In vitro and in silico studies of chalcone synthase variant 2 in Boesenbergia rotunda and its substrate specificity

Ragaventhan SANMUGAVELAN, Teow Chong TEOH*, Nurnadiah ROSLAN, Zulqarnain MOHAMED
Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia

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Abstract: In this study, transformation of BrCHS var 2 into B. rotunda cell suspension culture, followed by chalcone synthase enzymatic assay and HPLC analysis was conducted to investigate whether the substrate specificity for BrCHS var 2 is either cinnamoyl-CoA or p-coumaroyl-CoA. The HPLC profile showed an increase in the amount of pinocembrin chalcone when cinnamoyl-CoA and malonyl-CoA were added but not p-coumaroyl-CoA. Molecular docking was performed to explore the binding of cinnamoyl-CoA and p-coumaroyl-CoA to BrCHS var 2 receptor and the docking results showed that cinnamoyl-CoA formed numerous hydrogen bonds and more negative docked energy than p-coumaroyl-CoA. Cinnamoyl-CoA showed good interactions with Cys 164 to initiate the subsequent formation of pinocembrin chalcone, whereas the hydroxyl group of p-coumaroyl-CoA formed an unfavorable interaction with Gln 161 that caused steric hindrance to subsequent formation of naringenin chalcone. Docked conformation analysis results also showed that malonyl-CoA formed hydrogen bonding with Cys 164, His 303, and Asn 336 residues in BrCHS var 2. The results show that cinnamoyl-CoA is the preferred substrate for BrCHS var 2.

Key words: Chalcone synthase, cell suspension culture, homology modelling, molecular docking

1. Introduction

Ginger was used as a food, spice, and herbal remedy over 2000 years ago. It is a monocot plant from the family Zingiberaceae that can be found widely in Southeast Asia. Common members of this family include root ginger (Zingiber officinale), fingerroot (Boesenbergia rotunda), turmeric (Curcuma longa), and myoga (Zingiber mioga Roscoe). There has been increasing interest in its rhizome as a good source of medical treatment for humans and it has been utilized as a vital source to exhibit inhibitory activities as an anticancer, antimicrobial, antiviral, and antiinflammatory agent (Sohn et al., 2005; Kiat et al., 2006; Voravuthikunchai et al., 2006; Kirana et al., 2007).

In most plants, chalcone synthase (CHS; EC 2.3.1.74) is one of the key enzymes involved in the initiation of the flavonoid biosynthesis pathway. CHS is a plant-specific polyketide synthase type III (PKSIII) that forms chalcone by the condensation of one molecule of p-coumaroyl CoA with three molecules of malonyl-CoA to form metabolites such as an intermediate naringenin chalcone. To date, there are more than 2000 nonvolatile compounds detected using (LCMS) in fresh ginger and rhizome, but less than 100 have been structurally identified (Koo et al., 2013).

The CHS gene constitutes a multigene family in which it has irregular copy number of CHS in different plant species. Published studies have reported that only one CHS was found in Arabidopsis thalian (Feinbaum and Ausubel, 1988) and eight in Sorghum bicolor and Glycine max, while Vitis vinifera and Physcomitrella patens have three and seventeen copies, respectively (Goto-Yamamoto et al., 2002; Lo et al., 2002; Tuteja et al., 2004; Koduri et al., 2010). Initial results on gene isolation from B. rotunda CHS (BrCHS) revealed multiple BrCHS variants that were identified from different parts of the B. rotunda plant. BrCHS variant 2 (BrCHS var 2) was found to be expressed predominantly in the rhizome. As such, this variant was chosen to be introduced into B. rotunda cell suspension cultures for downstream HPLC analyses.

CHS has broad substrate preference toward aromatic and aliphatic CoA esters (Jez et al., 2002; Samappito et al., 2002; Abe et al., 2007). The first study on evaluating catalytic activity of the CHS enzyme was performed in cell suspension cultures of parsley (Petroselinum hortense) (Kreuzaler and Hahlbrock, 1975). p-Coumaroyl-CoA was reported to be the most preferential starter molecule for CHS in many plants as well as cinnamoyl-CoA, which is catalyzed at a considerable rate as compared to...
p-coumaroyl-CoA in few plants (Hatayama et al., 2006). Several plants prefer certain substrates and performed a typical CHS function such as in *Freesia* hybrid when using p-coumaroyl-CoA and malonyl-CoA as substrates (Sun et al., 2015). Besides that, CHS from *Scutellaria baicalensis* preferred both aromatic and aliphatic CoA esters such as benzyol-CoA, phenylacetyl-CoA, isovaleryl-CoA, and isobutyryl-CoA substrates to produce unnatural aromatic polypentide (Morita et al., 2000). More tests on enzyme isobutyryl-CoA substrates to produce unnatural aromatic as benzoyl-CoA, phenylacetyl-CoA, isovaleryl-CoA, and isobutyryl-CoA substrates that can produce diverse metabolites with different enzymatic function.

Computational simulations have been used to study the kinetics and binding modes of drugs (Tsou et al., 2012; Shen et al., 2016), homology modelling, and docking of chalcone synthase from *Coleus forskohlii* (Awasthi et al., 2016). As an added advantage, computational docking study provides further insight into the possible chemical interactions of a ligand to its receptor and hence the elucidation of the ligand–receptor binding mechanism. This is the first report on combinatorial in vitro and in silico studies of one of the CHS of *Boesenbergia rotunda* by using CHS substrate specificity enzymatic assay, followed by computational docking verification. The information obtained from this study could also be exploited for the future production of novel polyketides using cell suspension cultures.

2. Materials and methods

2.1. Transformation of *BrCHS var 2* into *B. rotunda* cell suspension culture

The pGEM-T vector harboring the entire CDS of *BrCHS var 2* was digested with restriction endonucleases, *Nco*I and *Spe*I (Fermentas, USA), followed by purification, ligation into pCAMBIA-1304 (GenBank accession no. AF234300.1), and transformation into *Agrobacterium tumefaciens*, strain LBA4404. Positive transformants were selected and subsequently transformed into *B. rotunda* cell suspension culture as described by Yu et al. (2007). After 3 days of co-cultivation, the resistant calli were then selected on semisolid Murashige and Skoog medium containing 300 mg/L cefotaxime (Duchefa Biochemie, Netherlands) and 15 mg/L hygromycin (Duchefa Biochemie, Netherlands) and were subcultured every 4 weeks. Calli that allowed inhibition of antibiotics were transferred into liquid media and subcultured for up to 2 months to get a sufficient number of cells. After 2 months, the cells were harvested and subjected to protein extraction and HPLC analysis.

2.2. CHS enzymatic assay and HPLC analysis

The total crude protein was extracted from the transformed and wild-type cell suspension cultures as described by Carpenter et al. (2005) with minor modification. At the last step, the pellet was dissolved in 50 mM Tris-HCl buffer, pH 7.6, containing 1 M urea in place of the CHAPS. To test the CHS enzyme activity, 100 µg of total crude protein extracted from cell suspension cultures was assayed using two sets of enzymatic reaction; Set 1: 40 µM malonyl-CoA (Sigma-Aldrich, USA) with 20 µM cinnamoyl-CoA (MicroCombiChem, Germany) and Set 2: 40 µM malonyl-CoA (Sigma-Aldrich, USA) with 20 µM p-coumaroyl-CoA (MicroCombiChem, Germany). The reaction mixture was prepared in a final volume of 500 µL of 100 mM potassium phosphate buffer (pH 7.2) containing 0.1% Triton X-100, incubated at 30 °C for 2 h and stopped by acidification using 7.5 µL of 1 N HCl.

The reaction products were partitioned with 800 µL of ethyl acetate and concentrated by N, flow. The residues were dissolved in 100 µL of methanol and separated by a reversed-phase HPLC system equipped with a Waters 1525 Binary HPLC pump and Waters 2998 photo diode array detector (Waters, USA) on a Kinetex RP C18 (150 mm × 4.6 mm) column (Phenomenex, USA). The solvent systems used for the elution were (i) solvent A: CH₃OH containing 0.01% H₃PO₄ and (ii) solvent B: H₂O containing 0.01% H₃PO₄. The gradient elution profile consisted of an isocratic step of 50% of solvent A for 1 min, and a linear gradient from 50% to 100% of solvent A for 10 min, followed by an isocratic step at 100% of solvent A for 10 min with a flow rate of 0.8 mL/min. Peak detection was monitored at 290 nm. Pinocembrin chalcone isolated from *B. rotunda* (kindly provided by NA Rahman and YK Lee), naringenin, naringenin chalcone, and pinocembrin (MicroCombiChem, Germany) were used as reference compounds.

2.3. Molecular modelling of ligands and CHS var 2 receptor

All the ligand molecular structures were retrieved from PubChem Compound at https://pubchem.ncbi.nlm.nih.gov/search/search.cgi. The ligand structures used were cinnamoyl-CoA (CID: 6440437), p-coumaroyl-CoA (CID: 5462161), and malonyl-CoA (CID: 644066). In addition, acetyl-CoA (CID: 444349) and Co-A (CID: 46936280) were docked as references. The ligands were then modelled by Discovery Studio Client v4.5.0.15071 and minimized by CHARMM force field (Accelrys Inc., Dassault Systèmes, BIOVIA Corp., San Diego, CA, USA).

Multiple sequence alignment was carried out by aligning in-house amino acid sequences of *BrCHS var 2* with amino acid sequences from *Cucuruma longa* (CIPKS9; JN017186.1), *Curcuma alismatifolia* (CaCHS; GU140082.1), *Musa acuminata* (MaPKSIII3; GU724609.1), *Oryza sativa* (OsCHS; AB000801.2), *Zea mays* (ZmCHS; NM_001155550.1), and *Medicago sativa* (MsCHS2; L02902.1) using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The homology model of *BrCHS var 2* receptor was modelled from the in-
house amino acid sequence (Supplementary material Figure A1) with template sequence of 4YJY (z-score: -0.814) (Supplementary material Figure A2) by using the YASARA Structure modelling software (Krieger et al., 2002). The stereochemical quality of BrCHS var 2 protein 3D receptor model was then checked by PROCHECK (Laskowski et al., 1993) webserver at https://services.mbi.ucla.edu/SAVES/. The homology model was minimized in GROMACS v5.1.4. (Abraham et al., 2015) initially with steepest descent and followed by conjugate gradient to energy convergence of 0.01 kJ/mol.

2.4. Molecular docking of BrCHS var 2
The minimized ligands were targeted on the BrCHS var 2 receptor binding site containing Cys 164, His 303, and Asn 336 as reported by Jez and Noel (2000) and confirmed by multiple sequence alignment. The Haddock 2.2 molecular docking software web server was used to perform the docking simulation using the protein–ligand module (de Vries et al., 2010; Wassenaar et al., 2012). The lowest docked energy ± standard deviation was extracted. The docked conformation graphics was generated using PyMOL 1.3 (Schrodinger, LLC) and the 2-D diagram was computed by using Discovery Studio Client v4.5.0.15071 and LigPlot+ v1.4. (Laskowski and Swindells, 2011) for the hydrogen bonding, electrostatic, van der Waals, and pi interactions.

3. Results and discussion
3.1. BrCHS var 2 substrates specificity
The flavonoid biosynthesis pathway at KEGG pathway ko00941 (http://www.genome.jp/dbget-bin/www_bget?ko00941) shows the formation of primary CHS products, which are pinocembrin and naringenin. These CHS products are produced when the substrates used are cinnamoyl-CoA and p-coumaroyl-CoA, respectively (Mazumdar and Chattopadhyay, 2015).

The retention times for all compounds and substrates identified in the HPLC chromatogram are listed in Table 1 and were referred prior to the CHS enzymatic assay. According to the results, there was an increase in the amount of pinocembrin chalcone (Figure 1a) when cinnamoyl-CoA and malonyl-CoA were added to the total crude protein extract. Pinocembrin chalcone (labelled as C1 in Figure 1a) was eluted at a retention time of 8.80 min. The estimated amount of C1 compound detected during HPLC in wild-type and transgenic cell suspension cultures were 64.67 mg/g (SE: 6.67E-02) and 112.20 mg/g (SE: 1.00E-01), differing significantly at P < 0.05, respectively. Cinnamoyl-CoA is another starter molecule that is catalyzed by CHS at comparable rate as compared to p-coumaroyl-CoA in some plants (Christensen et al., 1998; Fliegmann et al., 1992).

CHS has broad substrate specificity (Schüz et al., 1983; Jez et al., 2002; Samappito et al., 2002) with p-coumaroyl-CoA is the most preferential starter molecule found in many plants (Yamazaki et al., 2001; Hatayama et al., 2006). However, it was unpredictably found that BrCHS var 2 was inactive to p-coumaroyl-CoA and no naringenin chalcone was produced in our study (Figure 1b). Another abundant compound (labelled as C2 in Figure 1a and 1b) was eluted at a retention time of 11.70 min and detected in both sets of enzymatic reactions. This compound was a by-product of polyethyleneglycol (PEG), possibly derived from Triton X-100, after confirmation by LCMS (data not shown).

3.2. Binding site and homology model of CHS variant 2
The Ramachandran plot and PROCHECK results indicate that the homology model is of good quality as the number of residues in the favored region was 92.9% (>90%) as shown in supplementary material Figure B. As shown in Figure 2a, the multiple sequence alignment result of CHS receptors from different plant species indicates that the binding site of BrCHS var 2 receptor contains three main amino acids, Cys 164, His 303, and Asn 336, that form a catalytic triad. The catalytic triad is commonly found in all type III polyketide synthase of Zingiberaceae (Mallika et al., 2016). The surrounding active site residues MMYQQGC164-AGGT and GFGPG loop are as shown in Figure 2b.

Table 1. Retention time of four reference metabolites and substrates.

<table>
<thead>
<tr>
<th>Reference compounds</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolites</td>
<td></td>
</tr>
<tr>
<td>Naringenin</td>
<td>6.531 ± 0.10</td>
</tr>
<tr>
<td>Naringenin chalcone</td>
<td>6.571 ± 0.10</td>
</tr>
<tr>
<td>Pinocembrin</td>
<td>10.304 ± 0.10</td>
</tr>
<tr>
<td>Pinocembrin chalcone</td>
<td>8.913 ± 0.10</td>
</tr>
<tr>
<td>Substrates</td>
<td></td>
</tr>
<tr>
<td>Malonyl-CoA</td>
<td>2.231 ± 0.10</td>
</tr>
<tr>
<td>Cinnamoyl-CoA</td>
<td>5.068 ± 0.10</td>
</tr>
<tr>
<td>p-Coumaroyl-CoA</td>
<td>3.256 ± 0.10</td>
</tr>
</tbody>
</table>
Figure 1. a) HPLC chromatograms of CHS enzymatic activity assay in Set 1: malonyl-CoA with cinnamoyl-CoA of wild-type (top panel) and transformed cell suspension culture (bottom panel). b) HPLC chromatograms of CHS enzymatic activity assay in Set 2: malonyl-CoA with p-coumaroyl-CoA of wild-type (top panel) and transformed cell suspension culture (bottom panel). HPLC chromatogram detecting pinocembrin chalcone (C1) as shown in a) and nontargeted compound (naringenin and/or naringenin chalcone) detected in b). C2: Polyethylene glycol (PEG).
Figure 2. a) Alignment of CHS deduced amino acid sequences from B. rotunda (BrCHS var 1-var 5), Cucurma longa (ClPKS9; JN017186.1), Curcuma alismatifolia (CaCHS; GU140082.1), Musa acuminata (MaPKSIII3; GU724609.1), Oryza sativa (OsCHS; AB000801.2), Zea mays (ZmCHS; NM_001155550.1), and Medicago sativa (MsCHS2; L02902.1). b) The amino acids of the catalytic Cys^{164}-His^{303}-Asn^{336} triad of BrCHS var 2 with active site residues MMYQQGC^{164}-AGGT-H^{303}-N^{336} (magenta) and GFGPG loop (yellow) are shown in cartoon (left) and surface (right) rendering.
3.3. Molecular docking and docked conformation analysis

Based on the Table 2, cinnamoyl-CoA showed more negative docked energy compared to p-coumaroyl-CoA. Thus, cinnamoyl-CoA has higher binding affinity for BrCHS var 2 than p-coumaroyl-CoA. This result is also supported by several hydrogen bonds found in cinnamoyl-CoA compared to p-coumaroyl-CoA. In the docking simulations, CoA and acetyl-CoA were used as references. The binding of cinnamoyl-CoA to BrCHS var 2 receptor was dominantly stabilized by hydrogen bonding and electrostatic interaction, whereas the binding of p-coumaroyl-CoA to BrCHS var 2 receptor was hindered by the repulsive electrostatic interaction with less hydrogen bonding and electrostatic interaction. Gln 161 (2.63), Leu 258 (2.62), and Arg 259 (2.63) were the residues involved in hydrogen bond formation with cinnamoyl-CoA. On the other hand, Cys 164 (3.13) was the only residue that interacted with p-coumaroyl-CoA through hydrogen bonding.

Figures 3 and 4 show the docked conformations for cinnamoyl-CoA and p-coumaroyl-CoA, respectively. The main interaction types were hydrogen bonding and electrostatic with weaker interactions such as van der Waals, pi–pi, and pi–alkyl interactions. A similar result was reported by Awasthi et al. (2016) for CfCHS from Coleus forskohlii in which the preferred substrate was cinnamoyl-CoA compared to p-coumaroyl-CoA and it was found that cinnamoyl-CoA showed a more negative docking score than p-coumaroyl-CoA. They also indicated that Cys 164, His 304, and Asn 337 were identified to interact with the substrate ligand, including the two gatekeeper residues of CfCHS, Phe 266 and Phe 216 with π–π interactions. Moreover, Lys 270 also formed hydrogen bonding with cinnamoyl-CoA (Awasthi et al., 2016).

The only structural difference between cinnamoyl-CoA and p-coumaroyl-CoA is the extra hydroxyl group at the para position on p-coumaroyl-CoA or 4-hydroxycinnamoyl-CoA. As shown in Figures 3c and 3d, cinnamoyl-CoA interacts with Cys 164 to initiate the loading process of cinnamoyl moiety to Cys 164 as thioester monoketide intermediate, which will proceed to subsequent decarboxylation, elongation, and aromatization (Ferrer et al., 1999) to produce pinocembrin. On the other hand, the hydroxyl group of p-coumaroyl-CoA formed an unfavorable interaction with Gln 161 and a hydrogen bond with Cys 164 (Figure 4d), which constitutes a steric hindrance to the subsequent formation of thioester monoketide intermediate and hence the final production of naringenin. Therefore, molecular docking and docked conformation analysis enables the elucidation of reaction mechanism of BrCHS var 2 in using cinnamoyl-CoA as the preferred substrate instead of p-coumaroyl-CoA to produce pinocembrin.

The formation of hydrogen bonding by Cys 164, His 303, and Asn 336 with the thioester of malonyl-CoA is shown in Figure 5 and this will initiate subsequent decarboxylation, carbanion formation, and further attack of cinnamoyl thioester on Cys 164 (Ferrer et al., 1999). The more subtle van der Waals interactions from the gatekeeper residues Phe 215 and Phe 265 were also observed in Figures 3–5. Supplementary material Figures

Table 2. Docked energy for ligands to BrCHS var 2 receptor.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Docked energy (kcal/mol)</th>
<th>Electrostatic energy (kcal/mol)</th>
<th>van der Waals energy (kcal/mol)</th>
<th>Hydrogen bond length (Å)</th>
<th>Repulsive interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoA (Reference)</td>
<td>−83.7 ± 5.8</td>
<td>−289.1 ± 4.1</td>
<td>−21.0 ± 1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetyl-CoA (Reference)</td>
<td>−63.5 ± 3.6</td>
<td>−212.2 ± 2.0</td>
<td>−18.1 ± 6.8</td>
<td>Lys55 (2.62)</td>
<td></td>
</tr>
<tr>
<td>Malonyl-CoA</td>
<td>−62.7 ± 3.4</td>
<td>−223.9 ± 9.0</td>
<td>−18.0 ± 5.2</td>
<td>Lys62 (2.44, 2.56)</td>
<td></td>
</tr>
<tr>
<td>Cinnamoyl-CoA</td>
<td>−54.0 ± 3.0</td>
<td>−64.3 ± 14.7</td>
<td>−24.7 ± 5.8</td>
<td>Gln161 (2.63)</td>
<td></td>
</tr>
<tr>
<td>p-Coumaroyl-CoA</td>
<td>−49.3 ± 4.3</td>
<td>−33.5 ± 14.0</td>
<td>−27.3 ± 1.8</td>
<td>Cys164 (3.13)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3. Docked conformation of BrCHS var 2 with cinnamoyl-CoA rendering in (a) surface; (b) wireframe; (c) 2-D diagram; (d) interaction types. Cyan: CHS variant 2 receptor; yellow: amino acid around the binding site; CPK: cinnamoyl-CoA.
Figure 4. Docked conformation of BrCHS var 2 with p-coumaroyl-CoA rendering in (a) surface; (b) wireframe; (c) 2-D diagram; (d) interaction types. Cyan: CHS variant 2 receptor; yellow: amino acid around the binding site; CPK: p-coumaroyl-CoA.
C1 and C2 shows the 2-D interaction diagrams of acetyl-CoA and CoA, respectively, to BrCHS var 2 as reference.

In the present work, we found that the preferred substrate for BrCHS var 2 was cinnamoyl-CoA to produce pinocembrin chalcone, which has been successfully verified by HPLC analysis. Docked confirmation analysis further confirms the enzymatic assay results and we found that the binding of cinnamoyl-CoA to BrCHS var 2 receptor was with numerous hydrogen bonding and stronger electrostatic interaction with lower negative docked energy compared to p-coumaroyl-CoA. The findings of this investigation are useful for future in vitro production of novel polyketides by utilizing cell suspension culture as a plant host and it would also pave the way for further in silico investigation of chalcone synthase and flavonoid biosynthesis by using molecular dynamics and shed light on its system biology pathway modelling.

Acknowledgments
We are grateful for the University of Malaya’s research grants RP032-15AFR and RP032C-15AFR for supporting this research work. We thank Dr Teh Ser Huy for providing the amino acid sequences of the five variants of BrCHS. We also thank Ms Nurul Hana Mohd Yusof and Mr Md Arman Hossain for their assistance in the molecular docking experiments.

Figure 5. 2-D diagram of docked conformation of BrCHS var 2 with malonyl-CoA.
References


Supplementary material

A1)

>BrCHS_var2
MAKVQEIQRQRAREAGPAAILAIGTATPTNVYQADYAYYFYRTKSEHLELKEFKEKRMCDKSNIRKRYNHVTIELKENFNMCAYME
PSLDERQDIVVVEVPKLGEEAAAKAIKEWGGQPKSITHLIFCTTGVMGADYQITKLLGLRPSVNRNYQQGCFAGGTVRLAKD
LAEINRGRARVVLVVCSEITAVTFRGFSESHLDSLVQALGFQGAIIVGADPDLETERTPLFELVSAASQTILPDSEGAIIDGHLLREVGLT
FHLLKDVFGGLISKNIEKSLVEAFAPFLGIDDWSIFWIAYHPGPAILDQVEAKLALAEKMAATROVLYSEYGNMSSACVIFILDEMRK
SAEQGKATTGEGLNWGVLFGFPGPGLTVETVVLHSSPINHstop

A2)

>4YJY:A|PDBID|CHAIN|SEQUENCE
MAAATVEEVRAQRAEGPAATVLAAITPANCYQADYFYRTKSEHLELKEFKEKRMCDKSNIRKRYMLTEILQENFNMCAYME
YMAFSLDARQDIVVVEVPKLGEEAAAKAIKEWGGQPRSRITHLIFCTTGVMGADYQLAKNLGLRPNSLMMYQQGCFAGGTVRLVRV
AKDLAENRGRARVVLVVCSEITAVTFRGFSESHLDSLVQALGFQGAAAVIVGSDPDDEAVERPFLQMVSAASQIILPDSEGAIIDGHLLREV
GLTFHLLKDVFGGLISKNIEKSLVEAFAPFLGIDDWSIFWIAYHPGPAILDQVEAKV
GLDKEKRMATRHVLSELYGNMSSACVIFILDEMRKRSAEDGHAATGEGMDNWGVLFGFPGPGLTVETVVLHSVITAGAAAsstop

Figure A. 1) In-house amino acid sequence for BrCHS var 2 protein; 2) Template sequence for homology modelling.
Figure B. Ramachandran plot and PROCHECK result for BrCHS var 2 protein receptor homology model.
Figure C. Docked conformation rendering in 2-D diagram for (C1) acetyl-CoA and (C2) CoA, respectively.