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Structural characterization and substrate specificity of VpAAT1 protein related to ester biosynthesis in mountain papaya fruit

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ABSTRACT

The aroma in fruits is an important attribute of quality that influences consumer's acceptance. This attribute is a complex character determined by a set of low molecular weight volatile compounds. In mountain papaya fruit (*Vasconcellea pubescens*) the aroma is determined mainly by esters, which are produced through an esterification reaction catalyzed by the enzyme alcohol acyltransferase (AAT) that utilizes alcohols and acyl-CoAs as substrates. In order to understand the molecular mechanism involved in the production of esters in this fruit, an AAT gene which has been previously cloned and characterized from mountain papaya (*VpAAT1*) was expressed in yeasts, and the highest enzyme activity of the recombinant protein was obtained when the enzyme was tested for its ability to produce benzyl acetate. On the other hand, to gain insight the mechanism of action at the molecular level, a structural model for VpAAT1 protein was built by comparative modelling methodology, which was validated and refined by molecular dynamics simulation. The VpAAT1 structure consists of two domains connected by a large crossover loop, with a solvent channel in the center of the structure formed between the two domains. Residues H166 and D170, important for catalytic action, displayed their side chains towards the central cavity of the channel allowing their interaction with the substrates. The conformational interaction between the protein and several ligands was explored by molecular docking simulations, and the predictions obtained were tested through kinetic analysis. Kinetic results showed that the lowest K_M values were obtained for acetyl-CoA and benzyl alcohol. In addition, the most favorable predicted substrate orientation was observed for benzyl alcohol and acetyl CoA, showing a perfect coincidence between kinetic studies and molecular docking analysis.

1. Introduction

Mountain or highland papaya fruit is characterized by its intense aroma [1]. This fruit is native to South America and widely distributed throughout the Andes [2,3]. The specie was introduced to Chile more than 50 years ago and is currently cultivated in the Coquimbo and Valparaiso valleys and along the coast of the Maule Region. The papaya cultivated in Chile belongs to the *Caricaceae* family and corresponds to *Vasconcellea pubescens* (synonyms of *Vasconcellea cundinamarcensis*, *Carica pubescens*, initially called *Carica candamarcensis*) [2,3]. The fruit displays a climacteric behavior, and exhibits the characteristic rise in ethylene production during ripening, followed by softening, changes in color and rise in aroma production [1,4].

The aroma of fruits is determined by a large number of volatile compounds, and its biosynthesis depends on many factors, such as cultivar, ripening stage and postharvest conditions [5–7]. *V. pubescens*'s aroma is determined mainly by esters and in lower proportion by alcohols and aldehydes present at the ripe stage [1]. Alcohol acyltransferase (AAT), that catalyzes the esterification reaction between alcohols and acyl-CoAs, is the key enzyme involved in ester biosynthesis in this specie [8]. Recently, a gene encoding for an alcohol acyltransferase (VpAAT1) has been isolated and characterized in ripening mountain papaya fruit [8]. VpAAT1 is member of the BAHD superfamily and belongs to subfamily III [8], sharing the group with other AATs that participate in the synthesis of volatile compounds during ripening of fruits such as melon [9]. The first member from the BAHD superfamily which has a crystal structure is vinorine synthase, an acetyl transferase from *Rauvolfia serpentine* [10]. Members of the BAHD superfamily share the HXXXD motif, located in the middle of the protein sequence, which is highly conserved in higher plants and yeasts. The substitution of the histidine and aspartic residues from this motif causes the loss of enzyme function in vinorine synthase [11], which suggests that it could be

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involved in the transfer of the acyl group from acyl-CoA towards the alcohol.

Despite the knowledge generated in the past few years, we still have a limited understanding of the mechanisms controlling the synthesis of aroma volatiles. To clarify the molecular mechanism used by VpAAT1 it is important to know the interaction of the protein and its ligands, however no structural information has been reported for any AAT protein until now.

The understanding of the catalytic properties of the enzymes involved in the production of volatiles in a particular fruit could allow the design of additional strategies to improve its flavor during storage, shipping, marketing or processing. In this sense, the aim of this work was to go further in the structural and biochemical characterization of VpAAT1 protein, by using both bioinformatics (molecular modelling and docking simulations) and biochemical tools (determination of kinetics parameters).

2. Materials and methods

2.1. The 3D structure of VpAAT1 using homology modelling

The cDNA sequence of VpAAT1 was isolated and characterized recently (GenBank accession number FJ548611) [8]. The 3D model of VpAAT1 protein was built by homology modelling based on high-resolution crystal structures of homologous proteins. BLAST (Basic local alignment search tool) search and PSIPRED (Protein Structure Prediction Server) [12] were used for selecting the 3D models with the closest homology available in the Brookhaven Protein Data Bank (PDB). It resulted in the selection of the crystal structure of vinorine synthase protein (PDB: 2BGH) as template, that shares a moderate sequence identity with VpAAT1. The first requirement in the construction of VpAAT1 model is the sequence alignment with the template, focussed on the identification of structurally conserved regions (SCR) common to the template and the target. The pair-wise sequence alignment was obtained by using ClustalW software to build the initial model. The comparative model of VpAAT1 structure was generated by using the software MODELLER 9v5 (http://salilab.org/modeller/) [13]. Fifty models were generated and the model showing the lowest modeller objective function and rms deviation with respect to trace (Cα atoms) of the crystal structure of the template, was saved for further refinement and validation. Further, refinement was performed in order to obtain the best conformation of VpAAT1 structure resulting from MODELER. Structural refinements were accomplished by molecular dynamics simulations (MDS) and equilibration methods using Nano Molecular Dynamics (NAMD 2.6) software [14] and Chemistry of Harvard Molecular Modelling (CHARMM27) force field for lipids and proteins [15,16] along with the TIP3P model for water [17]. The tautomeric states of histidine residues in the model were assigned according to the local environment. After an initial minimization, the system was subjected to a short molecular dynamics run in order to remove wrong contacts and to fill empty pockets. All backbone atoms were restrained using a harmonic force constant of 5 kcal mol^{-1} $Å^{-2}$ and the loops were left free during relaxations. All MDS were done using a time step of 1 fs, with a 12 Å spherical cut off for non-bonding interactions and a switching function of 10 Å for the van der Waals term. The structure obtained was embedded into a periodic bordering condition (PBC) box (92 Å × 86 Å × 94 Å) contained in a 150 mM NaCl solution at 300 K. Under these conditions, a 2 ns MDS was performed using NAMD v2.6 program. Particle Mesh Ewald (PME) was employed to calculate long-range electrostatic interactions. Finally, to evaluate the accuracy of the model ProSA [18], PROCHECK (http://nihserver.mbi.ucla.edu/SAVS/) [19] and Verify3D [20] were employed.

2.2. Docking studies

Docking studies were performed to predict the putative binding of a group of acyl-CoAs and different alcohols. For this purpose, two different docking investigations using the Internal Coordinate Mechanics (ICM) program [21] (www.molsoft.com) were carried out. ICM is based on Monte Carlo simulations in internal coordinates to optimize the position of molecules using a stochastic global optimization procedure combined with pseudo-Brownian positional/torsional steps and fast local gradient minimization [21]. The scoring function includes electrostatic, hydrogen bond, hydrophobic, and two van der Waals terms, and the energies are computed using MMFF partial charges with the ECEPP/3 force field [21]. The active site of VpAAT1 enzyme was defined as the pocket that contained the HXXXD motif (H166, D170), which is located in the middle of the protein sequence and is highly conserved in superior plants and yeasts. Additionally, His166 of the HXXXD motif has been reported as a general base during catalysis [10,22].

The first docking study was done with acyl-CoA as acyl donor bound to the enzyme VpAAT1. The second docking run was performed with the alcohol. In order to verify that the order of entrance of ligands had no significant effect on the stability of the final complex, the docking study was performed in reverse order. The first one was done with alcohol bound to the enzyme VpAAT1 and the second docking run was performed with the acyl-CoA as acyl donor. In each case, 10 different docking arrangements were produced. The translation/orientation of all-atoms in the ligands was sampled in a completely unrestricted way. This conformation was obtained by Monte Carlo movements and local minimization using potentials of interaction pre-calculated on a 3D grid from the receptor (VpAAT1 protein). The conformations as result of Rigid-body docking were sorted by total binding energy, which include van der Waals, electrostatics, and hydrogen bonding potentials. The result was optimized by flexible docking. The VpAAT1 binding mode with each substrate pair was employed in docking simulations to assess the correlation between enzyme activity and the stability of the substrate–enzyme complex.

2.3. Expression of recombinant VpAAT1

The full-length sequence of VpAAT1 was initially cloned into pYES2.1 TOPO-TA cloning vector containing a C-terminal polyhistidine tag following the instructions provided by the manufacturer (Invitrogen). The construct was cloned in *Escherichia coli* TOP 10 One Shot (Invitrogen) and sequenced by Macrogen Inc. (Seoul, Korea). Once the right orientation of insert was confirmed, the construction was used to transform the *Saccharomyces cerevisiae* cell line INVSc1. Transformed yeasts were grown at 30 °C in selective medium (SC-U) with 2% galactose as inducer of the expression of recombinant protein, with constant stirring and bubbling of sterile air until OD_{600} of the culture reached <1 U.

2.4. Purification of recombinant VpAAT1

The purification of recombinant protein was carried out according to the method of El-Sharkawy et al. [9] with modifications [8]. Yeast cells collected were suspended in 2 mL of phosphate buffer (50 mM sodium phosphate (pH 7.5), 10% (v/v) glycerol, 0.3 M NaCl) containing 2 mM β-mercaptoethanol, and mechanically ground in liquid nitrogen. The crude extract obtained was purified through a Talon Metal Affinity column (BD Biosciences), an affinity column designed to purify polyhistidine-tagged proteins, according to the manufacturer's protocol. The recombinant protein was eluted with phosphate buffer containing 150 mM imidazole. Proteins were quantified according to Bradford [23] and visualized through 10% SDS-PAGE gels.

2.5. Assay of AAT activity and kinetic parameters

AAT activity of recombinant VpAAT1 protein was quantified by its ability to convert alcohols and acyl-CoAs into the corresponding ester [24,25]. AAT activity was assayed in 500 μL of total volume in the presence of 2 mM benzyl alcohol and 250 μM acetyl-CoA in 50 mM Tris–HCl (pH 7.5) buffer containing 10% (v/v) glycerol and 1 mM dithiothreitol (DTT) [8,9]. The reaction was initiated by addition of 35 μg purified protein, and the mixture was incubated at 30 °C for 2.5 h in sealed Eppendorf tubes. The reaction was stopped by the addition of 50 mg of citric acid and 185 mg of KCl, and after mixing during several minutes, the supernatant was transferred to a glass vial, which was sealed after the addition of 0.5 μL of 1,2-dichlorobenzene as internal control. The solution was stirred during 15 min at room temperature; meanwhile the volatiles produced during the enzymatic reaction were released into the headspace and adsorbed onto an SPME fiber (PDMS/DVB). The separation and quantification of each ester was done by a gas chromatograph fitted with a flame ionization detector (GC-FID) (Perkin-Elmer, Clarus 500) as described previously [1]. A calibration curve was prepared for benzyl acetate. AAT enzyme activity was expressed as pmol h^{-1} per milligram of protein. Determinations were performed in triplicate and expressed as mean ± SE.

For determination of K_M and *V*max values standard assay mixtures were prepared using 35 μg of purified protein. The Michaelis–Menten saturation curve was built using substrate concentrations between 0 and 8 mM for benzyl alcohol and between 0 and 4 mM for acetyl-CoA. The K_M and *V*max values were calculated from Lineweaver–Burk plots.

3. Results

3.1. The homology modelling of VpAAT1

The initial pair-wise alignment of the protein template (2BGH) against the target sequence (VpAAT1) with 21.5% of sequence identity was optimized manually, incorporating information on the secondary structure of the BAHD superfamily, especially in relation to conserved motifs (e.g., the catalytic HXXXDG and DFGWG motifs) which were spatially restricted during the modelling to avoid any distortion of them (Fig. 1). The final unperturbed conformations of the model were acquired by progressively relaxing parts of the initial model (Fig. 2). The model obtained showed that the protein structure is composed of 15 β-sheets, 14 α-helices (11 α-helices and three 3_{10}-helices) and 23 loops. Furthermore, the VpAAT1 structure consists of two approximately equal-sized domains (Fig. 3). The domains are connected with a large crossover loop (residues 210–232) that spans nearly 37 Å. Domain 1 contains a mixed 7-stranded β-sheets (β1–β7), which is covered on both sides by 7 helices (five α-helices, Hα1–Hα5; two 3_{10}-helices, 3_{10}H1–3_{10}H2), and domain 2 contains 7 helices (six α-helices, Hα6–Hα11; one 3_{10}-helice, 3_{10}H3) and a mixed 8-stranded β-sheets (β8–β15) (Fig. 3). Additionally, the model showed a solvent channel in the center of the 3D structure of VpAAT1 that is formed between the two domains by two loops which protrudes from domain 2 to contact domain 1 (Fig. 3). The first loop is located between the β-sheets 9 and 10 of domain 2. The second loop is situated between the β-sheets 12 and 13 also in domain 2. In this channel the important residues for catalytic action are located; these residues are in the HTMSD segment and located in the loop 13 between the β-sheet 7 and the α-helice 4 in the center of this solvent channel. The catalytic site is formed by the residues H166 and D170 with their side chains oriented to the central cavity of the channel in the protein, and they most probably interact with the substrates alcohols and acyl-CoAs (Fig. 2). Whereas the other three residues, located between the two catalytic ones, have their side chains oriented inward of the enzyme core and not exposed to the solvent channel.Another highly conserved region within the BAHD acyltransferases is the DFGWG motif near the C terminus. The analysis of VpAAT1 structure reveals that the DFGWG motif is remote from the active site, and therefore it is unlikely to have a direct role in either substrate binding or catalysis.

Table 1
Validation of VpAAT1 protein structure using PROCHECK program (Ramachandran Plot).

	Core (%)[a]	Allow (%)[b]	Gener (%)[c]	Disall (%)[d]
VpAAT1	90.1	7.8	2.1	0

[a] Most favorable region.
[b] Additional allowed regions.
[c] Generously allowed regions.
[d] Disallowed regions.

3.2. Model evaluation

To evaluate the geometric and energetic stability of the VpAAT1 model, 10 conformers with the lowest total energy were selected after energy minimization. The best conformer was determined by using different evaluation methods. The stereochemical quality of the VpAAT1 model was analyzed with PROCHECK. The most favored regions correspond to 90.1% of the structure and no badly modelled residues were found (Table 1) that validates the stereochemistry of the model. On the other hand, ProSA analysis displayed a local model quality by plotting energies as a function of amino acid sequence position *I*. This analysis showed a low ProSA energy in most structurally conserved regions, however there is a small region with unfavorable ProSA energy: this region corresponds to a loop distant from the active site and out of the protein solvent channel (Fig. 4a). Finally, an analysis using Verify3D program showed favorable scores for the entire structure of the three-dimensional structure of VpAAT1 protein and no residues showing values under 0 (Fig. 4b). The final structure of VpAAT1 protein was accepted for subsequent analysis.

3.3. Ligand binding analysis

The refined VpAAT1 model was used to study protein–ligand conformations by automatic docking for seven substrates (three acyl-CoAs and four alcohols). Substrates were chosen based on the results of activity previously described [8]. As shown in Table 2 the lowest ligand binding energies were found for acyl-CoAs (values between −203.5 and −212.0 kcal mol^{-1}), and binding energy for alcohols were less favorable (ranging from −41.4 to −22.8 kcal mol^{-1}). The strongest binding interaction was found between VpAAT1 protein and hexanoyl-CoA.

To obtain the ternary complex, the acyl-CoAs substrates were used to form a complex with VpAAT1 first, and then, the alco-

Table 2
Affinity energy of VpAAT1 protein model towards different acyl-CoAs and alcohols as substrates.

Substrate	Affinity energy (kcal mol^{-1})	rmsd (Å)[a]
Ethanol	−22.8	0.1
Butanol	−33.8	1.6
Benzyl alcohol	−35.7	2.7
Hexanol	−41.4	2.2
Acetyl-CoA	−203.5	1.8
Butanoyl-CoA	−206.9	0.2
Hexanoyl-CoA	−212.0	2.1

[a] rms deviation was calculated for the backbone (Cα, C, N) atoms.

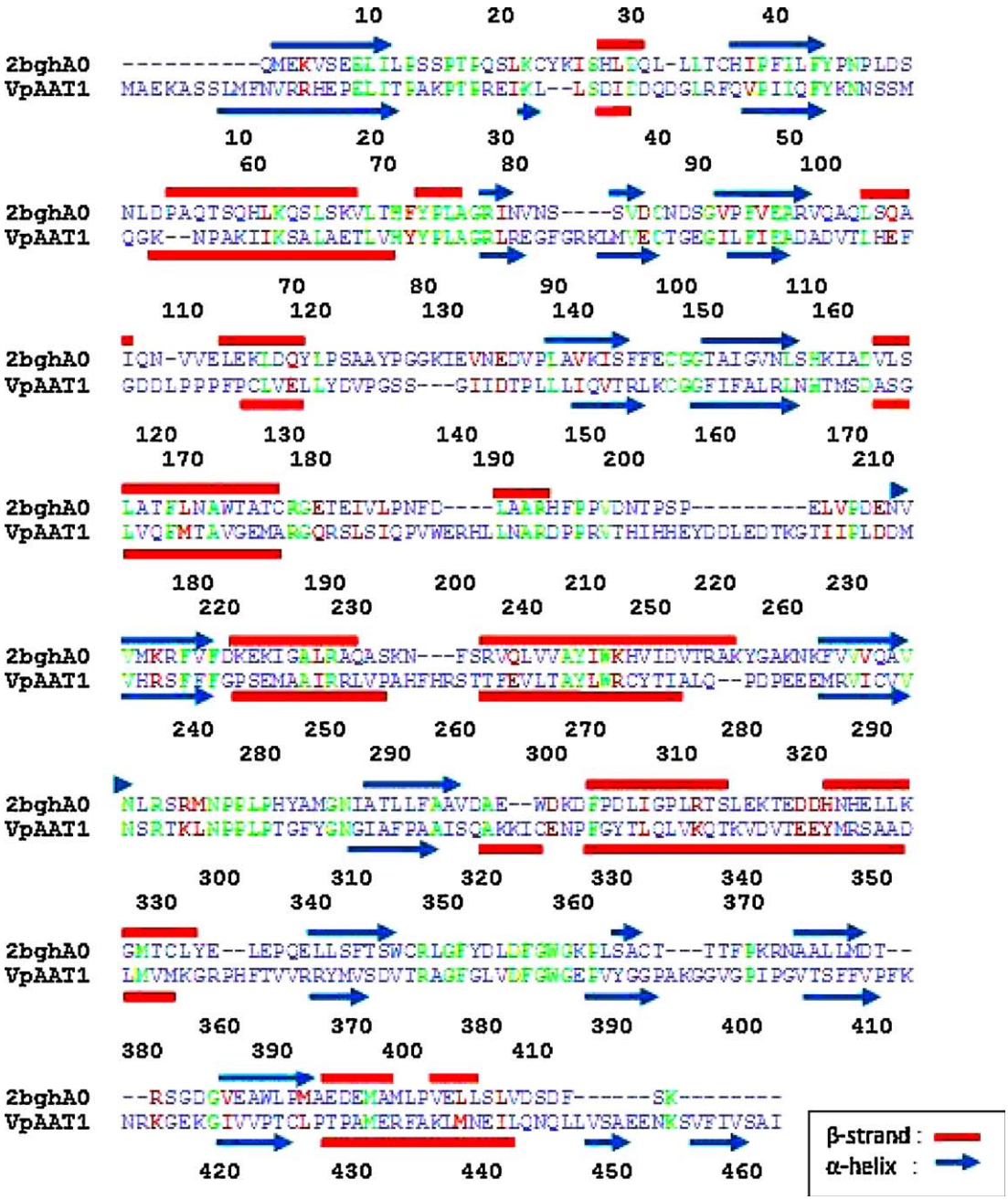

Fig. 1. Sequence alignment and secondary structure predictions of VpAAT1 protein. VpAAT1 amino acid sequence was aligned with that of vinorine synthase (PDB: 2BGH) from *Rauvolfia serpentina*. Gaps are indicated by dashes, letters in green color are identical residues, and letters in other colors are similar residues. Red sticks and blue arrows represent α-helix and β-strand structures, respectively.

hol was incorporated into the acyl-CoA_VpAAT1 complex. Fig. 5a shows a general view of the position of both ligands into the solvent channel and the interaction with the two catalytic residues (His 166 and Asp 170). In the acetyl-CoA_benzyl alcohol_VpAAT1 complex His166 is protonated at the nitrogen ε, which forms a hydrogen bond with the hydroxyl group of acetyl-CoA (Fig. 5b). Additionally, the distance between the carboxylic group of Asp170 and the hydroxyl group of benzyl alcohol was 2.73 Å, in an adequate position to stabilize this substrate through a hydrogen bond (Fig. 5b). In the case of hexanoyl-CoA and ethanol, a shorter distance could be observed for both substrates, suggesting a stronger interaction and high affinity (Fig. 5c). The main difference between acetyl-CoA_benzyl-alcohol_VpAAT1 complex and hexanoyl-CoA_ethanol_VpAAT1 complex is the alcohol localization into the solvent channel and the distance of the ligands with the residues His166 and Asp170. In the first complex, benzyl alcohol was found at 2.73 Å from residue D170 into the solvent channel. In the second complex, ethanol was located between the residues D170 and H166 at 1.6 Å of the carboxylic group of D170 and 1.8 Å of the nitrogen ε of H166. The acetyl-CoA and hexanoyl-CoA are oriented to similar distance from nitrogen ε of H166 (3.51 Å and 2.13 Å, respectively). Interestingly, another complex acetyl-CoA_ethanol_VpAAT1 showed a similar binding to the complex formed by acetyl-CoA_benzyl-alcohol_VpAAT1, with a distance between the carboxylic group of Asp170 and the hydroxyl group of ethanol of 3.02 Å, meanwhile the distance between the carbonyl group of acetyl-CoA and nitrogen ε of His166 was 2.6 Å (Fig. 5d).Interestingly, the

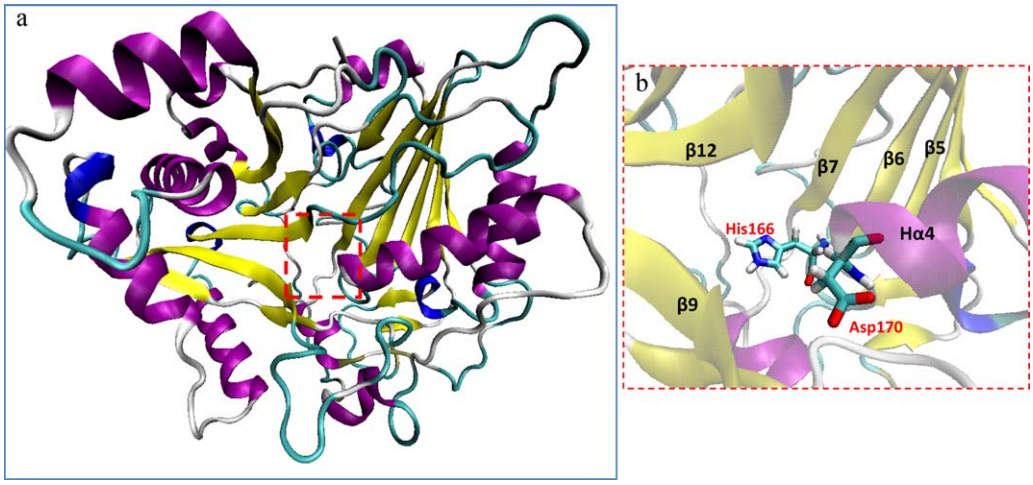

Fig. 2. (a) Structural model of VpAAT1. The α-helices are shown in purple, the 3_{10} helices are shown in blue, and the β-strands are shown in yellow. Image obtained with VMD software. NewCartoon representation. (b) The catalytic residues of VpAAT1 protein model: the H166 and D170 residues are exposed to the solvent channel of the enzyme, allowing their interaction with substrates.

two complexes acetyl-CoA_ethanol_VpAAT1 and acetyl-CoA_benzyl alcohol_VpAAT1, showed a similar binding position for the alcohol (which interacts with residue D170) and the acetyl-CoA (which interacts with residue His166) (Figs. 5b and d). Albeit, in the hexanoyl-CoA_ethanol_VpAAT1 complex, the ethanol is located between residues His166 and Asp170, showing a shorter distance to both residues than the other complexes. However, when the interaction energies were calculated for the complexes, there is a great difference between the complexes (Table 3). The acetyl-CoA_benzyl alcohol_VpAAT1 complex, that produces benzyl acetate, showed the lowest interaction energy (−266 kcal mol^{-1}), and the complex acetyl-CoA_ethanol_VpAAT1 (that produces ethyl acetate) has the highest energy value (−73.3 kcal mol^{-1}). When the acetyl-CoA_hexanol_VpAAT1 and butanoyl-CoA_ethanol_VpAAT1 complexes were modelled the alcohol took a position apart from His166 and close to Asp170, similar to what was observed for benzyl alcohol and ethanol when acetyl-CoA was the other substrate (data not shown).

3.4. Kinetic studies

Kinetic properties of the recombinant VpAAT1 enzyme were determined to validate '*in silico*' measurements. The K_M and *V*max values were determined for benzyl alcohol using acetyl-CoA as co-substrate at saturating concentration (1.5 mM acetyl-CoA), and for acetyl-CoA using benzyl alcohol as co-substrate at saturating concentration (2 mM benzyl alcohol) (Table 4). A big difference in K_M values was observed, with lower K_M value for acetyl-CoA (80 μM)

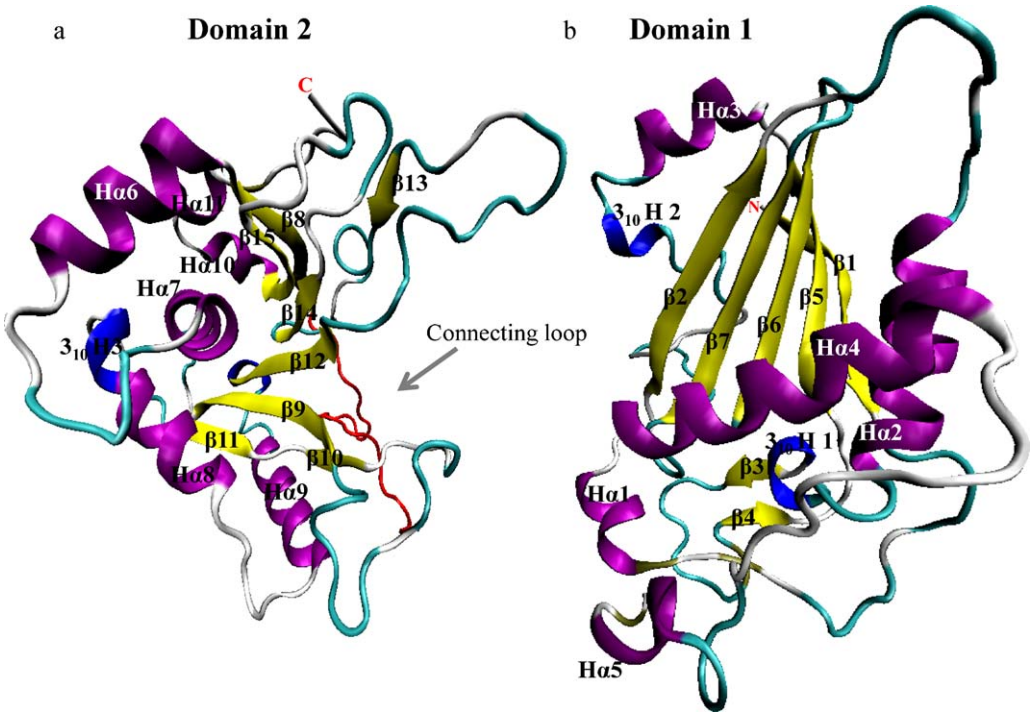

Fig. 3. The two domains of the VpAAT1 structure. (a) and (b) represent orthogonal views of the VpAAT1 structure as depicted in NewCartoon representation. N and C denote the ends of VpAAT1. The secondary structure elements are labeled (Hα1–Hα11, 3_{10}H1–3_{10}H3 and β1–β15), and domains 1 and 2 are indicated. The α-helices are shown in purple, the 3_{10} helices are shown in blue, and β-strands are shown in yellow. The large crossover loop (amino acids 210–232) that connects both domains is marked in red.

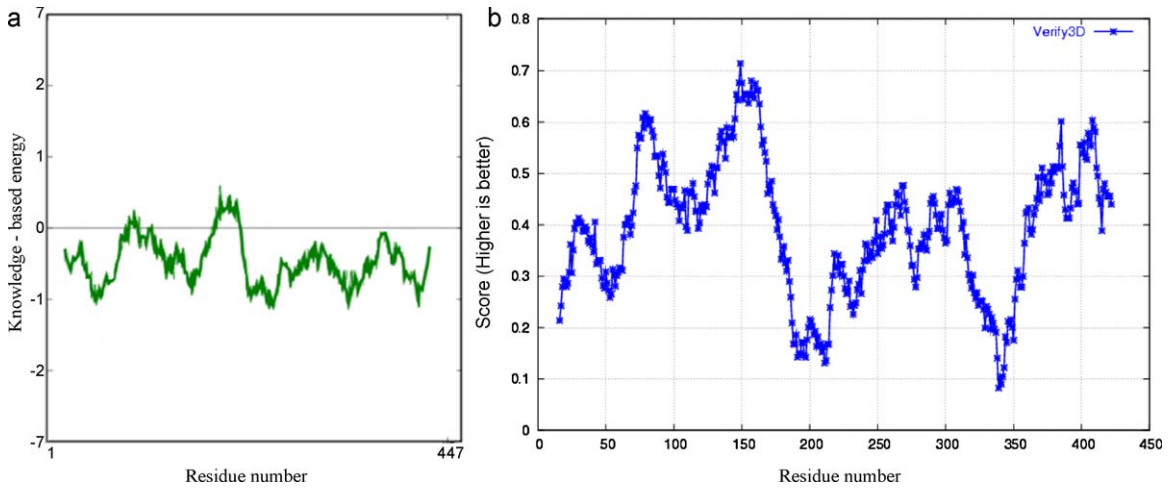

Fig. 4. Evaluation of structural model of VpAAT1 enzyme. ProSA energy profile (a) and Verify3D graph (b) drawn for the VpAAT1 model.

than for benzyl alcohol (130 μM), indicating a higher affinity of the enzyme for acetyl-CoA. This result is coincident with the information provided by docking simulations (Table 2).

4. Discussion

Mountain papaya is a climacteric fruit that develops an interesting and characteristic aroma during ripening, which is mainly due to esters [1], which are synthesized by alcohol acyltransferases. A gene (VpAAT1) has been isolated from mountain papaya fruit and its protein sequence shares all the conserved regions described for other acyltransferases belonging to the BAHD superfamily [8]. A large number of acyltransferases have been described in plants, nevertheless in fruit species such as melon and apple a small gene family has been identified [9,26,27].The BAHD superfamily members have been divided into three subfamilies (I, II and III) [27] and VpAAT1 has been clustered into subgroup III [8]. Members of subfamily III have been described for their great capacity to produce benzyl acetate [28], and interestingly, benzyl acetate has not been described as an aroma volatile in mountain papaya fruit [1],

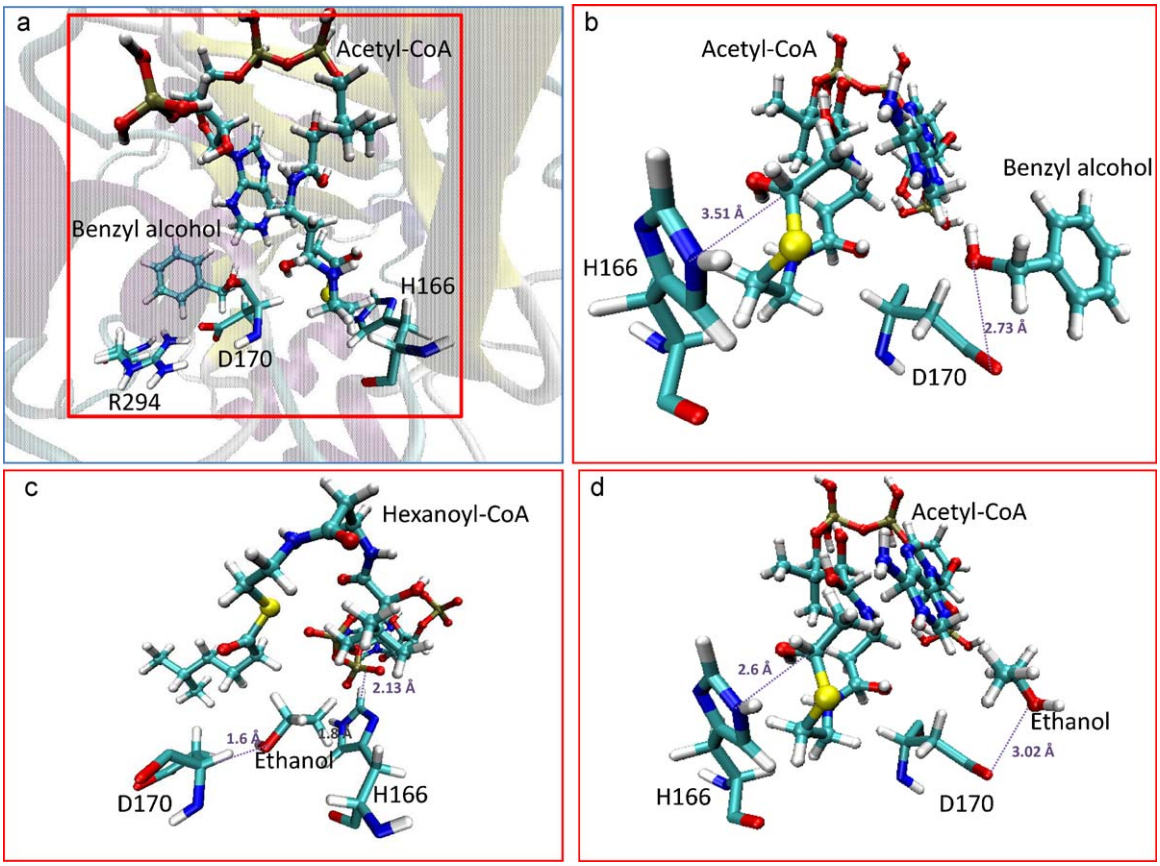

Fig. 5. Ligand binding analysis based on the refined VpAAT1 model. (a) The 3D structure of acetyl-CoA_benzyl alcohol_VpAAT1 complex is shown. (b) Docking of benzyl alcohol and acetyl-CoA to VpAAT1 model. (c) Docking of ethanol and hexanoyl-CoA to VpAAT1 model. (d) Docking of ethanol and acetyl-CoA to VpAAT1 model. Dash lines indicate distance between atoms.

Table 3
Affinity energies for the different acyl-CoA_alcohol_VpAAT1 modelled complexes.

Acyl-CoA	Alcohol	Ester produced	AAT activity (pkat mg^{-1})[a]	Affinity energy (kcal mol^{-1})	rmsd (Å)[b]
Acetyl-CoA	Benzyl alcohol	Benzyl acetate	29,275	−266.3	2.4
	Hexanol	Hexyl acetate	20	−222.9	4.1
	Ethanol	Ethyl acetate	tr	−73.3	4.4
Butanoyl-CoA	Ethanol	Ethyl butanoate	83	−230.9	5.7
Hexanoyl-CoA	Ethanol	Ethyl hexanoate	790	−250.2	1.9

[a] AAT activity values of VpAAT1 recombinant protein towards different acyl-CoAs and alcohols as substrates were obtained from [8].
[b] rms deviation was calculated for the backbone (Cα,C,N) atoms. tr, traces.

Table 4
Kinetic parameters obtained for purified recombinant VpAAT1 protein using cosubstrate at saturating concentration.

Substrate	K_M (μM)	*V*max (pmol h^{-1} mg^{-1})
Benzyl alcohol	130	27,950
Acetyl-CoA	80	30,578

Saturation curves were prepared for each substrate, while the other was remained constant at a saturating concentration (1.5 mM acetyl-CoA, 2 mM benzyl alcohol). 35 μg of purified VpAAT1 protein were employed in each enzymatic assay.

although the recombinant protein is able to synthesize this ester (Table 3).

To understand the structural and functional characteristics, the 3D structure of VpAAT1 protein was built by homology modelling and molecular dynamic simulations. The model obtained fulfils the structural characteristics proposed for the BAHD superfamily, including the solvent channel in where the active site is located, and the globular shape composed by two equally sized domains connected by an extensive loop (residues 210–232). The VpAAT1 structure presented in this work explains the functional importance of His166, a residue located in loop 13 between helix 5 and β-strand 7, and directly situated in the center of the solvent channel. This structural arrangement allows the incorporation of different acyl-CoAs and alcohols as substrates to approach the active site, at the front face (acyl-CoA binding) and the back face (alcohols binding) of the enzyme. In the HTMSD motif, the Asp170 is located close to His166 and it is part of the active site. The importance of the residues His and Asp in the HXXXD motif has also been demonstrated by chemical modification and site-directed mutagenesis experiments for other members of the BAHD superfamily [10,29]. The BAHD superfamily enzymes might therefore have a similar conformation of the catalytic His and use a similar reaction mechanism to that proposed for VpAAT1.Molecular docking analysis indicates that interaction between VpAAT1 enzyme with acetyl-CoA and benzyl alcohol showed the most favorable binding energy, and the recombinant protein displayed a high activity towards the synthesis of benzyl acetate, so the 3D models show specific interactions between the substrates benzyl alcohol and acetyl-CoA and key amino acid residues in the active site, and thus these interactions are consistent with the previously reported experimental data concerning the catalytic activity of VpAAT1 [8]. Moreover, the analysis performed supports the hypothesis that VpAAT1 enzyme shares similar structural and functional characteristics to members of the acyltransferase subfamily III with high activity for the production of benzyl acetate. Therefore, the fact that mountain papaya fruit does not produce benzyl acetate may be due to the absence of substrates during fruit ripening and not to the inability of VpAAT1 protein to interact with the substrates.

Recently, we described that VpAAT1 recombinant protein was able to use different alcohols and acyl-CoAs as substrates for the synthesis of esters [8]. We now show that the binding complex energies between the VpAAT1 protein and its different substrates (acyl-CoAs and alcohols) were significantly different. A good correlation was observed between the VpAAT1 activity recorded for the recombinant protein and the affinity energy determined for each protein–ligand complex: a higher VpAAT1 activity was measured when the complex has higher stability (lower affinity energy) (Table 3).

Hexyl acetate, ethyl butanoate and ethyl hexanoate are volatiles produced by mountain papaya fruit, and their production increased during ripening progress. These esters are characterized for its fruity notes and low odour threshold values in water (1–2 ppb), and in mountain papaya fruit ethyl butanoate and ethyl hexanoate are the most potent odourants [1]. Their increment during ripening is coincident with the increase in the expression level of VpAAT1 transcripts and total AAT activity in fruit extracts [8]. The low affinity energies determined for the complex between the VpAAT1 protein and each corresponding pair of ligands indicates that the esterification reaction could be performed by the VpAAT1 protein. In fact, the VpAAT1 recombinant protein is able to synthesize them, suggesting that VpAAT1 protein is directly involved in the biosynthesis of these important esters. On the other hand, ethyl acetate is also profusely produced by mountain papaya fruit, however its production starts late during ripening of the fruit and its increment continues even after the climacteric phase. The VpAAT1 recombinant protein is unable to produce ethyl acetate and also the complex VpAAT1_acetyl-CoA_ethanol has an unfavorable affinity binding energy. Therefore, it can be proposed that ethyl acetate is most probably produced *in vivo* by another alcohol acyltransferase and not by the VpAAT1 protein in mountain papaya fruit.

In summary, our study strongly suggests that the structural model of VpAAT1 enzyme is a useful tool to study the protein–substrate interaction, and reveals that the catalytic site is located into the solvent channel at the center of the protein structure. Moreover, docking simulations determine that the enzyme has a lower binding energy for benzyl alcohol and acetyl-CoA than for other pair of substrates, which correlates well with *in vitro* enzymatic activity data. As it has been shown, the combination of bioinformatics and kinetics assays are necessary to validate '*in silico*' predictions and to determine the more favorable interaction between an enzyme and its substrates.

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