Detection of substrate binding motifs for morphine biosynthetic pathway intermediates in novel wound inducible (R,S)-reticuline 7-O-methyltransferase of *Papaver somniferum*

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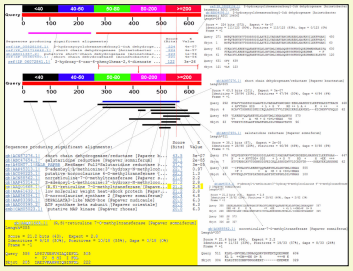
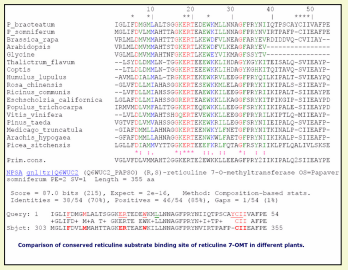
Abstract

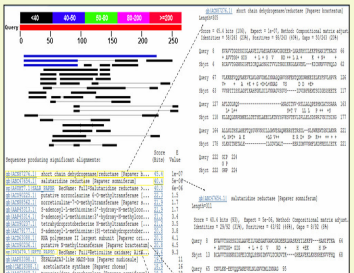
The benzylisoquinoline alkaloids (BIA) comprise a large and diverse group of nitrogen-containing secondary metabolites with about 2500 compounds identified in plants. BIA biosynthesis begins with the condensation of the tyrosine derived precursors dopamine and *p*-hydroxyphenylacetaldehyde to (*S*)-norcoclaurine. Subsequent regiospecific *O*- and *N*-methylations and aromatic ring hydroxylation lead to (*S*)-reticuline, which is the central intermediate for almost all BIAs. For morphinan alkaloid biosynthesis, (*S*)-reticuline undergoes an inversion of stereochemistry to (*R*)-reticuline, followed by C-C phenol coupling catalyzed by a unique cytochrome P450-dependent monooxygenase to yield salutaridine. The cDNA sequence of enzymes leading to (*S*)-reticuline, as well as those involved in the conversion of (*R*)-reticuline to salutaridine-7-*O*-acetate are already characterized. The inversion of (*S*)-reticuline to (*R*)-reticuline represent the important steps in morphine biosynthesis. Wound induced transcript accumulation in Papaver reveals a novel wound inducible EST (NCBI DbEST: GO238757) showing homology with (R,S)-reticuline 7-O-methyltransferase (ID: Q6WUC2) isolated from *Papaver somniferum*. We compare the substrate binding homology of this novel wound inducible (R,S)-reticuline 7-O-methyltransferase (7-OMT) using template of *P. somniferum* (Q6WUC2; gb|AAQ01668) as experimental control. Homology modeling with 70% identity & 85% similarity with catalytic site of template protein i.e., (Q6WUC2) short chain dehydrogenase/reductase (SDR), showed docking energy -69.9 and -75.8 kcal/mol with (S)-Reticuline (CID:439653) and (R)-Reticuline (CID:440586) respectively, which are comparable with experimental control binding site interaction energies. Docking of S- & R-reticuline into the active site revealed eight (F(5), E(18), W(24), C(47), F(44), P(45), C(46) and I(47) amino acids presumably responsible for the high substrate specificity of (R,S)-reticuline 7-O-methyltransferase.

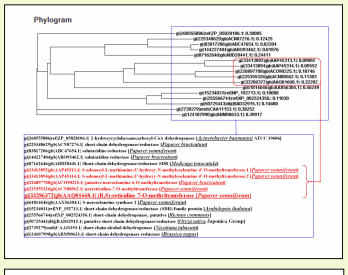
Introduction

- Poppies (*Papaver somniferum*, Papaveraceae) have long been used as medicinal plants, food plants, and drugs of abuse. Morphine alkaloids are found in many members of the genus Papaver, but codeine and morphine are only found in *P. somniferum*. Another species, *Papaver bracteatum* contains thebaine.
- Morphine alkaloids are derived from (-)- or (R)-reticuline by series of reactions involving an oxidative coupling reaction. Radioactive labelling experiments have established the series o reactions from thebaine to codeine to morphine.
- The most important compound from a biosynthetic point of view is (+)-reticuline. This alkaloid is a presursor of several other groups of alkaloids. (+)- or (S)-Reticuline is converted to (-)- or (R)-reticuline, which is, in turn, a major precursor of other alkaloid groups.
- For morphinan alkaloid biosynthesis, (*S*)-reticuline undergoes an inversion of stereochemistry to (*R*)-reticuline (an isoquinoline alkaloid) catalyzed by (R,S)-reticuline 7-O-methyltransferase (OMT), a member of the short chain dehydrogenase/reductase (SDR) protein family.
- (S)-reticuline formed in the poppy plant, is converted by means of 1,2-dehydroreticuline to (R)-reticuline, which in turn is then transformed into morphine.
- cDNAs have been isolated for all of the enzymes leading to (*S*)-reticuline, as well as those involved in the conversion of (*R*)-reticuline to salutaridine-7-*O*-acetate. Recently, the short-chain dehydrogenase/reductase (SDR) implicated in morphine biosynthesis was cloned from *Papaver somniferum*.
- The biosynthetic pathways and the participating enzymes or cDNAs are characterized only for a few selected members, whereas the biosynthesis of the majority of the compounds is still largely unknown (Pienkny et al., 2009).
- This study showed high expression of an expressed sequence tag (EST) of short-chain dehydrogenase/reductase (SDR) family protein (*Acinetobacter baumannii* and *Papaver bracteatum*).
- The open reading frame sequence analysis revealed that EST has similarity with salutaridine reductase, S-adenosyl-L-methionine, norreticuline-7-O-methyltransferase, S-norcoclaurine synthase and (R,S)-reticuline 7-O-methyltransferase (*Papaver somniferum*.
- Catalytic site domain showed similarity with O-methyltransferases from benzylisoquinoline biosynthesis (amino acid sequence showed 85% similar residues to (R,S)-reticuline 7-O-methyltransferase).
- OMT methylate (S)-reticuline at position 7- using S-adenosyl-l-methionine as a cofactor. Of all substrates tested, only (S)- & (R)-reticuline showed good binding affinity (kcal/mol).
- This enzyme represents a novel (R,S)-reticuline 7-O-methyltransferase. Expression analysis data revealed highly increased expression of ESTs in *P. somniferum* varieties containing papaverine, suggesting its involvement in the partially unknown biosynthesis of this pharmaceutically important compound.



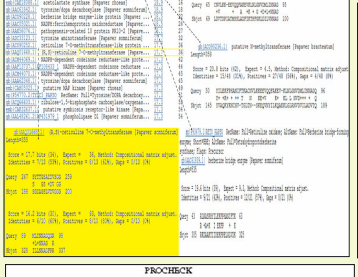


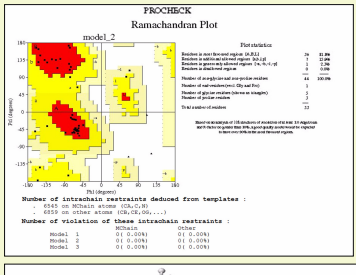


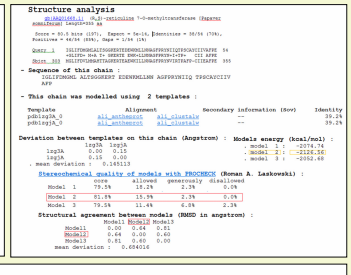


Methodology: Homology Modeling and Substrate Docking

- A homology model of the *P. somniferum* short chain dehydrogenase/reductase (SDR) catalytic region was created based on the x-ray structure of *P. somniferum* (R,S)-reticuline 7-O-methyltransferase [gb|AAQ01668; tr|Q6WUC2; Length=355 aa; Pdb:1zg3, 1zgj]. Model showed the typical α/ß-folding pattern of SDRs.
- Homology modeling of the catalytic region of SDR was based on the x-ray structure of (R,S)-reticuline 7-O-methyltransferase (AAQ01668/Q6WUC2) of *Papaver somniferum*.
- The structure was energy minimized using PM3 mechanics method. The resulting, slightly modified structure was used for the docking of S-reticuline, R-reticuline and other substrates of BIA pathway.
- For all subsequent docking arrangements the complex consisting of the ligand, cofactor and the protein was energy optimized.
- The stereochemical quality of the model was checked using Procheck. In the Ramachandran plot, 90% of backbone dihedral angles were located in the most favored area and 8% were found in additionally allowable regions. The only outlier occurred in a loop.
- The energy plot showed that almost all residues were in the negative energy range. Altogether these analyses are consistent with a reasonable protein model.
- Docking were performed through Scigress Explorer v7.7 (www.fujitsu.com)
- Crystal structure information were retrieved through NCBI-Pubchem (www.ncbi.nlm.nih.gov/pccompound) and PDB (www.rcsb.org/pdb/) database.





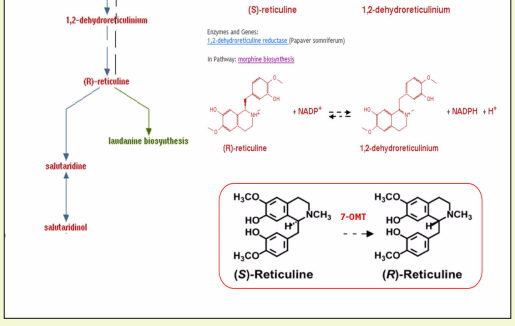


Results

- Docking of S- & R-reticuline into the active site revealed eight (F(5), E(18), W(24), C(47), F(44), P(45), C(46) and I(47) amino acids presumably responsible for the high substrate specificity of (R,S)-reticuline 7-O-methyltransferase.
- Site-directed mutagenesis of E(18) with N, resulted in enzyme variants with strongly reduced performance, showing the involvement of this residue in the methylation step.
- (R,S)-reticuline 7-O-methyltransferase converts reticuline to laudanine in tetrahydrobenzylisoquinoline biosynthesis in the opium poppy *P. somniferum*.
- A wound inducible gene expression study in *P. somniferum* suggest that partial amino acid sequences are homologous to those of (R,S)-reticuline 7-O-methyltransferase.
- Steady-state kinetic measurements data suggest that this enzyme is capable of carrying through sequential O-methylations on the isoquinoline and on the benzyl moiety of several substrates.
- The tetrahydrobenzylisoquinolines (R)-reticuline, (S)-reticuline, (R)-protosinomenine, and (R,S)-isoorientaline as well as guaiacol and isovanillic acid are O-methylated by this enzyme.
- A phylogenetic comparison of the amino acid sequences of these O-methyltransferases to those from other plant species suggests that these enzymes group more closely to isoquinoline biosynthetic O-methyltransferases (Ounaroon et al., 2003).

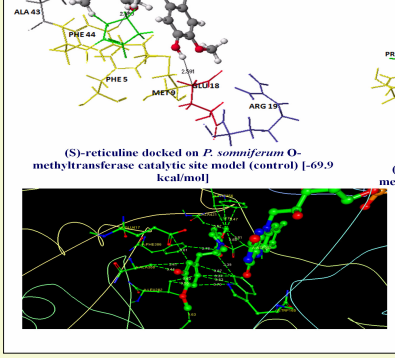
Substrate binding affinity decreases (red color) due to variation in the pocket residues (when 'E' replaced with 'N') of catalytic site of *P. somniferum* O-methyltransferase model.

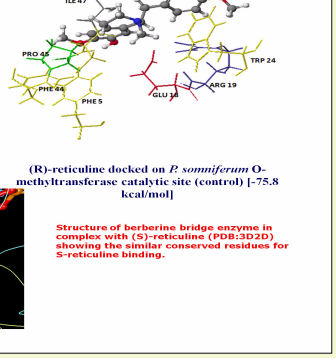
Catalytic site protein model	Docking energy (kcal/mol)	
	S-reticuline CID_439653	R-reticuline CID_440586
Control	**-69.881**	**-75.801**
Variant 1	-68.322	-70.42
Variant 2	-86.093	-77.678
Variant 3	-73.988	-72.888
Variant 4	-81.4	-88.897
Variant 5	-72.00	-73.715
Variant 6	-68.007	-73.655
Variant 7	-70.924	-70.802
Variant 8	-89.076	-93.027
Variant 9	-74.937	**-65.34**
Variant 10	-80.273	-90.686
Variant 11	-72.589	-81.536
Variant 12	**-63.977**	**-62.347**
Variant 13	-79.905	-82.658
Variant 14	-69.096	-70.899
Variant 15	-67.81	-73.976
Variant 16	-64.644	-74.765
Variant 17	-74.655	-85.707
Variant 18	-69.903	-72.923
Variant 19	-69.153	**-65.966**



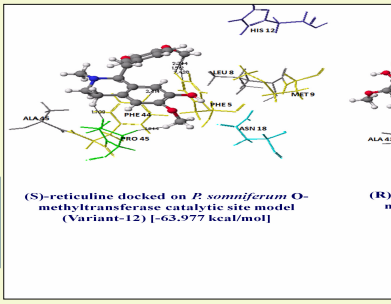
Conclusion

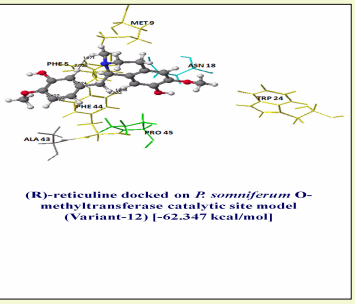
- Comparative analysis of the predicted catalytic site for S- & R-reticuline revealed that they share ~85% sequence similarity with *P.somniferum* (R,S)-reticuline 7-O-methyltransferase.
- Docking studies suggested highly conserved regions proposed as a signature for O-methyltransferases and believed to be involved in S-adenosyl-L-methionine, metal binding and (S)-& (R)-reticuline binding.
- Phylogenetic analysis of the amino acid sequences suggest that plant (R,S)-reticuline 7-O-methyltransferase may have arisen from common ancestral genes that were driven by different structural and/or functional requirements, and whose descendants segregated into different biochemical species.





Structure of berberine bridge enzyme in complex with (S)-reticuline (PDB:3D2D) showing the similar conserved residues for S-reticuline binding.





Acknowledgement

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