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Full Length Research Paper

Molecular cloning and characterization of strictosidine synthase, a key gene in biosynthesis of mitragynine from *Mitragyna speciosa*

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Mitragynine is one of the most dominant indole alkaloids present in the leaves of *Mitragyna speciosa*, a species of Rubiaceae. This alkaloid is believed to be synthesized via condensation of the amino acid derivative, tryptamine and secologanine by the action of strictosidine synthase (STR). The cDNA clone encoding STR from *M. speciosa* was cloned through reverse-transcription polymerase chain reaction (RT-PCR) and denoted as StrMs1. The clone is a full-length cDNA with a size of 1257 bp, which contains an open reading frame of 1056 bp starting from base pair 18 to 1076. Sequence analysis showed that StrMs1 has high homology with other *STRs* of TIA-producing plants. Nucleotide sequence of StrMs1 was deposited in GenBank with accession number ADK91432. The deduced amino acid sequence has 352 residues with a predicted molecular weight of 39 kDa and isoelectric point at pH 5.78. Southern blot performed showed that there is only one copy of *StrMs1* present in the genome of *M. speciosa*. Expression pattern on different tissues tested using RT-PCR revealed that besides leaf, the expression was also detected in root, stem and flower. Expression profiles under plant defense signal using salicylic acid (SA) was investigated on leaf tissues and the results showed that the transcript of StrMs1 were detected before and after treatment with salicylic acid. Result obtained from phylogenetic analysis suggested that StrMs1 is the most evolved protein among other STRs. However, the 3-D prediction of StrMs1 showed that there are alpha helices and beta propeller structures, which remain conserved with other STRs.

Key word: Strictosidine synthase, *Mitragyna speciosa*, StrMs1, semiquantitative reverse-transcription polymerase chain reaction (RT-PCR), molecular evolution, protein prediction.

INTRODUCTION

Plants are capable of synthesizing an overwhelming variety of low-molecular-weight organic compounds termed secondary metabolites. Currently, more than 100,000 compounds have been isolated from higher plants. Numerous plant secondary metabolites contribute to a wide variety of biological applications such as in pharmaceuticals and industries that produce insecticides, dyes, flavors and fragrances. Due to these overwhelming importance of secondary metabolites, we were interested in isolating the key gene responsible for the biosynthesis of the compound mitragynine which is described as having cough suppressant and analgesics properties (Jansen and Prast, 1988) and the ability to lessen the dependency on drug and alcohol (Kumarnsit et al., 2007), from Malaysian indigenous medicinal plants *Mitragyna speciosa* (Yamazaki et al., 2003).

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Abbreviation: STR, Strictosidine synthase; **SA,** salicyclic acid.

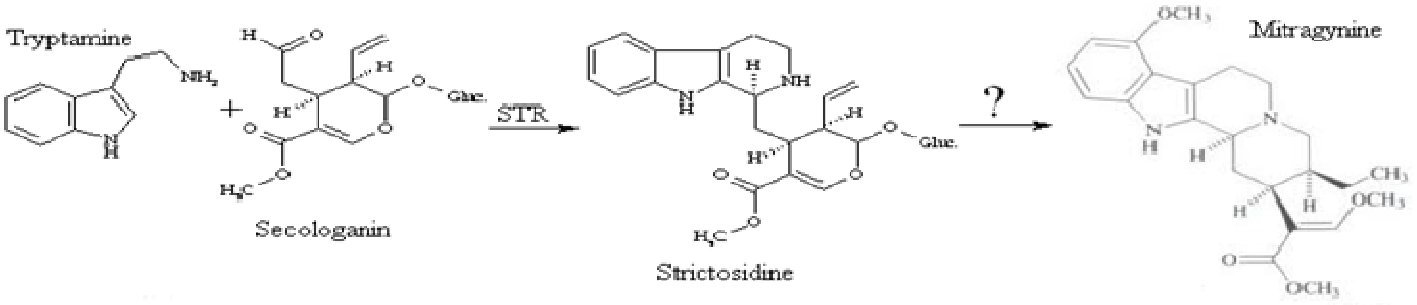

Figure 1. The compound mitragynine is proposed to have strictosidine as its precursor because of the comparative chemical structure of monoterpenoid indole alkaloid backbone.

The biosynthesis of mitragynine is a very complex process and thought to involve several enzymatic steps.

Moreover, the cloning of the genes in the biosynthetic pathway of mitragynine production has never been reported. Based on its chemical structure, it is suggested that mitragynine is derived from strictosidine (Figure 1), which is gotten from the condensation of the amino acid derivatives, tryptamine and secologanine. The regulatory enzyme in the biosynthesis of stricosidine is strictosidine synthase (STR) which catalyses the production of about 2000 monoterpenoid indole alkaloids (Ma et al., 2006) via a Pictet-Spengler (Chan et al., 2005) which is then utilized as a substrate for the production of other alkaloids such as ajmaline, raubasine, quinine, vinblastine and vincamine (Ma et al., 2006).

STR cDNAs were cloned from several plant species such as *Catharanthus roseus* (Miyamoto et al., 1995), *Rauvolfia serpentina* (Kutchan, 1995) and recently from *Ophiorriza pumila* (Lu et al., 2009). Nonetheless, until recently, there is no literature which reports on the cloning of *STR* genes involved in mitragynine biosynthesis from *M. speciosa*. Greater understanding of the mytragynine biosynthetic pathway will allow future improvements of mytragynine production via over-expression or down-regulation of key genes to optimize manipulated cultured cells or hairy roots.

In this study, cloning of cDNA for *StrMs1* gene from *M. speciosa* was carried out through reverse transcriptase polymerase chain reaction (RT-PCR) and the expression pattern was followed on different organ tissues. Furthermore, we have investigated the expression profile of *StrMs1* following the induction of salicylic acid (SA). In order to evaluate the evolutionary relationship with other known STRs, analysis on the deduced amino acid sequence using bioinformatics tools has led to the construction of a phylogenetic tree. The 3D structure of StrMs1 and its catalytic site were also predicted.

MATERIALS AND METHODS

Sampling of plant materials

Plants materials were collected from Pahang, Malaysia and grown in the green house of Faculty of Sciences and Technology at Universiti Kebangsaan Malaysia. In this experiment, 2 sets of samples were used for cloning of STR cDNA through RT-PCR and for transcript analysis. In order to study the effect of salicylic acid (SA) on *StrMs1*, the plant was treated with 5 mM SA by spraying it. The leaves were collected according to the time courses, which are before treatment, after 24, 48, 72 and 96 h, respectively.

RNA isolation and cDNA cloning of *M. speciosa STR*

RNA was isolated using Pateraki and Kanellis (2004) method of RNA extraction with slight buffer modification. The leaves were ground with mortar and pestle in liquid nitrogen and homogenized with 20 ml pre-chilled extraction buffer (Tris-HCL pH 8.5, 300 mM LiCl, 10 mM EDTA, SDS 1% w/v, 5 mM Thiourea, 1% β-mercaptoethanol) and 6 ml of 20% PVP 40. Then, the homogenate was spun for 15 min at 10000 g and 4°C. The supernatant was mixed with sodium acetate 3 M and ethanol and incubated at -20°C for at least 2 h. Then, the samples were spun for 20 min at 10000 g and 4˚C. The pellet was re-suspended in 6 ml of extraction buffer and phenol : chloroform (1:1). The mixture was vortexed and spun at 10,000 *g* for 10 min at 4°C. The upper phase was incubated in 65°C in a final concentration of 0.7 M NaCl and 2 M CTAB for 15 min. Next, equal volume of chloroform : isoamyl alcohol (24:1) was added to it followed by vortexing of the mixture. The mixture was centrifuged at 10,000 g for 10 min at 4°C. The upper phase was collected and incubated overnight in 3 M LiCl. The mixture was spun and the pellet was resuspended in 50 µl of DEPC water. The total RNA obtained was treated with DNase (Promega, USA) and the RNA was converted to single stranded cDNA using first strand cDNA synthesis kit (Promega, USA). Multiple sequence alignment of *STR* genes from different plants was conducted to identify the conserved regions of *STR*. PCR was conducted with temperature of 51°C and the primers were as follows: Forward, 5' TCGAAAA TCACACCTAACATGA 3' and reverse primer, 5' AGAAACAAAA TGTTCAAGTATT 3' according to cDNA sequence with accession EU288197.1 on NCBI database. The PCR product was cloned into pGEMT Easy and sent for sequencing.

Semi-quantitative RT-PCR from different tissues and induction with salicylic acid (SA)

RNA was extracted from 4 different tissues, which were leaves, roots, stem and flower. The PCR was done using 612 bp sized STR amplicon with primer pairs Forward- 5' GTTCCTCAAGTCACCC TA'3 and Reverse- 5' GGACCCTTTAACCAATAC '3. For SA treatment, *M. speciosa* leaves were sprayed with 5 mM SA and sampling was carried out within 4 consecutive days including before

treatment. The samples were labeled according to the day (before treatment; 0 h; and after treatment, 24, 48, 72 and 96 h). PCR was carried out using the same primers and the PCR product was electrophoresed through 1% agarose gel.

Southern blot analysis

Genomic DNA was isolated according to Doyle and Doyle (1987) and 20 µg DNA was digested with restriction enzymes and electrophoresed on 1% agarose gel for 16 h with 12 V 400 mAmp. The gel was transferred onto a Hybond-N^+ nylon membrane (Amersham) and Southern blot was performed as per manufacturers' instructions (Roche Science). The probe used was a 612 bp sized STR amplicon from purified PCR product. Membrane was washed under low-stringency conditions (2 × SSC, 0.1% SDS, at room temperature for 5 min followed by 65°C for 15 min).

Bioinformatics analysis

The sequence was searched for homology using BLASTX program at National Centre of Bioinformatics (NCBI). Prediction of protein localization and presence of signal peptide were predicted using Expasy tools. The phylogenetic tree was constructed using Molecular Evolution MEGA software.

Prediction of 3-D structure via comparative modeling

The X-ray diffraction structure of the native strictosidine synthase is available at PDB: 2fp9B and was used as template structure to generate 3-D model for structure of StrMs1. The X-ray 3-D structure of template was retrieved from http://www.rcsb.org/pdb/explore/explore.do?pdbId=2fp9. The 3-D structure of targeted protein was generated by SWISS-MODEL (Arnold et al., 2006) tool using comparative modeling approach and visualization of 3-D structure was done with UCSF Chimera 1.4.1.

Evaluation and validation of the 3-D structure

The evaluation and validation of generated protein 3-D structure was done using software tools PROCHECK and Errat 2.0. The PROCHECK (Laskowski et al., 2003) and Errat 2.0 (Colovos and Yeates, 1993) were used for validation of 3-D structure of strictosidine synthase of *M. speciosa*. The overall of stereochemical quality of the protein and the amino acid residues in the allowed and disallowed regions were assessed by Ramachandran plot analysis.

RESULTS AND DISCUSSION

Molecular cloning of STR cDNA from *M. speciosa*

STR is thought to be the regulatory gene in mitragynine production. Cloning the gene will be one of the steps in understanding the secondary metabolite production in *M. speciosa*. Based on the sequence deposited in GenBank under accession EU288197.1, a primer pair was designed to amplify the strictosidine synthase (STR) from *M. speciosa*. A single fragment was obtained from RT- PCR and cloned into pGEMT for sequencing. The resulting sequence analysis revealed that the cDNA clone showed a high degree of homology with other STR sequences from other plant species deposited in the NCBI database. The sequence analysis also revealed that *STR* of *M. speciosa* has a size of 1257 bp with an open reading frame of 1056 nucleotides that encode 352 amino acid residues with a predicted protein of ~39 KDa. This cDNA clone was designated as *StrMs1* and deposited in GenBank with accession number ADK91432. The deduced protein had a predicted isoelectric point (pI) at pH 5.78 with a protein formula of $C_{1754}H_{2662}N_{460}O_{519}S_6$. Homology search using BLASTX at NCBI showed that StrMs1 has high similarity with *Ophiorrhiza japonica* and *O. pumila* with 71 and 72% identity, respectively followed by Rauvolfia and *Catharanthus roseus* with 59 and 56%, identity at amino acid level, respectively. Comparatively, amino acid sequences of STR are variables and conserved at only their catalytic site.

Prediction of sub-cellular localization of StrMs1

Determining sub-cellular localization is important as a first step towards studying its function. To predict the localization of StrMs1, Expasy tools were used to analyze the presence of signal peptide. Signal peptide targets a protein for translocation across the endoplasmic reticulum (ER) membrane in eukaryotes (von Heijne, 1990). Through analysis using SignalP software, one signal peptide within StrMs1 amino acid sequence was identified inferring that StrMs1 is targeted to the ER where the nascent peptide is cleaved at the site between amino acid alanine and glutamic at position 28 and 29. It is known that STR is translocated from ER to vacuole of the cell through secretory pathways. This is supported by the presence of both strictosidine compound and STR enzyme in the vacuole (Luijendijk et al., 1998).

Expression in different tissues and salicylic acid (SA) induced plants

In order to investigate the level of expression of *StrMs1* transcript, we employed RT-PCR on several tissues: leaf, stem, flower and root. Under normal condition, the transcripts were detected in all tissues investigated. This is in agreement with previous observation reported on *STR* of *O. japonica* (Lu et al., 2009). We have also investigated the effect of SA on the pattern of *StrMs1* expression on the mitragyna plants treated with 5 mM SA. SA has been reported as an elicitor that could induce secondary metabolite production (Yan et al., 2004). Moreover, it was reported that exogenous application of SA could trigger *de novo* transcription of the gene involved in secondary biosynthetic pathway (Rhoads and Mclntosh, 1992). It is observed that the transcript was first detected even before the leaf was treated with SA (Figure 2a). This finding suggests that the leaf tissues

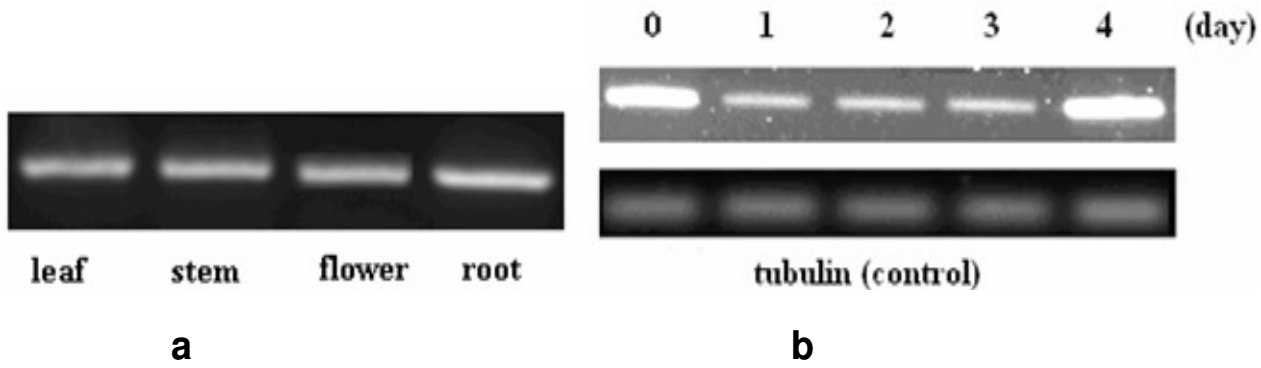

Figure 2. (a) Expression analysis of *StrMs1* in leaves, stem, flower and root with tubulin as the control (tubulin not shown); (b)*StrMs1* expression was noted when the SA treated leaves was collected at 4 consecutive days (day 0-control, 1, 2, 3 and 4) with tubulin as the control.

used for the analysis were picked at their mature stage where the mitragynine biosynthetic pathway is already activated. Whereas, in the treated leaf tissues, the transcript was low after 24 h of treatment and remained at a steady state and then increased abruptly at 96 h (Figure 2b). From this observation, it is suggested that SA might have a significant role in inducing the expression of *StrMs1.*

Southern blot

In order to identify the gene copy number of StrMs1 in *M. speciosa*, Southern blot was performed. Southern blot analysis with *STR* probe gives one hybridization signal with *Hind*III enzymes, implying that only one copy number of *STR* is present in the genome of *M. speciosa* (data not shown). This finding is in conformity with *C. roseus* and *R. serpentina* (de Waal et al., 2005; Pasquali et al., 2006) that have only 1 copy number of *STR* found in their genome.

Phylogenetic analysis

In order to determine the evolutionary distance between different STRs, phylogenetic tree was constructed. Phylogenetic tree construction was done using MEGA 4.1 (Molecular Evolutionary Genetics Analysis) and Expasy tools software at www.expasy.org. Eleven taxa were aligned using ClustalW and the phylogenetic tree was constructed using MEGA software (Figure 3). The maximum parsimony tree diagram was constructed by putting bootstrap value to show the strength and reliability of the tree (Claverie and Notredame, 2006). Figure 4 shows the evolution of StrMs1 where StrMs1 is found to be the most evolved as compared to the other STRs since StrMs1 is represented as an outgroup. From the tree, it can be concluded that the closest evolution of StrMs1 is to STR of *O. japonica* since *O. japonica* belongs to the same genus of *M. speciosa* which is rubiaceae. Partially, during evolution, STR maintains homology of important sequences in order to stabilize the protein structure.

The evolutionary analysis was carried out on StrMs1 protein through 2 Tajima tests on MEGA software. The evolution rate was analyzed by choosing an outgroup for each test where the first test compares StrMs1 and STR *O. japonica* with *C. roseus* as the outgroup, while the second test compares StrMs1 and STR *O. japonica* with *R. serpentina* as the outgroup. The output showed that similar sequences between StrMs1 and STR *O. japonica* is only about 161 to 163 aa from a total of 352 aa. This contributes to a low percentage of similarity which is only 45%. This test supports the tree constructed that explains the evolutionary rate of StrMs1 between other STR where StrMs1 is the most evolved between other species. It also shows that although *O. japonica* and *M. speciosa* are of the same genus, the homology among STR genes is still very low.

Prediction and evaluation of StrMs1 3D structure

From the information on STR family protein, the 3D structure of StrMs1 was predicted by utilizing the software Geno3D (Figure 5). The study on StrMs1 protein structure enables us to predict its active site and substrate binding site in order to understand reaction mechanism. It is also useful in reconstruction of many other STR variants that can react with more substrates to produce different alkaloids (Ma et al., 2006) such as in the study of the crystal structure of STR of *R. serpentina* by Stöckigt et al. (2007). As the crystal structure of STR from *R. serpentina* is already known, the structure of *StrMs1* was predicted using STR *R. serpentina* as the template where the STR protein of *R. serpentina* has 60% identity with StrMs1. From the data, the 3D structure and Ramachandran plot were generated. The result indicates the existence of 2 α helices and 8 β barrels. It is

Figure 3. StrMs1 was aligned using ClustalW and the catalytic site is boxed in red. The catalytic site is on the 299th position which is glutamic acid.

noted that this structure is different from Str native protein that has a shape of 6 bladed β propeller. Although, there are similarities in 3D structure, the number of β propellers is different since β propellers are very diverse with different functions. The low homology and functional diversities are referred to characteristics of β propeller (Jawad and Paoli, 2002). It is also believed that the difference in propeller structures is due to dissimilar precursor substrate. The change in 3D structure in many organisms is also associated with few modifications that allow diversity in functionality (Stöckigt et al., 2007).

Based on the generated Ramachandran plot, it is suggested that the predicted structure of STRMs1 is compromised. The percentage of residues in the allowed region is 71.1%, while percentage of residues in allowed additional zone is 24.8%, percentage of residues in good areas is 2.4% and percentage of residues in the disallowed region is 1.6%. The inaccuracy of StrMs1 conformation was thought to be as a result of the scarcity of information on other STR protein structures besides the low homology between STR proteins, the inaccuracy may be caused by the bias towards existing model

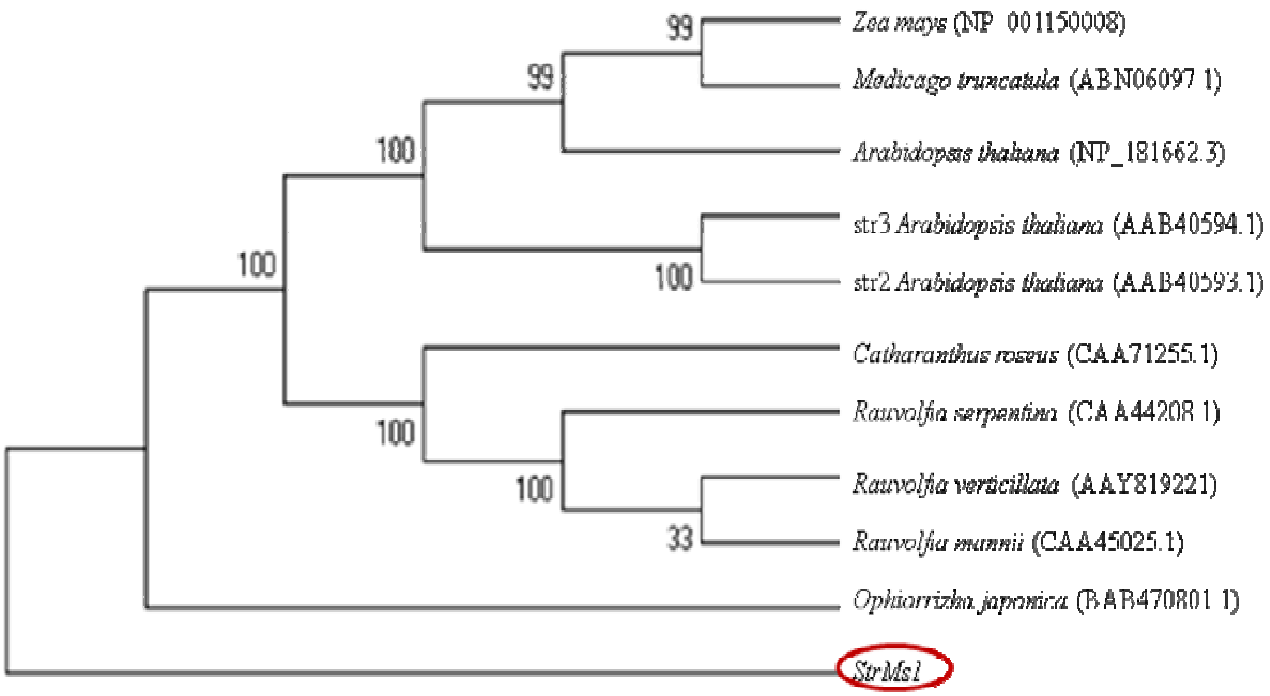

Figure 4. Maximum parsimony phylogenetic tree was constructed to compare the evolution of *StrMs1* with *Zea mays* (NP_001150008), *Medicago truncatula* (ABN06097.1), *Arabidopsis thaliana* (NP_181662.3), str3 *Arabidopsis thaliana* (AAB40594.1), str2 *Arabidopsis thaliana* (AAB40593.1), *Catharanthus roseus* (CAA71255.1), *Rauvolfia serpentina* (CAA44208.1), *Rauvolfia verticillata* (AAY819221), *Rauvolfia mannii* (CAA45025.1), *Ophiorrizha japonica* (BAB470801.1); *StrMs1* was assigned as an outgroup because it is the most evolved gene as compared to others.

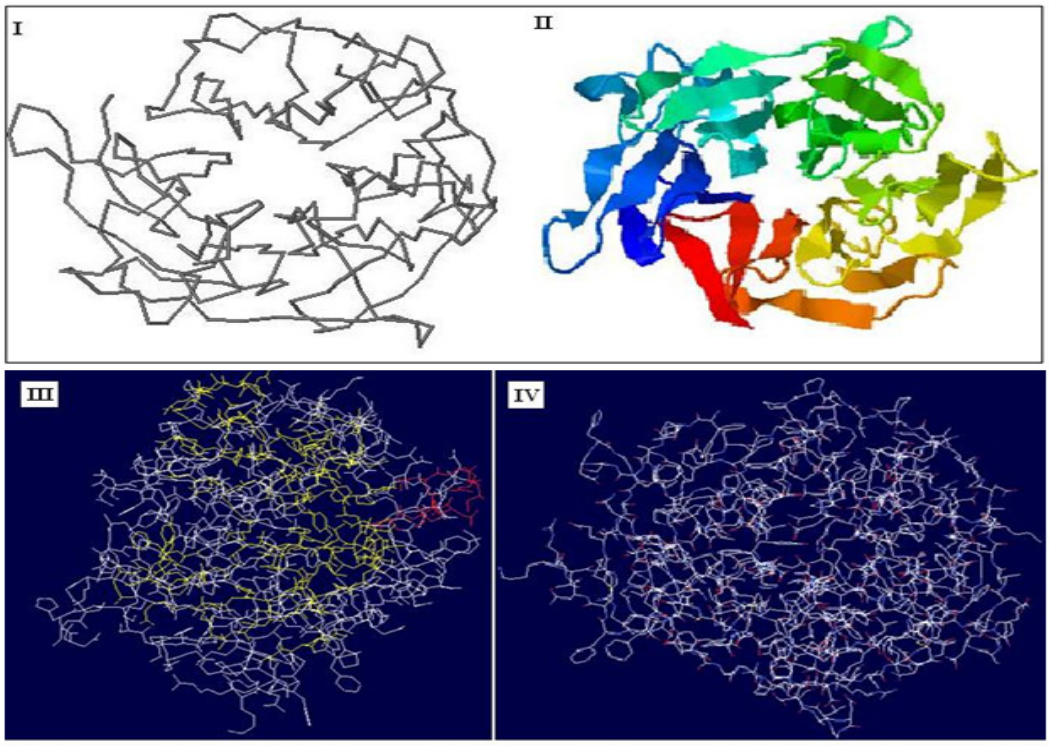

Figure 5. 3D Structure prediction of strictosidine synthase of *M. speciosa* (I) backbone of StrMs1; (II) ribbon structure; (III) secondary structure; and (IV) *StrMs1* ribbon structure.

(Bertini et al., 2003). Therefore, this 3D prediction is tolerable although, not entirely accurate.

Structure validation was also done using Errat 2.0 software found at http://nihserver.mbi.ucla.edu/ERRATv2/. The result shows that this structure has 95.07% of quality factor. The result obtained indicated that the predicted structure had generally acceptable three-dimensional profile. Despite the phylogenetically distant relationship of the target-template sequences, the sequence structure alignment yielded sufficient information of structurally conserved regions to facilitate a functionally probable structure for strictosidine synthase of *M. speciosa.*

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