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Cloning, Expression in *Escherichia coli* and Purification of the Resveratrol Synthase Gene from *Arachis hypogaea*

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Resveratrol synthase (RS) is the key and rate-limiting enzyme in resveratrol biosynthesis pathway. In this paper, the RS gene was cloned from *Arachis hypogaea* and was identified as an open reading frame of 1,170 bp encoding a protein of 389 amino acids. The predicted protein of RS has a calculated molecular mass of 43 kDa and an isoelectric point of 7.0. A prokaryotic expression vector containing *Bam*H I and *Sal* I restriction sites, pET-30a/RS, was constructed and transformed to *Escherichia coli* BL21(DE3). Then the recombinant bacteria were identified by bacteria liquid PCR, enzymes digestion and sequencing. The inducible expression of the target protein, a soluble protein, was detected and analysized by SDS-PAGE and Western Blot. Finally, the object protein was purified by Ni-NTA affinity chromatography after the bacteria cells disruption. Based on current research, the overproduction and purification system for the RS developed in this study would be invaluable in future studies of the functions of RS and biosynthesis of resveratrol.

Key words: Resveratrol synthase, *Arachis hypogaea,* Soluble protein, Resveratrol.

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene, Res), a member of the non-flavonoids family, is a low molecular weight phenolic compound produced in some higher plant species, e.g., grapes (*Vitis vinifera*), peanuts (*Arachis hypogaea*), and mulberry fruits (*Fructus mori*). With damage, fungal infection or ultraviolet radiation on plants, the Res was produced as a phytoalexin and could effectively play a role in resilience[1,2]. Luhua14 is a special and important provenance of the *Arachis hypogaea* species and mainly distributes in Shangdong Province, China. Some studies have found that the Res has many benefits for human health, i.e., anti-oxidation[3,4], anti-cancer[5], anti-aging[6], anti-diabetic[7], cardiovascular protection[8,9], and neuroprotection[10] Therefore, the Res is becoming known as another new green anti-cancer drug similar to paclitaxel. In plants, the Res was derived from the general phenylpropanoid pathway[11-16]. A *de novo* biosynthetic pathway of the Res is shown in Fig. 1. In this synthetic pathway, resveratrol synthase (RS) is the rate-limiting and key enzyme, and has a high specificity for the substrate[17]. The last step of this pathway is the stepwise condensation of one molecule of *p*-coumaroyl-CoA with three molecules of malonyl-CoA catalyzed by RS[18,19]. In this paper, we described cloning of the RS gene from *Arachis hypogaea*, construction of a prokaryotic expression vector, and inducible expression of the object protein. Finally, we analysized the target protein with western blot and

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constructed the means to purify it. Studying the renatured protein can lay the foundation for large-scale fermentation of the Res.

MATERIALS AND METHODS

Plant material, strains, plasmids and reagents

The plant material used in this study was luhua14 (*Arachis hypogaea*), which purchased from Shandong Academy of Agricultural Science. The bacterial strains and plasmids used in this study were the following: pMD18-T vector (Takara, Tokyo, Japan); pET-30a (+) (Invitrogen, Carlsbad, CA, USA); *Escherichia coli* TOP10 and *E. coli* BL21 (DE3) (TransGen Biotech, Beijing, China). The reagents used in this study were the following: DNA markers, protein markers and 2×Taq PCR MasterMix (Biomed, Beijing, China); T4 DNA ligase, *Bam*H I-HF and *Sal* I-HF (New England Biolabs, Beijing, China) and all other reagents were of analytical grade. Unless otherwise stated, all bacteria were routinely cultured at 37! in LB broth (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) in a shaking incubator or LB agar plates supplemented with kanamycin sulfate (30 mg/L).

Extraction of total RNA and synthesis of first-strand cDNA

Due to containing rich polyphenols and polysaccharides of peanuts, the quality of RNA using trizol was poor. Therefore, a MiniBEST Plant RNA Extraction Kit (TaKaRa, Dalian, China) should be considered to extract the total RNA in this research. cDNA was synthesized with a reverse transcription kit (TransGen Biotech, Beijing, China) and immediately preserved at -20!.

Cloning of the RS gene

According to the full-length sequences of the RS gene from *Arachis hypogaea* in the NCBI (GenBank accession no. AY170347), the primers used for PCR amplification are lised in Table 1. The RS gene was synthesized with cDNA as a template, RSF_1 and RSF_2 as primers for PCR amplification. The reaction solutions were the following: cDNA, 2 ¼L; RSF_1, 1 ¼L; RSR_1, 1 ¼L; 2×Taq PCR MasterMix, 10 ¼L; ddH_2O, 6 ¼L. Reaction conditions were the following: 94! 5 min; 94! 30 s, 58! 30 s, 72! 120 s, 30 cycles; 72! 10 min. The amplified fragments were detected by agarose gel electrophoresis and then recovered. The recovered fragments were connected to the pMD18-T vector and transformed to *E. coli* TOP10.

Construction of pET-30a/RS

To connect the RS gene to the pET-30a (+) vector, *Bam*H I and *Sal* I restriction sites were introduced into upstream primer and downstream primer, respectively. The gene fragment I, *Bam*H I + RS + *Sal* I, was synthesized with the pMD18-T/RS as a template, RSF_2 and RSR_2 as primers. Reaction solutions and conditions were identical to those previously mentioned. The gene fragment I and pET-30a (+) vector were digested using *Bam*H I and *Sal* I enzymes. The digested fragments were detected by agarose gel electrophoresis, and then recovered. The recombinant plasmid, pET-30a/RS, was constructed by ligating the two recovered fragments with T4 DNA ligase and transformed to *E. coil* BL21(DE3).

Identification of pET-30a/RS

In the first place, the recombinant plasmid, pET-30a/RS, was identified by bacteria liquid PCR. A single colony of the *E. coil* BL21(DE3) harboring pET-30a/RS was grown overnight in fresh medium. In this study, we designed a negative control (i.e., pET-30a (+) as a template) and also designed a positive control (i.e., the final ligation of product as a template). In this study, the T7 primers were selected for bacteria liquid PCR (Table 1). In the next place, the pET-30a/RS was identified with enzymes digestion. Plasmids were extracted from positive strains identified by bacteria liquid PCR and digested with *Bam*H I, *Sal* I and both of them. Finally, the above bacteria identified as positive were sequenced.

Inducible expression and western blot of the RS

Recombinant strains were inoculated into fresh medium in a proportion of 1:50 and grown for 4 h (OD_{600} approximately 0.5). Then IPTG (Isopropyl-²-D-Thiogalactopyranoside) was added at a final concentration of 0.3 mM at 28!. In induction time experiments, these strains were induced for 2 h, 4 h, 6 h, and 8 h. The bacterial cells were collected with hypothermia centrifuge and then disrupted with hypothermia ultrasonic. We analyzed the induced protein to determine whether it was soluble or not.

Protein obtained at optimum condition was electrophoresed on 12% SDS-PAGE and transferred to PVDF (Millipore, Boston, USA) which had immersed in methanol for 5min and then transfer buffer for 30 min by electroblotting. The

PVDF membrane was blocked with 0.05% skimmed milk diluted in PBS buffer (2 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, and pH 7.4) at room temperature (RT) for 2 h, and incubated with ProteinFind anti-His mouse monoclonal antibody (TransGen Biotech, Beijing, China) against the expressed proteins at 4! overnight. After washed for three times with TBST (20 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween20), the membrane was incubated with ProteinFind goat anti-mouse IgG(H+L) conjugated HRP (TransGen Biotech, Beijing, China) at RT for 1 h. After washed for five times with TBST, the membrane was drained on the filter paper and immersed in the superECL plus detection reagent (Millipore, Boston, USA) at RT for 3 min. Protein bands were revealed using a PageRuler™ Prestained Protein Ladder (Fermentas, Shenzhen, China).

Purification of the expressed RS

The recombinant strains were diluted 50-fold into fresh medium under optimized culture condition, and prepared for purification by a *ProteinIso*™ Ni-NTA Resin (TransGen Biotech, Beijing, China). The total protein was isolated from the culture supernatant by precipitation with saturated solution of ammonium sulfate in an ice bath for 30 min, and dissolved in 50 mL of equilibrating buffer (300 mM NaCl, 50 mM NaH_2PO_4, 10 mM imidazole, 10 mM Tris base, and pH 8.0). The solution was then filtered through a 0.45 ¼m filter and applied to the *ProteinIso*™ Ni-NTA resin column, which was equilibrated with equilibrating buffer for 30 min. After elution of proteins lacking the His-tag with equilibrating buffer, the fusion protein containing the His-tag was eluted from the resin using elution buffer (300 mM NaCl, 50 mM NaH_2PO_4, 80 mM imidazole, 10 mM Tris base, and pH 8.0). The eluted protein was collected and ananlyzed by SDS-PAGE.

RESULTS

Total RNA and the RS gene

Total RNA was detected by agarose gel electrophoresis (Fig. 2). It showed the total RNA had two apparent bands, and the 28S band was twice the brightness of the 18S band. The 5S band was not observed, which because the 5S was probably degraded for the extraction kit. The results indicated the quality of the extracted RNA was good.

Agarose gel electrophoresis (Fig. 3) showed that the size of the amplified fragment was 1,170 bp, which was consistent with the RS gene in size. The amplified fragment was recovered and ligated to the pMD18-T vector. After sequencing and analyzing, the cloned RS gene contained 1,170 nucleotides, including the initiation codon and termination codon, as a complete ORF (open reading frame) encoding 389 amino acids (Fig. 4). Sequence alignment of nucleic acids showed that the cloned gene was similar to the known RS gene in the NCBI database at a rate of 94% to 99%. The homologous alignment of proteins showed that the amino acid sequences were similar to the known amino acid sequences at a rate of 95% to 99%.

Identification of pET-30a/RS

(1) Bacteria liquid PCR identification: results of bacteria liquid PCR were detected by agarose gel electrophoresis (Fig. 5). The amplified fragment of the negative control was 410 bp in size, which showed that the sequences between T7 promoter and terminator in pET-30a (+) were 410 bp in size. The amplified fragments of bacteria

Table 1. Sequence of primers for PCR.

primer	(5''!3') sequence
RSF_1	ATGGTGTCTGTGAGTGGAATTCGCAAG
RSR_1	TTGTATGGCCATGCTGCGGAGGACAAC
RSF_2	CGCGGATCCATGGTGTCTGTGAGTGGAAT*
RSR_2	ACGCGTCGACTTATATGGCCATGCTGCG**
T7 promoter	TAATACGACTCACTATAGGG
T7 terminator	TGCTAGTTATTGCTCAGCGG

* The underlined sequences are *Bam*H I restriction site.
** The underlined sequences are *Sal* I restriction site.

liquid and the positive control were 1,580 bp in size, which showed that pET-30a/RS plasmid had been successfully constructed.

(2) Enzymes digestion: digestion results were detected by agarose gel electrophoresis (Fig. 6). The recombinant plasmids after single-enzyme digesting were 6,579 bp in size, while after double-enzyme digesting were 5,409 bp and 1,170 bp in size. The 5,409 bp fragment was consistent with pET-30a (+) digested with the same enzymes, while the 1,170bp in size was consistent with the RS gene fragment. The results further verified that the prokaryotic expression vector, pET-30a/RS, was constructed successfully.

(3) Sequencing: the above bacteria that were identified as positive were sequenced. Sequence analysis showed that the RS gene had been successfully connected to the pET-30a by *Bam*H I and *Sal* I restriction sites.

Inducible expression and purification of the RS

The induced protein was detected by SDS-PAGE (Fig. 7a). The results showed that the molecular weight of the induced protein was approximate 43 kDa, which matched the predicted

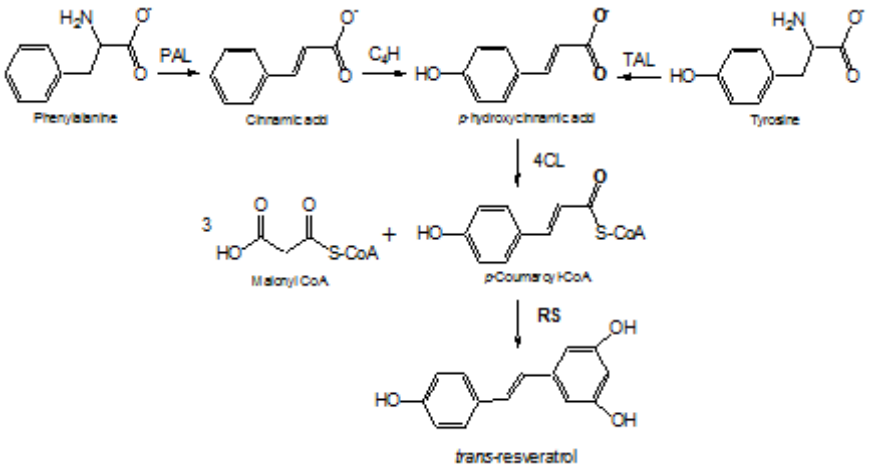

Fig. 1. Biosynthetic pathways of the Res. Res can be sythesized either starting with phenylalanine or from tyrosine, both pathways giving rise to *p*-hydroxycinnamic acid. The 4CL attaches the *p*-hydroxycinnamic acid to the pantetheine group of coenzyme A to produce *p*-coumaroyl-CoA, which is then transformed into the Res. PAL: phenylalanin ammonia lyase, TAL: tyrosine ammonia lyase, C_4H: cinnamate-4-hydroxylase, 4CL: *p*-coumaroyl-CoA ligase.

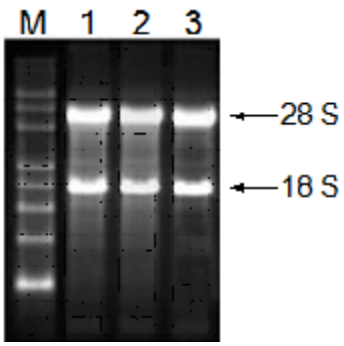

Fig. 2. Agarose gel electrophoresis of total RNA from luhua14 (Arachis hypogaea). Lane M, DNA molecular weight markers: BM5000Marker; Lanes 1-3, total RNA.

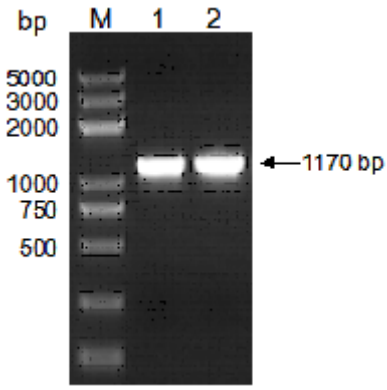

Fig. 3. Agarose gel electrophoresis of the RS gene from luhua14 (Arachis hypogaea). Lane M, DNA molecular weight markers; Lanes 1-2, RS gene.

molecular weight of the object protein. The bacterial cells after inducible expression were collected and disrupted. The product was centrifuged for 30 min at 4!, 13,000 rpm. The supernatant was analyzed by SDS-PAGE (Fig. 7b), and showed that the induced protein was a soluble protein. This provided a theoretical basis for the Res biosynthesis in prokaryotic microbial fermentation. Our results also showed that the target protein had obviously begun to express after 6 h and the expression levels changed little with time after 8 h, therefore, we determined the optimal time of inducible expression as 8 h. The western blot (Fig. 8) showed a major cross-reaction polypeptide of 43 kDa that was coincident with the predicted size range of the RS protein.

The supernatant was also purified with ProteinIso Ni-NTA Resin. The results (Fig. 9) showed that the object protein was purified after nickel affinity column based on the presence of 6×His tag in the recombinant protein. The activity of the puried protein needs further study. We hope that the purified protein after being renatured can be used for resveratrol synthesis.

Fig. 4. Nucleotide and deduced amino acid sequences of RS gene (GenBank accession no. **AY170347**). Deduced amino acid sequences were shown below the nucleotide sequences. ATG with frame was the initiation codon of the RS gene. A 'Cys' with circle was the active site of RS enzyme. The underlined amino acids, GVLFGFGPGLT, were the characteristic sequences of the RS family. The stop codons are marked with an asterisk.

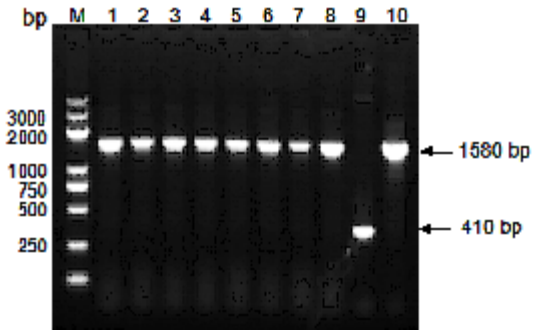

Fig. 5. Identification of bacteria liquid PCR for *E. coli* BL21 (DE3) transformed with pET-30a/RS. Lane M, DNA molecular weight markers; Lanes 1-8, Bacteria liquid; Lane 9, Negative control; Lane 10, Positive control.

DISCUSSION

As an important plant secondary metabolite, the Res has a variety of biological functions and beneficial functions for human health. From "French Paradox" phenomenon in-depth study, the Res was the most beneficial ingredient in red wine for human health[20,21]. At present, there are ways to obtain the Res using plant formulations, chemical synthesis, and biosynthesis. Biosynthesis can not only overcome the problem of ecological destruction and resource scarcity associated with plant formulations, but can also solve environmental pollution problems through chemical synthesis.

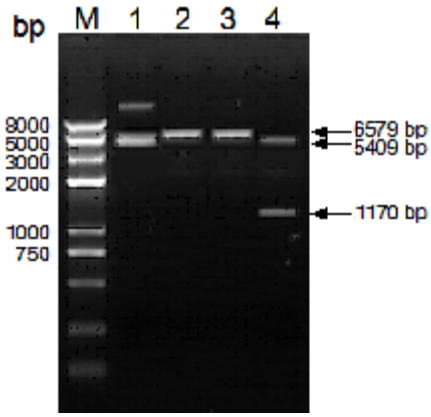

Fig. 6. Identification of enzyme digestion for the pET-30a/RS. Lane M, DNA molecular weight markers: BM8000Marker; Lane 1, Indigestion of plasmids; Lane 2, *Bam*H I digestion of plasmids; Lane 3, *Sal* I digestion of plasmids; Lane 4, *Bam*H I & *Sal* I digestion of plasmids.

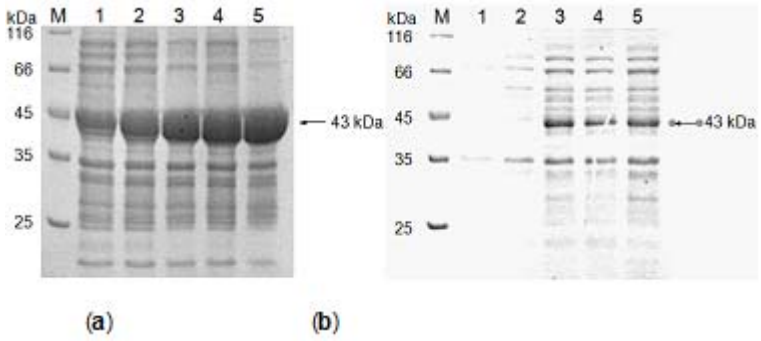

Fig. 7. SDS-PAGE analyses of total proteins (**a**) and supernatant proteins after disruption and centrifugation (**b**). RS was obtained from *E. coli* BL21 (DE3) transformed with pET-30a/RS with 0.3 mM IPTG induction at 28!, and was analyzed on 12% SDS-PAGE gels. Broad range protein molecular weight markers were used as the standard of proteins for the determination of molecular weights. Lane M, Protein Marker; Lanes 1-5, induction of the pET-30a/RS at 2 h, 4 h, 6 h, 8 h, and 10 h, respectively.

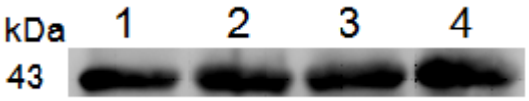

Fig. 8. Western blot analyses of the RS expression. The membrane was cut lengthaise direction between the two lanes and incubated with anti-His antibody. Note the induced RS is about 43 kDa. Lanes 1-4, Loading volume of the protein lysate at 5¼L, 10¼L, 15¼L, and 20¼L, respectively.

The RS is the last key enzyme in the Res biosynthetic pathway. In this study, we showed the RS gene from luhua14 (*Arachis hypogaea*) contained a complete ORF after sequencing and homology comparison. In addition to the partial nucleotide differences due to different varieties, the RS gene was highly conserved in comparison with nucleotide sequences of stilbene synthase and chalcone synthase. Meanwhile, the sequences of the RS have some similarities with other members of the stilbene synthase family and contain a characteristic sequence 'GVLFGFGPGLT' and active site 'Cys'[18,22]. A prokaryotic expression vector was constructed and then inducible expressed to obtain a soluble protein. Finally, we separated and purified the target protein using protein purification and separation technology. The combinant trains in this study may be used for the Res biosynthesis in large-scale fermentation.

CONCLUSION

We successfully cloned, expressed in *E. coli* and purified the resveratrol synthase from luhua14 (*Arachis hypogaea*). The purified RS could be used for many studies such as structures and functions of the RS. Overproduction and purification system for the RS developed in this study would be invaluable in future studies of the functions of RS. We firmly believe that a large-scale biosynthesis of resveratrol can become a reality in the near future.

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