site machinery, and enzyme–substrate interactions of *B. juncea* myrosinase.

2. Theory and methods

2.1. Retrieval of the target protein sequence and alignment with template sequences

The protein sequence of myrosinase (thioglucoside glucohydrolase) from *B. juncea* [31] was retrieved from the NCBI protein sequence database with accession number CAA11412 (http://www.ncbi.nlm.nih.gov/protein). A search of the RCSB Protein Data Bank confirmed that the X-ray crystal structure of myrosinase from *B. juncea* is not publically available. Myrosinase from *B. juncea* is 547 amino acids in length. Primarily, the search began with finding of a number of related sequences by the PSI-BLAST program to reveal myrosinase related three dimensional structures as a template [32]. Three most suitable templates were selected for the study. High-resolution X-ray crystallography structure of the myrosinase (1MYR-A, 1E4M-M and 1DWA-M) from *Sinapis alba* were the selected template proteins [33]. The multiple sequence alignment was performed by using dynamics programming based Align2D module in Modeller [34].

2.2. Protein homology modeling

Program Modeller9v7 was employed to construct the 3D structure of myrosinase. Modeller is simply an implementation of automated approach to comparative modeling by satisfaction of the spatial restraints. After aligning with the help of Align2D, the query and template sequences were used as input in Modeller program and 5 models were generated for myrosinase protein. Modeller derives the restraints automatically from related known structures existing in the database. 3D models were generated by optimization of molecular probable density function. The model with the highest score was validated by the probable density functions. The validated model was chosen for further studies and refinements.

2.3. Model optimization and evaluation

Modeled structure often produces unfavorable bond lengths, bond angles, torsion angles and contacts. Therefore, it was essential to minimize the energy to regularize local bond and angle geometry as well as to relax close contacts in geometric chain. It must, however, be noted that extensive energy minimization should not move coordinates away from the real structure. Therefore, the energy minimization steps should be kept to a minimum. The goal of energy minimization was to relieve stearic collisions and strains without significant alterations in the overall structure. During the course of refinement, key conserved residues and those involved in cofactor binding have to be restrained if necessary. Each model was optimized with the variable target function method (VTFM) with conjugate gradients (CG) followed by further refinement by using the molecular dynamics (MD) with simulated annealing (SA) method [35] in Modeller itself. Eventually, refinement and energy minimization were achieved by using Amber force field. Among the above models, the most acceptable model was finalized with fit data and known parameters such as bond angles, bond lengths, etc. according to Ramachandran plot. After the optimization procedure, the final 3D model of myrosinase was verified by using the Structural Analysis and Verification Server (SAVES) which has various programs such as PROCHECK, WHAT CHECK and VERIFY 3D (http://nihserver.mbi.ucla.edu/SAVES). The stereochemical excellence of the protein structure and overall structural geometry were confirmed by PROCHECK program [36]. Many stereo-chemical parameters of the residues in the model were ensured for their authenticity by WHAT CHECK program [37,38]. The VERIFY 3D program was used to determine the compatibility of an atomic model (3D) with its own amino acid sequence (1D) by assigning a structural class on the basis of its location and environment (alpha, beta, loop, polar, non-polar, etc.) as well as comparing the results with good database structures [39].

2.4. Active site analysis

Usually, active site structure is highly conserved among distantly related enzymes. The catalytic activity of an enzyme is performed by a small, highly conserved constellation of residues within the active site. Active site identification included the superimposition of the model with template which provided integrity of the homology model and assisted in positioning conserved active site residues. Active site information was obtained for template structure from Catalytics Site Atlas (CSA) database of European Bioinformatics Institute (http://www.ebi.ac.uk/thornton-srv/databases/CSA/) [40]. The model was overlapped with the template to obtain active site information for the modeled structure and conserved active site residues were also verified manually in multiple-aligned-sequences.

2.5. Docking study of modeled myrosinase

The docking study was performed by using AutoDock4.2 program. AutoDock is a Monte Carlo simulated annealing based technique for the configurational exploration with the rapid energy evaluation using grid based molecular affinity potentials. Thus, it combines the dual advantages of a large search space and a robust energy evaluation [41,42]. The AutoDock scoring function is a subset of the AMBER force field that inclines the molecules by using United Atom model. Sinigrin (2-propenylglucosinlate) was prepared for docking study by adding the polar hydrogens and

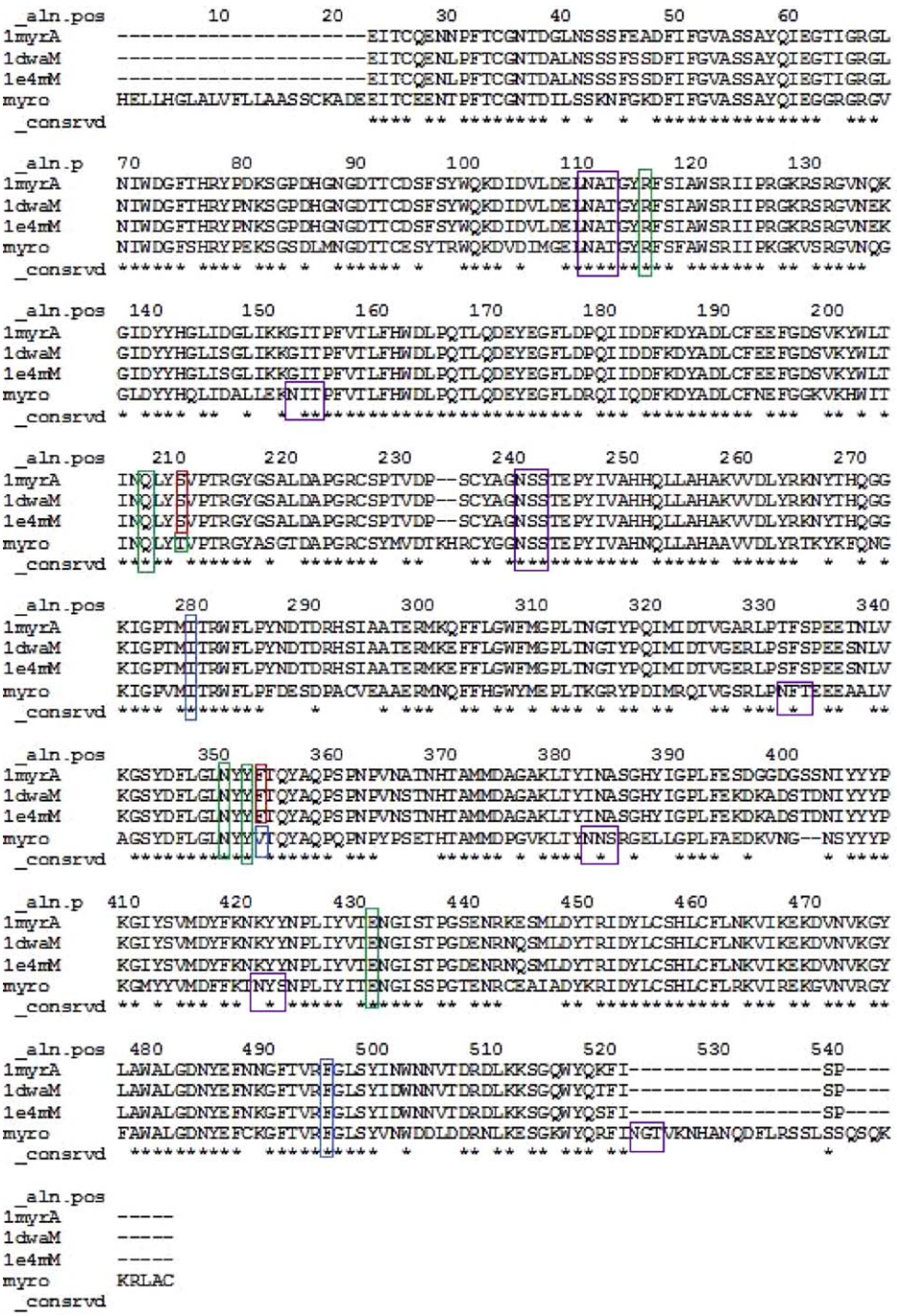

Fig. 1. Multiple sequence alignment of target (myro) and templates (1 myr-A, 1 dwa-M and 1 e4m-M). The important residues from active site point of view are highlighted with green rectangles, hydrophobic residues with blue rectangles and residues constituting N-glycosylation sites with violet rectangles. The residual variations in active site are highlighted with red rectangles.

partial charges, and defining the 11 rotatable bonds and atom types that would be explored during docking. Modeled protein was also prepared by adding polar hydrogens and merging non-polar hydrogens. Ultimately, Kollman united atom charge and atom type parameter were added. Grid map dimensions were set to the surrounding of active site and also to the significant portion of the surrounding surface. Lamarckian genetic search algorithm was employed and docking run was set to 30. All other parameters were set to default values such as maximum number of energy evaluation was 25,000,00 per run and maximum number of generation in the genetic algorithm was increased to 27,000.

3. Results and discussion

3.1. Protein homology modeling

Homology search for *B. juncea* myrosinase resulted into a large number of sequences by running PSI-BLAST against PDB database. The target sequence showed high identity (72%) with 1MYR-A, 1E4M-M and 1DWA-M chains of myrosinase from *S. alba* used as templates in multiple sequence alignment performed using align2D module in the Modeller as described in Fig. 1. The large sequence similarity of *B. juncea* myrosinase with *S. alba* strongly advocates that these two

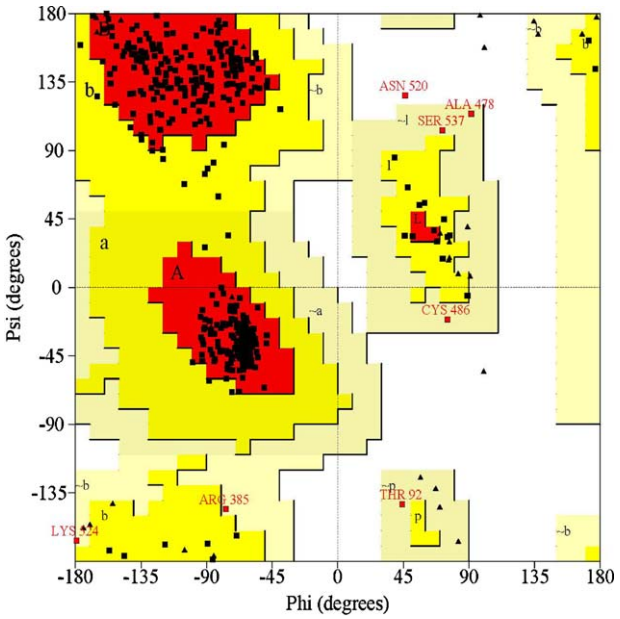

Fig. 2. Ramachandran plot of myrosinase model obtained by PROCHECK validation package.

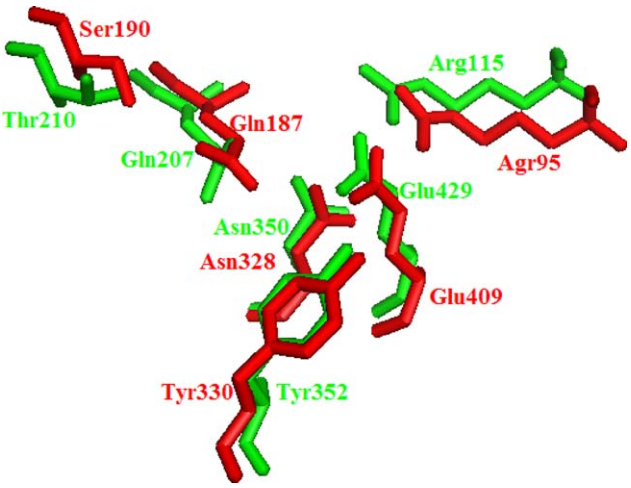

Fig. 3. Superimposition of active site residues of template 1 MYR-A and modeled protein. Green and red color sticks represents modeled and template proteins, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

enzymes have evolved from a common ancestor, particularly O-glycosidase.

3.2. Myrosinase structure

The other reason to select myrosinase from *S. alba* as template for structural analysis was the availability of its X-ray crystallographic structure. After the optimization and energy minimization process, the best model was selected among five 3-D models generated for *B. juncea* myrosinase on the basis of Modeller score. Energy minimization of 3-D structure is vital for providing the maximum stability to the protein. Ramachandran plot drawn through PROCHECK program validated the model with 98.5% of the total residues in the allowed region and 1.3% in the generously allowed region (Fig. 2). This stipulates that protein backbone dihedral angles phi (φ) and psi (ψ) occupied reasonably accurate positions in the selected 3-D model. Only one residue, Thr92 was located in the disallowed region, which constituted 0.2% of the total protein. Further, it was not a part of the active site under investigation. The selected 3-D model structure of *B. juncea* myrosinase was also compared against protein database (PDB) using DaliLite V.3 server [43] illustrated RMSD 0.3, identity 72% and Z score value 64.6 with template 1MYR-A. Like other enzymes particularly in several families of related O-glycosidases, the 547 amino acid residues of *B. juncea* myrosinase were folded into a single $(\beta/\alpha)_8$ barrel [44–46]. Three disulphide bridges (Cys226-Cys236, Cys26-Cys458 and Cys34-Cys454) were evident in the conceptual model of myrosinase. These bridges probably confer additional stability to the enzyme. The carbohydrate chains were scattered over the entire surface of myrosinase dimer. The overall structure of myrosinase from *B. juncea* was found to be extremely similar to that of *S. alba* as revealed by superimposing 3-D structures. These features conclude that the two myrosinases of different origins have evolved from a common ancestor and play a pivotal role in plant defense mechanisms.

3.3. Active site analysis

Active site information was obtained through superimposing 3-D model structure of the target enzyme with that of template protein 1MYR-A from *S. alba*, which provided reliability of homology between the structures, and also aided in positioning the conserved active site residues. Active site information of template structure was deduced from Catalytic Site Atlas (CSA) database of European Bioinformatics Institute (http://www.ebi.ac.uk/thornton-srv/databases/CSA/) and overlapped with that of target protein (Fig. 3). Active site of modeled myrosinase was constituted by amino acid residues Arg115, Gln207, Thr210, Asn350, Tyr352 and Glu429 corresponding to Arg95, Gln187, Ser190, Asn328, Tyr330 and Glu409 of template protein. Only one residue, i.e. Ser was found to be replaced with Thr, an amino acid from the same group. Thus, active site forming residues were found to be highly conserved among myrosinases (Figs. 1 and 3). Active site analysis also showed the presence of a hydrophobic pocket in the target protein constituted by residues Val353, Phe393, Phe495, Ile279 and Tyr352. The amino acid residue Phe331 of *S. alba* was found to be replaced by Val353 in target protein. Hydrophobic pocket constituting residues were also observed to be strictly conserved among myrosinases (Fig. 1). Tyr352 was also conserved, not only in myrosinases but also in a large number of related O-glycosidases [47].

The catalytic nucleophile Glu429 in target protein was corresponding to the catalytic nucleophile Glu409 in template protein whereas the second catalytic residue Gln207 was corresponding to Gln187 of template protein. Myrosinase belongs to family 1 of the glycoside hydrolases [45,48,49], but is an unusual member of this family because of lacking the second catalytic glutamate or acid/base residue of the O-glycosyl hydrolases in its active site [33]. The fact that the glutamate or acid/base residue of O-glycosidases is replaced by a glutamine and no other residue in all known myrosinase sequences advocates for a possible specific role of this residue. In catalytic site of target protein, Arg115 was corresponding to Arg95 of template protein. Burmeister et al. [33] reported that the charge of Arg95 residue in *S. alba* myrosinase becomes buried upon formation of glycosyl–enzyme complex and may destabilize the complex. It has been revealed that superimposition of active site residues from the two enzyme structures was perfect (Fig. 3).

Sequence comparison between target and template protein has also provided useful information about the active site residues (Fig. 1). The amino acid residues Asn186 and His141 of template protein which recognized the O_2 atom of glucose in sinigrin substrate molecule were also found to exist in target protein as Asn206 and His161, respectively. The sulphate group of the substrate is

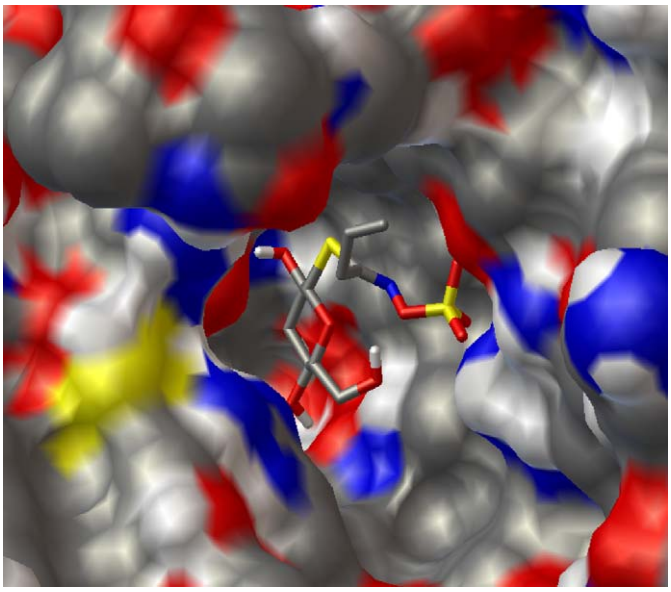

Fig. 4. Best fitted, docked conformation pose of sinigrin into active site cavity of modeled protein.

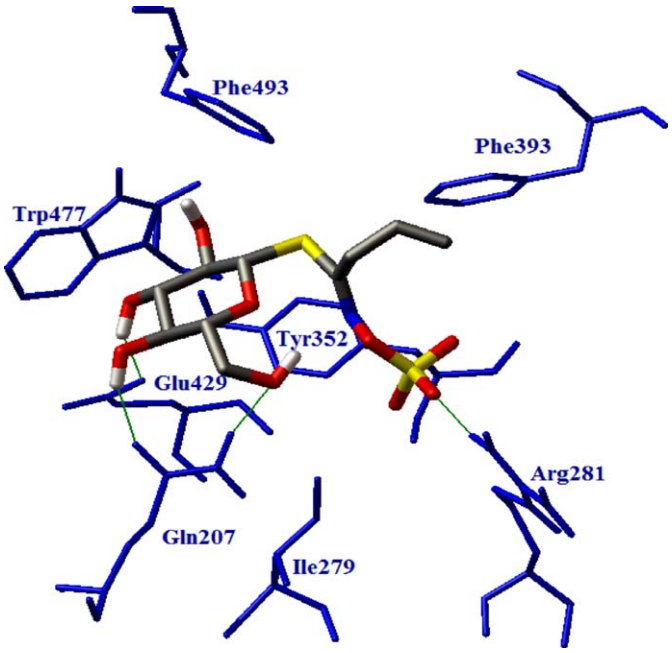

Fig. 5. Ligand–protein interactions as obtained by docking studies. Blue color sticks represents the active site residues whereas various atoms of sinigrin are represented by multicolored sticks. H-bonding between active site residues and sinigrin is shown by green lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

probably the site of a specific recognition which is most likely to be achieved by residues Arg194 and Arg259 in case of *S. alba* myrosinase which are strictly conserved among myrosinases but absent in the related β-glucosidases [33]. These residues were present at positions Arg214 and Arg281 in target protein and their interaction was addressed with sulphate group of substrate under docking studies. Myrosinase is a dimer and the two subunits are stabilized by a Zn^{2+} ion existing in a tetrahedral coordination with four residues, His56 and Asp70 from each subunit (33). The residues corresponding to His56 and Asp70 of *S. alba* myrosinase were also found to be conserved in the target protein as His76 and Asp90 as specified in Fig. 1.

3.4. Glycosylation sites

In conceptual myrosinase model, seven N-glycosylation sites were reported at residues 110, 152, 240, 331, 382, 419 and 520. These sites were found to be conserved with Asn-x-Thr/Ser amino acid sequence and displayed electron density for carbohydrates (Fig. 1). The number of glycosylation sites (seven) was virtually close to that predicted for other myrosinases (five to six) but less than that predicted for *Arabidopsis thaliana* (nine) and *S. alba* (ten). The surface of myrosinase was highly glycosylated which is needed to maintain molecular stability and solubility in the dehydrated environment.

3.5. Docking of substrate into the active site

Docking studies provided valuable information about the residues involved in enzyme–substrate interaction. For such interaction studies, the most important requirement was the proper orientation and conformation of substrate (sinigrin in this case) which fitted to the enzyme (myrosinase from *B. juncea*) active site appropriately and formed enzyme–substrate intermediate complex where optimum interactions took place between the two followed by breaking of the complex into products. Therefore, optimal interactions and the best autodock score were used as criteria to interpret the best conformation (Fig. 4) among the 30 conformations, generated by AutoDock program. Sinigrin molecule with a twisted boat conformation for the glucose ring fitted the active site in a perfect manner (Fig. 5). Our findings of docking studies were in accordance with earlier results of Sulzenbacher et al. [50] and Davies et al. [51].

Interaction studies also showed the presence of a hydrophobic pocket in the active site formed by residues Val353, Phe393, Phe495, Ile279 and Tyr352. These amino acid residues were found to be conserved among myrosinases (Fig. 1). This pocket was occupied by hydrophobic part of substrate, i.e. allyl in case of sinigrin as depicted in Fig. 5. Sulphate group of sinigrin, which has a specific recognition site for myrosinase, interacted with Arg214 and Arg281 (Fig. 5), which were also found to be conserved among myrosinases (Fig. 1) as described earlier. Hydrogen bonding of sinigrin with Gln207 (two H bonds) and Glu429 (one H bond) amino acid residues of target protein was also reported as shown in Fig. 5.

Docking studies provided the best conformation of sinigrin in the active site of modeled protein with estimated free binding energy of −7.33 K cal/mol at temperature 298.15 K. Various interactions took place between different groups of substrate and enzyme residues included –SO–HN Arg281, HO–HN Gln207, OH–O=C Gln207, and OH–O=C Glu429 were obtained with bond length 2.14 Å, 2.16 Å, 1.98 Å and 2.23 Å, respectively.

3.6. Probable mechanism of catalysis

Myrosinase catalyzed reaction involves two steps, i.e. glycosylation and deglycosylation of the enzyme. In glycosylation step, Glu429 residue of target protein corresponding to Glu409 of template protein was predicted to be involved in nucleophilic attack on the anomeric carbon of the sinigrin and led to the formation of a glycosyl–enzyme. In deglycosylation step, the glutamine residue that replaces the catalytic glutamate residue of the classical β-glucosidases might ensure the correct positioning of a water molecule, without deprotonating it to hydrolyze the glycosyl–enzyme. This positioning of water molecule as well as more reactive glycosyl–enzyme of myrosinase are sufficient to be hydrolyzed by water molecule without any base assistance [33]. Further, the excellent ability of the aglycon of glucosinolate to act as

a leaving group seems to make acid catalysis unnecessary [52]. The role of catalytic acid/base (second glutamate of β-glucosidases) is believed to have been replaced by ascorbate that enters the empty active site left by the aglycon and activates the water molecule [53]. Therefore, myrosinase catalyzed reactions in *B. juncea* has not seemed to take place via. acid–base catalysis. Similar findings have been reported for myrosinase from *S. alba* by Burmeister et al. [33]. Many researchers have also reported similar mode of catalysis in myrosinase catalyzed reactions [54–56].

4. Conclusion

Higher sequence and structural similarity is obtained between myrosinases from *B. juncea* and *S. alba* indicate that both have evolved from a common ancestor. Modeled myrosinase-structure-backbone dihedral angles phi (φ) and psi (ψ) occupied reasonably accurate positions. Only one residue, Thr92 was found to be present in the disallowed region, which merely constituted 0.2% of the total protein. The structural stability to the enzyme has been provided by: dimeric form, a massive glycosylation and three disulphide bridges (Cys226-Cys236, Cys26-Cys458 and Cys34-Cys454). Active site of modeled myrosinase has been constituted by amino acid residues Arg115, Gln207, Thr210, Asn350, Tyr352 and Glu429. Within modeled myrosinase structure, seven N-glycosylation sites were reported at the residues 110, 152, 240, 331, 382, 419 and 520. These sites were found to be conserved with Asn-x-Thr/Ser amino acid sequence and illustrated electron density for carbohydrates. Myrosinase of *B. juncea* has specific recognition and catalytic sites for the sinigrin. Myrosinase–sinigrin interaction studies also depicted the presence of a hydrophobic pocket in the active site that was formed by residues Val353, Phe393, Phe495, Ile279 and Tyr352. This pocket was occupied by hydrophobic part of substrate, i.e. allyl in case of sinigrin. Sulphate group of sinigrin, which had a specific recognition site for myrosinase, interacted with Arg214 and Arg281, which were also reported to be conserved among myrosinases. Modeled protein tended to form hydrogen bonding with Gln207 (two H bonds) and Glu429 (one H bond) amino acid residues of sinigrin. Further, myrosinase catalyzed reactions didn't take place via. acid–base catalysis in *B. juncea*. Conclusively, the present study has furnished a first important *in silico* insight into the 3-D structure, active site machinery as well as enzyme–substrate interactions of *B. juncea* myrosinase.

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