

Molecular Characterization of Novel form of Type III Polyketide Synthase from *Zingiber Officinale* Rosc. and its Analysis using Bioinformatics Method

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Abstract

Enzymes of Type III polyketide synthase superfamily play an important role in the biosynthesis of medicinal natural products in plants. The PKSs generate the diversity of polyketide derivatives by changing their preference for starter molecules, the number of acetyl additions catalysed and the cyclisation of the polyketide intermediates. The amazing structural features of gingerol and related compounds of ginger (*Zingiber officinale* Rosc., Zingiberaceae) provide a genomic insight in to the presence of novel forms of PKS. The current study describes the isolation and characterisation of a novel of PKS from *Z. officinale* using degenerate oligonucleotide based PCR method. The inducible expression of recombinant ZoPKS in *E. coli* resulted in the formation of a protein with approximate molecular weight of 43kD. The comparative sequence and phylogenetic analysis of ZoPKS shows its significant variation from already identified PKSs. The novelty of the ZoPKS was further confirmed by homology modeling based comparative structural bioinformatics analysis. The novel form of PKS identified in the study has very remarkable amino acid substitutions at the key residues determining the starter substrate selectivity and condensation reactions and forms a genomic basis of PKS from *Z. officinale* to explore its potential in biosynthesis of gingerol and related compounds.

Introduction

The amazing diversity of polyketide derivatives in plants are generated by a group of structurally related enzymes called as the type III polyketide synthases (PKSs). The most well known and widely distributed member of the PKS superfamily is the chalcone synthase (Winkel-Shirley, 2001). In the typical reaction mechanism, it forms the chalcone by the stepwise decarboxylative condensation of coumaroyl CoA with three malonyl coA followed by the claisen type cyclisation of the tetraketide product (Jez et al., 2001). Extensive gene duplication followed by the functional divergence is believed to have played an important role in generating the biochemical diversity of PKS superfamily. The

expanding members of the family, as shown by the identification of 2-pyrone synthase, stilbene synthase, benzalacetone synthase, valerophenone synthase, acridon synthase etc., from various sources indicate that the biosynthetic potential of the PKS is just begin to be explored and many more members are to be identified from plants especially from medicinal plants (Austin and Noel, 2003).

Zingiber officinale Rosc. (Ginger, Zingiberaceae) is well known for its use in traditional therapeutic and preventive medicine. The major pharmacologically active component present in *Z. officinale* is of the gingerol group (Ramirez-Ahumada Mdel et al., 2006). Molecular insight in to the gingerol biosynthesis suggested that enzymes similar to type

III polyketide synthases are having important role (Denniff et al., 1980; Schröder, 1997). Considering the pharmacological potential of gingerol and the distribution of structurally similar compounds in other members of the family, identification and characterisation of the PKS from *Z. officinale* can provide a genomic basis for elucidation of biosynthetic pathway. In the present work, we are reporting the cloning and characterisation of a novel form of type III PKS from *Z. officinale*. The comparative structural bioinformatic analysis suggests that the PKS identified in the study may be a prime candidate for the biosynthesis of phenylbutanone derivatives of *Z. officinale*.

Materials and Methods

Zingiber officinale var. Rio de Janeiro was used as the experimental material for the study. The plants were collected from Kerala Agricultural University, Thiruvananthapuram, Kerala and were grown and maintained in the experimental plant garden at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala.

PCR and Cloning

Total RNA was isolated from young leaves of *Z. officinale* by Trizol method and reverse transcribed using the MMLV RT (Promega). Amplification of core cDNA fragment of PKS was carried out by nested RT-PCR using the inosine containing degenerate oligonucleotide primers as reported earlier (Abe et al., 2001). The sequences of the primers are as follows: PKS1 5'-RARGCIITIMARGARTGGGGICA-3', PKS2 5'-GCIAARGAYITIGCIGARAAAYAA-3', PKS3 5'-CCCMWITCIARICCITCICCIGTIGT-3' and PKS4 5'-TCIAYIGTIARICCIGGICCRAA-3'. The primers PKS1, 2, 3 and 4 represents the 112, 174, 368 and 380 amino acid position of typical CHS. The conserved amino acid residues at these areas provide opportunities for nested PCR based approach for isolating core sequence of PKSs from taxonomically diverse plants. The introduction of degenerate bases and inosine makes the primers to bind to functionally divergent members of PKS family. The core fragment (major part of the second exon) of PKS contain most of the residues contributing to the catalytic architecture. So the changes observed on conserved residues at these areas may have remarkable impact on reaction mechanism of PKS. The primary PCR reaction (50 µl) contained the cDNA template, 30 pmoles of each primers (PKS1 and PKS2), 1.25 units of *Taq* DNA polymerase (Promega), 1.5 mM MgCl₂ and 200 µM of each dNTPs. The reaction was carried out for 35 cycles in a Bio-Rad iCycler using the following conditions; initial denaturation was done for 3 min at 94 °C, the cyclic denaturation at 94°C for 30 sec, annealing at 42 °C for 1 min and extension at 72 °C for 1 min with a final

extension at 72°C for 7 min. The secondary PCR was carried out using primer sets of PKS3 and PKS4 using 1 µl of the primary PCR product as template under the same PCR conditions as for the primary PCR. The PCR product was analysed in a 2% agarose gel and was purified using the GFX gel band purification system (Amersham). The purified fragment was ligated to pGEMT easy vector (Promega) and propagated in *Escherichia* strain JM109 (Promega). Plasmid isolation and purification was done using the Wizard Plus SV Minipreps DNA purification system (Promega). Automated sequencing of recombinant clones was carried out using Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit Version.3.1 (Applied Biosystem) in the ABI 3730 DNA sequencer.

3' and 5' RACE

Gene specific primers designed from the core fragment of PKS from *Z. officinale* were used for the RACE experiments. The RACE ready cDNA was prepared using the First Choice RLM RACE kit (Ambion). The primers used for the 3' RACE were PKS31 5'-ATCGCCAAGGACCTGGCGGAGA -3' and PKS32 5'-ACAACCGCGGCGCGCGCTCCTCG -3'. The primers used for the 5' RACE experiments were PKS51 5'-GATGTTGCTCGCAATGATCGACGGAA-3' and PKS52 5'-ATGATCGACGGAAGCTGGCTCTTGAGG-3'. For the 3' RACE, the primary PCR was carried out with the primer set of PKS31 and 3' RACE outer primer and the secondary PCR with PKS32 and 3' RACE inner primer. The PCR condition used were; initial denaturation at 94 °C for 3 min, cyclic denaturation at 94 °C for 30 sec, annealing at 65 °C for 30 sec and extension at 72 °C for 1 min with a final extension of 7 min at 72°C. The product formed in the secondary PCR was cloned and sequenced. The 5' RACE ready cDNA was used as template for the 5' RACE PCR using the primers PKS51 and PKS52. The primary PCR was carried out using PKS51 and 5' RACE outer primer followed by the secondary PCR using the primer sets PKS52 and 5' RACE inner primer. The conditions for both the PCR experiments were; initial denaturation at 94°C for 3 min, cyclic denaturation at 94 °C for 30 sec, annealing at 65 °C for 30 sec and extension at 72°C for 1 min with a final extension of 7 min at 72°C. The PCR product formed was cloned and sequenced.

Cloning and Expression of Full-length cDNA

Full-length cDNA of PKS from *Z. officinale* (ZoPKS) was isolated by RT-PCR using N-terminal gene specific primer NPKSF 5' CGCTCGAGCATATGCATCA CCATCACCATCACATGGGTAGCCTGCAGGCGATG 3' containing the site for *Nde* I and six histidine residues and

the C-terminal gene specific primer CPKSR 5' CGGGATCCCTACGGTATTGGTAGACTGCGTAG 3' containing the site for *Bam* HI . The PCR was carried out with an initial denaturation for 3 min at 94 °C, cyclic denaturation at 94 °C for 30 sec, annealing at 65 °C for 30 sec and extension at 72 °C for 2 min with a final extension of 7 min at 72 °C. The PCR product formed was cloned, sequenced and analysed by BLAST programme. The sequence was submitted to NCBI under the accession number DQ486012. The phylogenetic analysis of ZoPKS was carried out along with other PKSs by NJ and MP methods implemented in the MEGA3 (Kumar et al., 2004).

For the prokaryotic expression, the ZoPKS was cloned at the *Bam*HI/*Nde*I site of pET-32b (Novagen). The recombinant clones were confirmed for the sequence and transformed in to *E. coli* BL21(DE3)pLysS. The cells with the recombinant plasmid were cultured to an OD600 of 0.6 in Luria-Bertani medium containing 100 mg/ml of ampicillin at 28 °C. The expression of the recombinant protein was induced by the addition of 0.5 mM isopropyl thio- β -D-galactoside by incubating at 28 °C for 16 h. Western blotting of the recombinant ZoPKS was carried out using antibodies specific to His-tag (Sigma) (Towbin et al., 1979).

Multiple Sequence Alignment and Homology Modeling

Sequences of 14 plant PKSs were collected from the NCBI database and was used along with ZoPKS for the comparative structural analysis. Most of the selected PKSs were biochemically studied and were shown to have role in biosynthesis of plant specific metabolites. The sequences selected were *Ruta graveolens* - *Acridone synthase* (S60241), *Aloe arborescens* - *Octaketide synthase* (AAT48709), *Aloe arborescens* - *Pentaketide chromone synthase* (AAX35541), *Arabidopsis thaliana* - *Chalcone synthase* (CAI30411), *Gerbera hybrida* - *2-pyrone synthase* (CAA86219), *Humulus lupulus* - *Valerophenone synthase* (BAB12102), *Hydrangea macrophylla* - *Coumaroyl triacetic acid synthase* (BAA32733), *Iris germanica* - *Chalcone synthase* (BAE53636), *Oryza sativa* - *Chalcone synthase* (CAA61955), *Rheum palmatum* - *Benzalacetone synthase* (AAK82824), *Vitis vinifera* - *Stilbene synthase* (ABJ97068), *Wachendorfia thrysiiflora* - *Polyketide synthase* (AAW50921), *Medicago sativa* - *Chalcone synthase* (P30074) and *Dendrobium nobile* - *Chalcone synthase* (ABE77392). The multiple sequence alignment of the sequences was done by using the Clustal W programme (Thompson et al., 1994).

Homology model of ZoPKS was generated based on the crystal structure of CHS from *M. sativa* (PDB id – Ibi5)

and 2-PS from *G. hybrida* (PDB id –1qlv) and were taken as the representatives of the typical chalcone forming and nonchalcone (Pyrone) forming members of the PKS super family. X-ray crystallographic information of both the templates was collected from the Protein Data Bank (Berman et al., 2000). The alignment between the sequence of ZoPKS and the structure of selected templates was carried out using Clustal W as the starting point for modeling the tertiary structure of ZoPKS.

The HOMOLOGY and DISCOVER module (Insight II User Guide 1997, MSI San Diego, CA) of the Insight II molecular modeling package were used in the construction and optimisation of structures of ZoPKS and other selected PKSs. The computed models were verified for stereochemical quality by using PROCHECK at RCSB web site (<http://rcsb-deposit.rutgers.edu>). The graphical images of the models were viewed and generated by using Pymol graphical interface (<http://pymol.sourceforge.net>). The structural impact of amino acid substitution on the shape and size of substrate binding cavity of ZoPKS was investigated on a comparative basis. The amino acid residues forming the substrate binding region were mapped on all the models generated and the images were generated using the Pymol.

Results and Discussion

Gingerols, the major active components of ginger (*Z. officinale*), has received a great deal of attention because of its broad spectrum of biological activities including the anti-inflammatory, anticarcinogenic, and antitumor activities. Some initial investigations on gingerol biosynthesis identified the potential of enzymes of the PKS family as designers of its basic structural skeleton, but the lack of genomic information of PKS is a limiting factor to unravel the complex biosynthetic process. Degenerate oligo nucleotide primers targeting the conserved region of PKS was used for isolating the 600 bp core fragment of PKS by RT-PCR. This gene specific sequence was used for designing primers for the RACE experiments. The full length cDNA of PKS from *Z. officinale* (ZoPKS) was found to have 1173 bp length and was predicted to form a polypeptide of 43 kD containing 391 amino acid residues [Figure 1]. The BLAST analysis of ZoPKS gave a maximum identity of 64% to the PKS identified from *W. thrysiifolia*. The low identity can be taken as an indication of its novel functions since the members exhibiting typical chalcone forming reactions are shown to have more than 85% identity (Brand et al., 2006).

The phylogenetic analysis of PKS superfamily showed clustering based on their biochemical functions [Figure 2]. The ZoPKS was found to cluster distinctly with the

nonchalcone forming PKSs like the benzalacetone synthase from *Rheum palmatum* (Abe et al., 2001), 2-pyrone synthase (2PS) from *Gerbera hybrida* (Eckermann et al., 1998), 4-coumaroyltriatic acid lactone synthase (CTAS) from *Hydrangea macrophylla* (Akiyama et al., 1999), valerophenone synthase (VS) from *Humulus lupulus* (Paniego et al., 1999) and acridone synthase (ACS) from *Ruta graveolens* (Springob et al., 2000). The nonchalcone forming PKSs are involved in the biosynthetic processes other than typical tetraketide derivatives and because of their specific role, they are considered as species-specific or metabolite specific. Thus the characteristic clustering of ZoPKS may support for considering its role in nonchalcone forming reactions and possibility in the biosynthesis of pehnlybutanone derivatives of *Z. officinale*. The ZoPKS was cloned at the *NdeI* and *BamHI* site of pET32b for studying its expressivity in the prokaryotic system. The SDS-PAGE analysis showed the formation of recombinant protein of approximately 43 kD up on IPTG induction and was confirmed by the western blotting using the anti-His antibody [Figure 3].

Multiple sequence alignment of ZoPKS with the other PKSs showed that the conserved residues are located almost same position in all [Figure 4]. The sequence identity analysis of ZoPKS with the PKSs selected for the comparative analysis shows that it is identical to other PKSs selected for the analysis at a range of 49-64%. The maximum identity is towards the PKS from *W. thrysifolia* (64%) and the minimum is towards the *A. arborescens* pentaketide chromane synthase (49%). The multiple alignment analysis also revealed that the ZoPKS shows significant variations of catalytic residues at the highly conserved pockets. The catalytic triad forming the active site of the typical CHS like Cys 164, His 303 and Asn 336 are maintained as such in ZoPKS showing that it belongs to the PKS superfamily. The ZoPKS also showed the presence of stretches of conserved residues G(368)VLFGFGPGLT(378) considered as the signature sequence of PKS superfamily [Figure 1].

Structural insight into the catalytic machinery of polyketide biosynthesis was explained by the crystal structure studies of CHS from *Medicago sativa* (Ferrer et al., 1999). The studies showed that in addition to the catalytic triads, stringent conservation of amino acid residues constituting the catalytic pockets is also essential for the typical chalcone forming reactions. These include the substrate binding pocket (Ser133, Glu192, Thr194, Thr197 and Ser338) which forms a 16Å tunnel to the active site cavity and the cyclisation pocket (Thr132, Met137, Phe215, Ile254, Gly256, Phe265 and Pro375). The overall architecture is maintained by the conserved amino acid residues like the Pro138,

Gly163, Gly167, Leu214, Asp217, Gly262, Pro304, Gly305, Gly306, Gly335, Gly374, Pro375 and Gly376. A change in the amino acid residues at these pockets was found to have remarkable impact on the reaction mechanism leading to metabolic diversity (Abe et al., 2001). The ZoPKS showed significant amino acid substitution at these positions which makes it to be analyzed in detail to unravel the effect of these changes.

Among the amino acid residues constituting the substrate binding pocket, ZoPKS showed variation from typical CHS by the substitution of Thr (197) to Ser and Ser (338) to Gln. Two amino acid substitutions are observed at the amino residues forming the cyclisation pocket by replacing Thr (132) with Ileu and Ileu (254) with Val. Furthermore, three amino acid residues are replaced at the strongly conserved residues shaping the geometry of the active site by changing Gly (306) to Asn, Leu (214) to Gly, and Gly (163) to Ala. The similar amino acid substitutions were observed in other members of the nonchalcone forming PKSs and these changes can be considered to have determining effect on the functionality of PKS. The amino acid substitution of S(338)I and T(197)L as observed in 2-Pyrone synthase when compared to the CHS was found to reduce the size of active site cavity to accept smaller substrates (Jez et al., 2000). Very interestingly, site directed mutagenesis studies revealed that substitution of just three amino acid residues (T197L / G256L / S338I) can make the CHS to have 2-PS activity showing the potential of key catalytic residues to regulate the reaction mechanism (Jez et al., 2002). Similarly, the PKS from *W. thrysifolia* showed the replacement of T197 and Ser 338 with more bulky Met and Phe. This also have the characteristic Thr 132 to Gly substitution to provide the additional rotational freedom resulting in the size reducing effect (Brand et al., 2006). The active site analysis of Aleosine synthase showed a downward expanding T197A replacement, when compared to the typical CHS, illustrating the significance of Thr 197 as the controller of the polyketide chain length (Abe et al., 2006). Thus it is very probable that the amino acid substitutions observed in ZoPKS can alter the size and shape of cavity to favour the production of compounds other than the tetraketides. The structural impact of these substitutions on substrate binding pocket of ZoPKS was analysed in detail on a comparative basis.

Homology model of ZoPKS was generated based on the crystal structure of CHS (*M. sativa*) and 2-PS (*G. hybrida*) as templates. The energy minimisation of the modeled structure was carried out in the DISCOVER using the steepest descents algorithm (200 cycles) and conjugate gradient (1000 cycles) until a low energy derivative of around 1 kcal.mole⁻¹ had been reached. At this point the calculated

total energy of the molecule was consistent with the relaxed and stable conformation. The superposition of the ZoPKS model structure with the template structures showed very small deviation with an RMSD less than 0.5 Å and 0.8 Å respectively respectively towards the 2-PS and CHS [Figure 5]. Similarly the homology models were also generated for PKSs selected for the comparative structural analysis. The quality of all the models was assessed by Ramachandran plot analysis using PROCHECK. It helps to check the stereochemical quality of the optimized structures. By the inspection of phi/psi angle, we can analyse the quality of the Ramachandran plot and can determine whether the protein conformation belongs to the allowed region or not. The Ramachandran plot analysis of the ZoPKS showed that 92.9% of the amino acid residues come under the most favorable region, 6.2% amino acid residues come under the additional allowed region and 0.6% amino acid residues come under the generously allowed region. The same analysis for the crystal structure of CHS (*M. sativa*) and 2-PS (*G. hybrida*) gave the result of 93.4% and 92.6% amino acid residues at the most favourable region which strongly support the stereochemical quality of the model. The Ramachandran plot analysis of other PKSs selected for the study also showed the distribution of 91-93% of amino acid residues in the most favourable region [Table 1].

Although the PKSs have promiscuous substrate specificity, the amino acid residues constituting the substrate binding region was found to have mechanistic and steric effects on the substrate selectivity (Brand et al., 2006). This is also supported by the results of the multiple sequence alignment in which the conserved residues forming the substrate binding region were maintained as such in the CHSs from *Arabidopsis thaliana*, *Iris germanica*, *Oryza sativa* and *Dendrobium nobile*. But characteristically the amino acid residues were substituted in other PKSs specifically for their specific functions. So these residues were selected for the comparative structural analysis of ZoPKS for making predictions about its novel functions possibly as a nonchalcone forming PKSs. The RMSD values of the C α atoms of all the amino acid residues forming the substrate binding pocket like the Ser 133, Glu 192, Thr 194, Thr 197 and Ser 338 were calculated for the homology models by overlay of the C α atoms of generated models with the CHS of *M. sativa*. A number of substantial differences were observed for the RMSD values of ZoPKS and these differences along with the changes in the positioning of the side chain indicate the distinct structural characters of ZoPKS [Table 2]. Very interestingly the amino acid residues at the 338 position of ZoPKS showed an RMSD value of 1.76 Å where the Ser is substituted by more bulky Glu. The Ser at the 338 position

was found to have role in starter substrate selectivity and is more prone to substitution in members of nonchalcone forming PKSs for the specific function (Abe et al., 2006). In order to understand the combined effect of amino acid substitutions at these well studied positions, the substrate binding residues were mapped on modelled structures of ZoPKS and other PKSs selected for the comparative analysis. A significant variation is observed at the substrate binding region of PKSs and the changes observed in ZoPKS supports its novel functions [Figure 6]. The proposed reaction mechanism for the gingerol biosynthesis demands the PKS to have altered substrate selectivity, altered condensation and cyclisation reactions when compared to the typical CHSs. So the amino acid substitutions observed for the ZoPKS may contribute the changes in the structural architecture of ZoPKS to perform this novel function. But it has to be demonstrated by in vitro studies to confirm and the current study forms a genomic basis for this. As the molecular fascination of PKSs is just begun to explore, the remarkable changes observed on the PKS identified in the study show that it forms a potential member of PKS superfamily.

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