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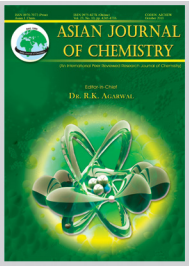

Characterization of Partial cDNA Sequence for *Gnetum gnemon* Resveratrol Synthase Encoding Gene

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Gnetum gnemon is known to be a potential natural source for resveratrol. *Gnetum gnemon* resveratrol synthase encoding gene then was considered to be one of the targets to be cloned. This work describes the characterization of partial cDNA sequence coding for resveratrol synthase by RT-PCR amplification. A pair gene specific primers (5'-GCAACCGTCCTGGCAATCGC-3') and (5'-GTTCCACCTGCGAAGC AGCC-3') was successfully used to amplify a 450 bp fragment that codes for a partial length of *Gnetum gnemon*. The DNA sequences were shown to share a high level of identity to resveratrol synthase from other plants at about 70 % identity. This homology is higher compared with a common 66 % identitiy level of resveratrol synthase among known resveratrol-producing plants.

Key Words: Resveratrol, cDNA, *Gnetum gnemon*, Resveratrol synthase.

INTRODUCTION

Resveratrol is well-known for its anticancer and cancer chemo-preventive activities[1]. It has been suggested that resveratrol acts as an effective anticancer agent for certain types of cancers such as pancreatic cancer[2], gastrous cancer[3], prostate cancer[4], liver cancer[5], lung cancer[6] and servix cancer[7]. Another potential health benefit of resveratrol that has been reported is related to their cardio-protection ability[8].

In plants resveratrol is synthesized and accumulated in non-specific organs of various plant species. The key step in resveratrol biosynthesis is the formation of the stilbenoid backbone by the condensation of a molecule of *p*-coumaryl-CoA and two molecules of malonyl-CoA. This one-step reaction[9] is catalyzed by resveratrol synthase (RS) as shown in Fig. 1. cDNAs encoding resveratrol synthase have been isolated and characterized from various plants including *Vitis riparia, Arachis hypogaea* and *Pinus strobes*[10-12]. Plant resveratrol synthase genes are highly regulated and present as a multigene family that some of the members are regulated by different signals[13]. The resveratrol synthase genes were grouped according to their responsiveness to external signals, including abiotic stresses or biotic signals originating from fungal cells[14].

The resveratrol synthase gene has been considered to be one of the main targets for genetic engineering of resveratrol production[15] as well as attempts to genetically modify the resveratrol content in transgenic plants in which resveratrol synthase is absent, resveratrol synthase genes were transferred in order to provide a defense system against fungal infections. Production of resveratrol are associated with an increased resistance to various fungal pathogens in transgenic tobacco[16], rice[17], wheat[18] and sweet potato[19]. Recently, the development of genetically modified *Rehmannia glutinosa* with three copies of peanut resveratrol synthase gene (AhRS3) has been reported. The resveratrol content in the plant was expressed constitutively in the leaf, root and flower at similar levels[19]. The use of peanut resveratrol synthase has substantially increased resveratrol accumulation in the transgenic plants. The results indicated that the use of different resveratrol synthase source with potentially different activity or effectiveness could result in a better accumulation of resveratrol in the transgenic plants.

Gnetum gnemon is known to be a unique natural source for resveratrol and can be found in a large number in Indonesia. Resveratrol and its derivates were found in *Gnetum gnemon*[20]. Fifteen derivates of resveratrol including viniverin, gnetol, isrhapontigenin, gnetifolin latifolol have been identified present in this plant *Gnetum gnemon,* which make this species could be potentially exploited its resveratrol synthase gene for genetic engineering. However, details concerning resveratrol synthesis in *Gnetum gnemon* are very limited due to the lack of studies to elucidate the pathways. To date, full length of cDNA encoding the *Gnetum gnemon* resveratrol synthase has

not been reported. The current work was aimed at obtaining the full length of cDNA codes for the resveratrol synthase from *Gnetum gnemon*. Here, we report the characterization of a cDNA encoding the partial length of *Gnetum gnemon* resveratrol synthase gene. Our results indicate that the gene is highly conserved in the *Gnetum gnemon*. To our best of knowledge, this is the first report on the isolation and characterization of *Gnetum gnemon* resveratrol synthase.

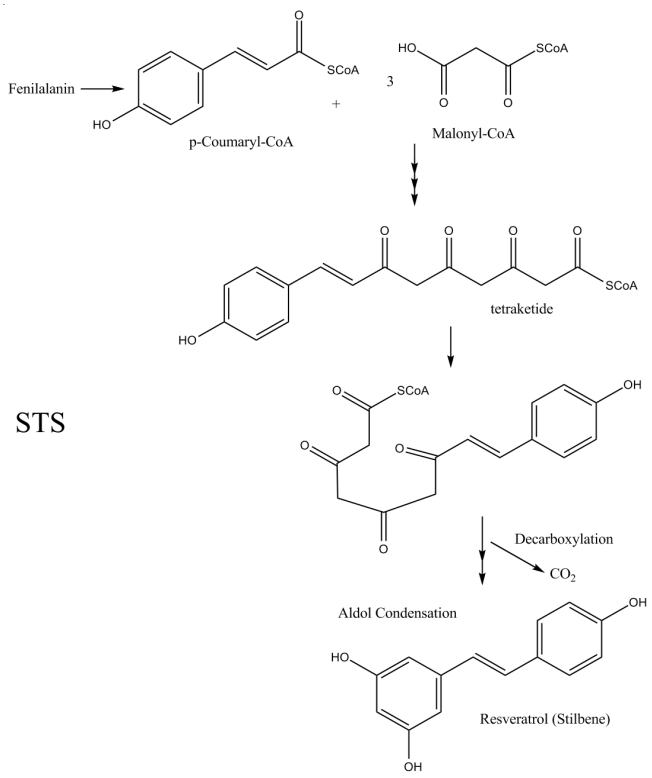

Fig. 1. Resveratrol biosynthesis

EXPERIMENTAL

Leaf of *Gnetum gnemon* was harvested from garden near by Department of Biology, Gadjah Mada University. Trizol RNA isolation kit (Invitrogen), synthesis kit was Transcriptor First Strand cDNA Synthesis Kit (Roche) and iLLustra™ puReTaq Ready-To-Go polymerase chain reaction beads were the main reagents.

Primer design: Polymerase chain reaction primers for reverse transcription-polymerase chain reaction were designed by using conserved regions of resveratrol synthase sequences from other plants that are available at the gene bank (http://www.ncbi.nlm.nih.gov) including *Arachis hypogaea* (Accession # L00952 and DQ124938) *Vitis vinifera* (Accession # DQ459351, AF274281, DQ366302 and DQ366301) *Vitis riparia* (Accession # AF128861), *Polygonum cuspidatum* (Accession # DQ900615 and DQ459349), *Rheum tataricum* (Accession #AF508150). The homology analysis of the amino acid sequence is done using the Clustalw software (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The designed primers were then synthesized at 1stBASE Singapore.

Isolation of total RNA: A 100 mg frozen tissue of young *Gnetum gnemon* leaf was ground to powder in liquid nitrogen using mortar and pestle. The powder was digested in a 1.5 mL tube using Trizol kit (Invitrogen) according to manufacturer protocol with modification. Cell fragments were removed by centrifugation at 12000 g for 5 min at room temperature. The supernatant (800 µL) was extracted once with 200 µL chloroform and shaken well. To precipitate RNA, the mixture is incubated at room temperature for 10 min. RNA was collected by centrifugation at 12,000 g for 5 min. The supernatant (500 µL) was extracted once with 0.5 mL isopropyl alcohol and incubated in room temperature for 10 min. Total RNA was collected by centrifugation, rinsed with 75 % ethanol, dried to completion and dissolved in RNase free water. Gel electrophoresis and visualization of 5 µL total RNA was performed on 1 % agarose gel stained with etidium bromide.

Reverse transcription: Reverse transcription was carried out using kit transcriptor first strand cDNA synthesis kit (Roche) in a total volume of 20 µL. One µg total RNA treated with DNAse (1 µg) was used together with 1 µL primer oligo (dT) 18 (2.5 µM), 2 µL primer random hexamer 60 µM and polymerase chain reaction graded water to obtain a volume total of 13 µL. The template-primer mixture is then heated for 10 min at 65 ºC in a polymerase chain reaction machine and then cooled in ice bath. Into similar tube, 4 µL trancriptor reaction Buffer 5X, 0.5 µL protector RNase 40 U/µL, 2 µL dNTP 10 mM and 0,5 µL enzim trancriptor mix and reverse trancriptase 20 U/µL are added to reach final volume of 20 µL. The product was carefully homogenized, centrifuged and then incubated in polymerase chain reaction at 55 ºC for 0.5 h.

Polymerase chain reaction: The polymerase chain reaction was conducted using iLLustra™ puReTaq Ready-To-Go polymerase chain reaction Beads. Into a polymerase chain reaction tube containing 2 beads (beads consists of enzyme, dNTP and buffer solid mixture), 2 µL primer forward (GGF15'-GCAACCGT CCTGGCAATCGC-3') 10 µM and 2 µL primer reverse (5'- GTTCCACCTGCGAAGCAGCC-3') 10 µM, 2 µL cDNA from reverse transcription step and 21 µL polymerase chain reaction-grade water were added to get a total volume of 25 µL. The mixture was incubated, homogenized and shortly centrifuged. Amplification was carried out with an initial denaturation at 95 ºC followed by 30 cycles at 95 ºC for 1 min, annealing at 52 ºC for 1 min, polymerization at 72 ºC for 1 min and a final extension at 72 ºC for 5 min. Gel electrophoresis and visualization of 20 mL reverse transcription-polymerase chain reaction products was performed on 2 % agarose gels stained with ethidium bromide.

DNA sequence analysis: The sequencing of DNA was carried out on polymerase chain reaction fragment using an ABI PRISM 310 Instrument DNA Analyzer. DNA sequence homology analysis was carried out using Clustalw software (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

RESULTS AND DISCUSSION

Primers for reverse transcription-polymerase chain reaction: The primers used to amplify *Gnetum gnemon* resveratrol synthase gene were designed based on the conserved regions of the genes. This was mainly due to the lack of information available on *Gnetum gnemon* resveratrol synthase. The conserved sequence of amino acids in the positions of 17-23 = ATVLAIG, 112-118 = KAIKEWG were

TABLE-1
INFORMATION ON DESIGNED PRIMERS

No	Primer pair	Amplification region*	Fragment size (pb)	Tm (°C)	GC (%)
1	GGF1: 5' GCAACCGTCCTGGCAATCGC 3'	49-506	450	66.5	65
2	GGR1:5' GTTCCACCTGCGAAGCAGCC 3'			66.5	65

taken as primers. This conserved sequenced have been successfully used for amplification of other polyketide synthase genes in other plant[21]. By considering the preference codon of *Gnetum gnemon* as well as other critical primer parameters: % GC 45-55 %, 18-22 pb primer length and 500-700 pb targeted fragment length, the forward primer GGF1, 5'-GCAACCGTCC TGGCAATCGC-3' and the reverse primer GGR1, 5'- GTTCCACCTGCGAAGCAGCC-3' were derived from the amino acid sequences ATVLAIG and KALKEWG, respectively. Based on the positions of the primers relative to the known sequences for resveratrol synthase from *Arachis hypogaea* the information regarding the size of the expected amplified fragment is shown at Table-1.

Total RNA: Total RNA isolated from leaf tissues of *Gnetum gnemon* was used as a template for reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was recovered at around 0.25 mg/mg fresh weight tissue. Gel electrophoresis and visualization of total RNA products was performed on 1.5 % agarose gels stained with ethidium bromide as shown in Fig. 2. Several bands appeared during electrophoresis could be representation of ribosome RNA.

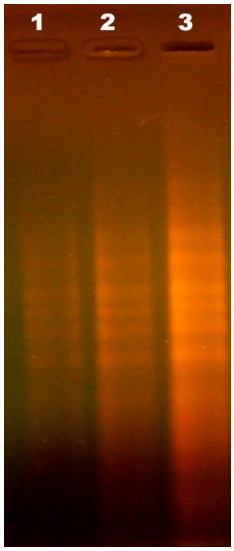

Fig. 2. Electrophoresis of isolated total RNA from *G. Gnemon* leaf. Lane, 1,2,3 are different of the volume of the loaded sample: (1) 1.12 mg, (2) 3.36 mg (3) 5.60 mg

Reverse transcription-polymerase chain reaction of *Gnetum gnemon* resveratrol synthase cDNA: Reverse transcription-polymerase chain reaction was carried out to the total RNA instead of mRNA considering the fact that resveratrol along with its derivates is significantly available. Therefore sufficient amount of resveratrol synthase mRNA could be found in the total RNA and more than enough to be amplified by reverse transcription-polymerase chain reaction. All condition presented here was a result of optimization of previous with considering primers information. OligoDT and hexamer primers were used in the reverse transcription step to produce a collection of resveratrol synthase cDNA.

The partial DNA sequence was obtained for representative reverse transcription-polymerase chain reaction products. In this work, a primer combination (GGF1/GGR1) was successfully optimized to produce a single amplified fragment of about 450 bp (Fig. 3). Based on the positions of the primers relative to the known sequences for resveratrol synthase from *Arachis hypogaea* (Table-1), this fragment is of the expected size. However the DNA bands could still consist of many fragment with similar size but different nucleotide sequence. Ideally cloning of the fragments in *E. coli* using T-plasmid vector followed by characterization the resulted clones is the most reasonable approaches. Instead of cloning, reverse transcription-polymerase chain reaction fragment was then purified using PureLink™ Quick gel extraction kit (Invitrogen) for further analysis and directly sequence. This approach has been reported to successfully polyketide synthase gene in *Cannabis sativa*[22].

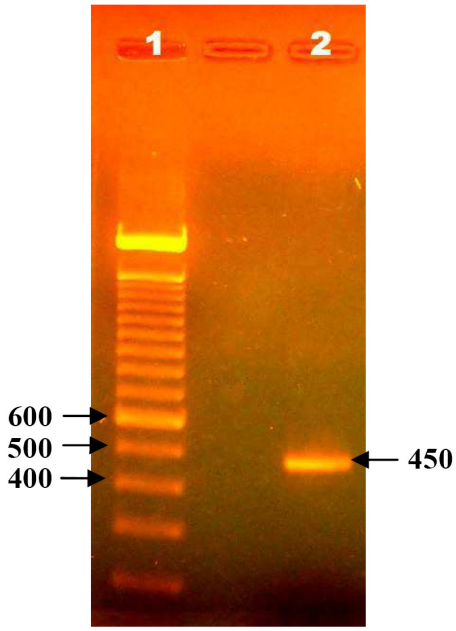

Fig. 3. Reverse transcription-polymerase chain reaction amplification of *Gnetum gnemon* resveratrol synthase gene using degenerate primers. A combination of GGF1/GGR1 primers was shown to successfully produce a single amplified fragment of the expected size. Lane 1: DNA marker; lane 2: 450 bp fragment

DNA sequence analysis: The direct sequence of the polymerase chain reaction product provides evidence that the isolated DNA fragment is single band. This evidence comes from the electrophoregram of the sequence that shown clear resolution of the nucleotide sequence. The complete DNA sequence was obtained for representative fragment from reverse transcription-polymerase chain reaction products. Since the sequencing was performed directly to the purified polymerase

chain reaction fragments, the size of the obtained sequence is shorter than the size of the DNA fragment. It is due to sequence of the primer at both side as well as a few bases located next to the primer is difficult to be analyzed. The sequence analysis revealed that this fragment's size was 403 bp and shown in Fig. 4.

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TACCATCTTT AGGCAGTCGC ATACTCGAGT GGATTGCCCT ATGTATCAGA
CCAAGAACCG AATTTCCCAA CAGGAGCTCT TGGTCTACTA CTAGGGATTC
CAAATCCACC ATAACCAAAA CATAAGGAGA GGAGGTAGAG CAATAGCTTG
GGTTGACCCT CTTAATGAAG ATCGATCTGG CAGAGGAGAA GTAGATCCAT
TCAAGTCGAA AGCAAGGTGG AAAGTCTCAA TTCAATGCTG CGGTTGATGA
CCGTAGCAAT AGTCAATGGC ACTGGAGGGA AGCCGCTTGG AAGGAAGGTG
GCTTATGCTG TTGAAAGCCT AGCTCATCCT AGCATGGCCT ATCTTTATCC
CCTGATAATG ATCAATCATA TTCAATATCA TGAATAGGTC TGCCTGTCTA
CCA 403
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Fig. 4. Nucleotide codes of fragment obtained from GGF1/GGR1 primers

Results from Clustalw analysis indicated that the DNA sequence was highly identical to resveratrol synthase sequence from other plants (*Arachis hypogaea* and *Vitis vinifera* released in Genbank databases) at about 70 % identity (more than average homology among resveratrol synthase gene sequences) and the position of the homolog sequences implying that it is highly probably a part of a resveratrol synthase gene[23]. The nucleotide sequence of GGF1/GGR1 resveratrol synthase gene fragment has been deposited in GenBank databases under the Accession # AB628073.

Conclusion

Reverse transcription-polymerase chain reaction using primer designed based on the published resveratrol synthase to the total RNA isolated *Gnetum gnemon* have succeeded to amplify part of resveratrol synthase gene present in *Gnetum gnemon*. It is confirmed by the homology of the sequence to the other resveratrol synthase gene, which is 70 % (higher than average homology among resveratrol synthase gene) and the position of the homology sequence which indicate that reverse transcription-polymerase chain reaction has amplied expected cDNA fragment.

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