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Construction of Escherchia coli strain for producing recombinant antioxidative peptide from longan seeds

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Abstract

Antioxidants, substances that prevent damage from free radicals ,have originally been discovered in various natural sources including fruit seeds. In longan seeds, a potential antioxidative peptide with amino acid sequence as ISYVVPVYIAEITPKT - FRGGF, was found. In this research, to overcome protein hydrolysate preparation problems, genetic engineering was used to produce arecombinant version of this antioxidative peptide in Escherichia coli. For the easier genetic manipulation, the DNA fragment for encoding the target peptide was designed by containing 4 copies of the interested peptide and each copy was linked by a codon of Aspartic acid. After IPTG induction, the recombinant peptide was successfully expressed and purified. The recombinant peptide was verified by Endoproteinase AspN digestion and MALDI - TOF-MS. The antioxidant activity of this recombinant peptide will be further studied

Keywords : *antioxidant / longan/overexpression / peptide.*

1. Introduction

Free radicals potentially cause many diseases due to the ability to damage biomolecules. Although human body contains mechanisms to eliminate free radicals [1], it may not enough. Therefore, taking additional sources of antioxidants, such as fruits, vegetables and vitamins, is necessary. Currently, natural antioxidative peptides become interested due to potential toxicity from synthetic antioxidants, including butylated hydroxyl toluene (BHT), butylated hydroxyl anisole (BHA) [2-3].

Previous researches have suggested health benefitsfrom phytochemicals that found in edible plants and served as antioxidants [4-7]. Moreover, they are potentially applicable as functional ingredients in food industry to reduce oxidative deterioration, and as active ingredients in nutraceuticals and cosmeceuticals [8]. However, direct extraction from natural sources usually encounters some problems such as high amount of samples , low yield of peptide, and relatively difficult extraction process.

To overcome these problems, the peptide named as Longan1 (ISYVVPVYIAEIT-PKTFRGGF) from the longan seed protein hydrolysate (Dr. Aphichart Kanchanatat, personal communication) was attempted to be producedfrom recombinant *E.coli*. However, as the target peptide was short and difficult to genetically manipulate, the DNA fragment was designed to contain four repeats of the target peptide linked by single aspartic acid residues. This design with repetitive copies was reported previously for synthesizing small recombinant peptides , such as the small recombinant beefy meaty peptide [9]. In this work, the recombinant peptide was successfully produced. The antioxidant of this peptide will be tested. It could be a candidate for further applications as antioxidant. Furthermore, the technique to express this recombinant peptide should be useful for producing other short peptides.

2. Materials and Methods

DNA fragment synthesis: The DNA fragment encoding the target peptide characterized from the longan seed hydrolysate was synthesized by Integrated DNA Technologies (IDT), Inc., Coralville, IA, USA. Owing to the short length of the peptide, the DNA fragment was designed to have four repeats of the target peptide with each peptide linked by a single aspartic acid residue (Figure 1).

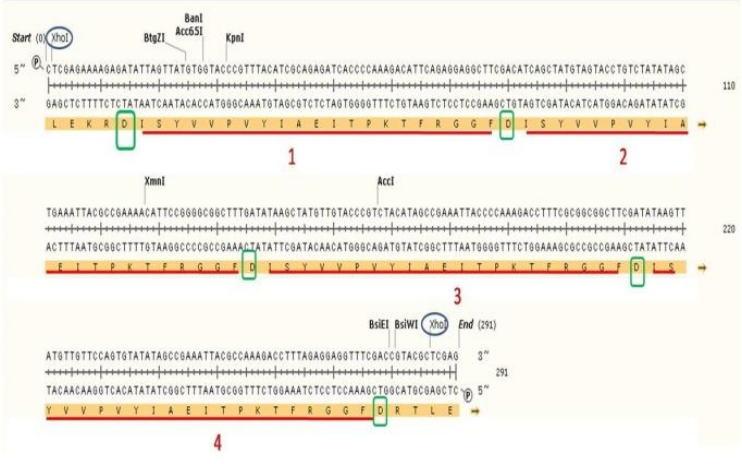

Figure 1. The DNA fragment designed for recombinant Logan 1 peptide production.

The synthesized DNA fragment was flanked by *XhoI* restriction sites, in which the 5′ and 3′ ends were further changed to *BamH* I and *Hind* III sites, respectively. during amplification by PCR. The 279 bp DNA fragment was amplified by Neo DNA polymerase (TOYOBO, Tokyo, Japan) with the primers: L1F (5'-GGATCCAAAA-GAGATATTAGTT- ATGTGGT-3' and L1R (5'-AAGCTTCG-AAACTCCTCTAAA-3') introducing *BamH* I and *Hind* III sites, respectively. The PCR product was digested with *BamH* I and *Hind* III (New England Biolabs, Ipswich , MA, USA) at 37°C overnight , and cloned into the same sites of the pQE 30-Xa vector . The ligation reaction was performed at 16°C overnight with 20 U of T4 DNA ligase (New England

Biolabs). Ten microliters of the ligation reaction was transformed into *E. coli* MG 1655 by chemical transformation and selected by the blue /white screening method on LB agar containing 8µg/ml of X-gal and 100 µg/ml of ampicillin [10]. The recombinant plasmid was further verified by *BamH* I and *Hind* III double digestion , PCR withL 1F and L1R primers, and DNAsequencing (Macrogen, Seoul ,Korea).

Expression and purification of the recombinant peptide : The verified recombinant *E. coli* strain was cultured in 2 ml of LB broth with 100 µg/ml ampicillin and incubated at 37°C with 200 rpm shaking overnight. One hundred microliters of overnight culture was transferred into 50 ml of LB medium containing 100 µ g/ml of ampicillin and incubated at 37°C and 200 rpm until the OD 600 value reached 0.6–1.0 (~3 h). Isopropyl β-D-1- thiogalactopyranoside (IPTG) was added to the culture to induce peptide expression. Samples were harvested every 2 h for 6 h. The cell pellet after centrifugation at 8,000 × g for 15 min was kept at 20°C until use for cell extraction under denaturing conditions.

The cell pellet was thawed on ice and 1 g of the wet cell pellet was resuspended in 5 ml of lysis equilibrium wash (LEW) buffer (50 mM Na2PO4 and 300 mM NaCl, pH 8.0) on ice. The resuspension was mixed by pipetting until no visible cell aggregates were observable and 1 mg /ml of lysozyme was added . After stirring on ice for 30 min, cells were further lysed by sonication (10 ×15 s bursts at high intensity with a 15 s cooling period between each burst). The soluble protein fraction was harvested by collecting the supernatant after centrifugation at 10,000× g and 4°C for 30 min. The remaining pellet was resuspended in 5 ml of LEW buffer for washing and harvested by centrifugation at 10,000 × g and 4°C for 30 min. The pellet was resuspended in 3 ml of denaturation solubilization (DS) buffer (50 mM Na 2PO 4, 300 mM NaCl and 8 M urea, pH 8.0). After centrifugation at 10,000 × g and 4°C for 30 min, this supernatant was marked as the insoluble protein fraction. All samples were stored at 4°C until use. The target peptide band was analyzed by loading collected samples onto an 18% SDS-PAGE gel.

Owing to expression using the pQE-30 Xa vector , the recombinant peptide included a polyhistidine-tag and the Protino® Ni-IDA packed column (MACHEREY - NAGEL, Germany) was used to purify the recombinant peptide under denaturing condition according to the manufacturer mannual. The purification profile of each collected fraction was analyzed by 18% SDS-PAGE.

Verification of the recombinant peptide:The purified recombinant Longan 1 was digested by Endoproteinase AspN (New England Biolabs, Ipswich, MA, USA) at 37°C for 3 h. The molecular weight s of the chemical synthesized and digested recombinant Longan 1 peptide were determined by an autoflex (MALDI - TOF - MS (Bruker Daltonics, Leipzig, Germany). One microlitter of each sample was spotted onto the MTP 384 grondsteel target plate (Bruker Daltonics),air dried, and then 1µl of thebead - suspension - SA- matrix mixture (1:1, v/v) was directly spot ted onto the MTP 384 massive aluminium target plate. The external calibration of the instrument was performed using a standard/peptide

mixture kit according to the mannual of the manufacturer. Twenty individual

sprectra were averaged to produce a single mass spectrum in each analysis

3. Results and Discussions

The antioxidati vepeptide named as Longan 1 (ISYVVPVYI AEITPKT-FR-GGF), found in longanseeds hydrolysate, was obtained from Dr. Apichart Kanchanat at, Chulalongkorn University. The synthesized DNA fragment was in the plasmid IDT -blue (IDT, USA) named as pTWLG1 was used as the templte for PCR. The roducts with the expected size of 279 bp was obtained (Figure 2). Ths DNA fragment contains 4 copies of nucleotides encoding Longan 1 peptide and each is linked by a codon of Aspartic aci d. The PCR product was then digested with *BamH* I and *Hind* III and cloned into the same sites of pQE 30-Xa plasmid vector. The recombinant p lasmid was transformed into *E. coli* MG 1655 by chemical transformation and selected by the blue /white screening method on LB agar containing 8µg/ml of X-gal and100µg/ml of Ampicillin. Four white clones were selected and named as TWLP1-4.Plasmids, named as pTWLP 1, pTWLP 2,pTWLP 3 and pTWLP 4, were extracted from the four previously mentioned clones and further checked by *BamH* I and *Hind* III digestion. The expected DNA bands with approximately 280 bp to 3500 bp, as shown in Figue 3, was obtained from the digestion.

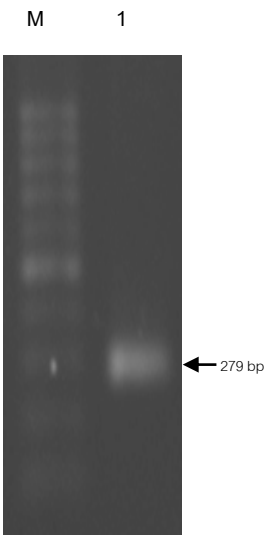

Figure 2. The amplification of DNA for Longan 1 peptide production by PCR. M: 100 bp Marker, 1: PCR productfrom pTWLG1 plasmid

The selected clones were also verified by PCR with primers L1F and L1 R to detect the DNA inserts. As in Figure 4, the expected 279 bp PCR products were obtained. Furthermore, the correct DNA sequences were revealed from these 4 plasmids when verified by DNA sequencing (data not shown)

The selected TWLP strains were then cultured and the target peptide expression was induced by IPTG . the expected 10 KDa protein bands were revealed after puried

by Protino® Ni - IDA Packed Columns (Figure5). However, there were still unspecific proteins obtained after purification. The 10 KDa protein band was extracted from the SDS-PAGE gel, and then was digested by Endoproteinase AspN. With this digestion, Aspartic acid linkage should be cut, and short peptide, as the original, with the molecular weight 2358 Da should be obtained. The moelcular weight of the digested recombinant Longan 1 peptide was determined by MALDI-TOF-MS. The chemical synthesized Longan 1 peptide was used as control. As expected, the sprectrum of 2358 Da was revealed from the digested recombinant, as well as, chemical synthesized one (data not shown). It could be suggested that the recombinant Longan 1 peptide was ssuccessfully produced from the recombinant *E. coli* strain.

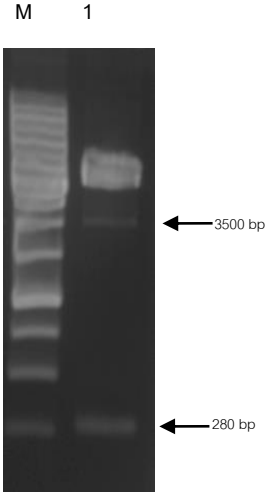

Figure 3. Digestion of pTWLP1 plasmid with with BamHI and HindIII enzymes providing approximately 280 bp to 3500 bp DNA bands.
M: 1Kb, 1: pTWLP1 vector digested with BamHI and HindIII

4. Conclusion

A potential antioxidative peptide , with amino acid sequence as ISYVVPVYIAEI-TPKTFRGGF, named as Longan 1, was found in longan seeds. In this research, recombinant *E. coli* strains were construct-ed to improve this antioxidative antioxidative peptide production. The DNA fragment encoding 4 copies of Longan 1 peptide linked each by a codon of Aspartic acid was designed. This DNA fragment was then inserted into the expression vector, pQE-30Xa and transformed into *E.coil* MG1665. The four selected clones were verified and further used for recombinant peptide expressionby IPTG induction. The expected 10 KDa of recombinant peptide was expressed. However, some unspecific proteins were still obtained after purifying the target peptide by Protino ® Ni-IDA Packed Columns. Therefore, other suittable purification step are required to improve the recombinant peptide production. The recombiant Longan 1 peptide was verified Endoproteinase AspN digestion and MALDI-TOF-MS. The further study will be focus on antioxidant activity characterization of this recombinant Longan 1 peptide.

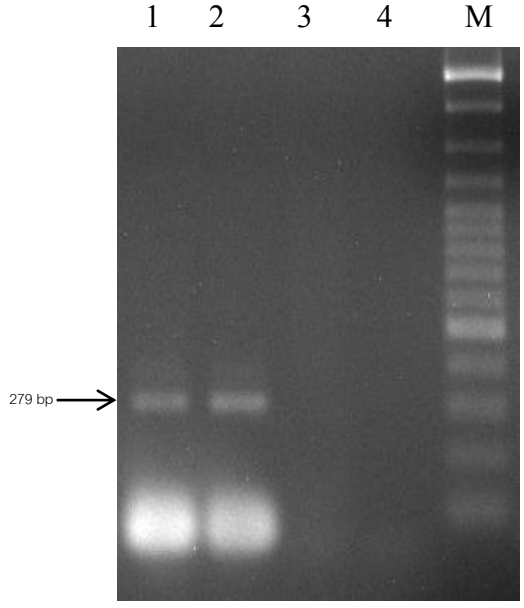

Figure 4. Verification of selected clones by PCR with primers L1F and L1R. The expected 279 bp DNA bands were revealed in lane 1 and 2 which pTWLP1 (lane1) and pTWLP 2 (lane 2) were used as DNA templates . The negative controls were empty pQE-30 Xa (lane3) and water (lane 4). M: 100bp Marker

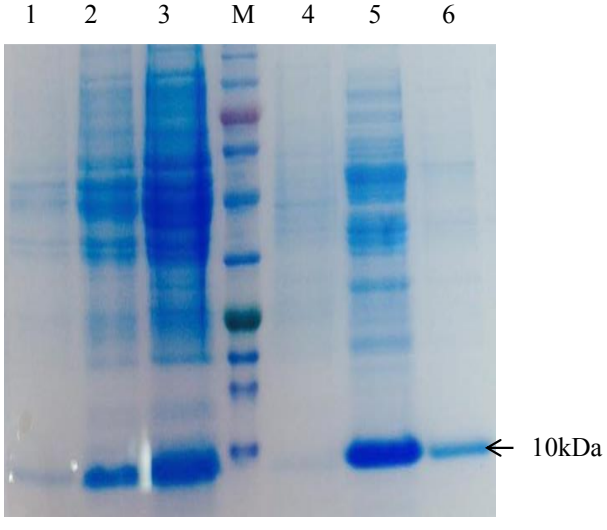

Figure 5. Cell extracts and purified protein by Protino® Ni-IDA Packed Columns Cell extracts (lane 1) and purified proteins (lane 4) from strain without DNA insert (6 h induction), Cell extract from TWLG1 strain without IPTG induction (lane 2), Cell extract (lane 3), unbinding proteins (lane 5) and purified protein from the TWLG1 strain after 6 hour IPTG induction.

5. Acknowledgements

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6. References

(1) Barber SC, Mead RJ, Shaw PJ, Oxidative stress in ALS : A mechanism of neurodegeneration and a therapeutic target. Biochimica et Biophysica Acta. 2006 Nov-Dec 1762:1051–1067.

(2) Kahl R, Kappus H, Toxicology of the synthetic antioxidants BHA and BHT in comparison with thenatural antioxidant vitamin E. ZLebensm Unters Forsch. 1993 Apr 196: 329–338.

(3) Lindberg-Madsen H, Bertelsen G, Spices as antioxidants. Trends in Food Science Technology. 1995, 6:271-278.

(4) Torres-Fuentesa C,Contreras M, RecioI, Alaiza M, Vioque J, Identification and characterization of antioxidant peptides from chick-pea protein hydrolysates. Food Chemistry 2015 Aug 180 :194–202

(5) Lin K,Yeh H, Lin S, Yang C, Tsai S, Tsai J, Chao P, Antioxidant activities of methanol extracts from selected Taiwanese herbaceous plants. Journal of Food and Nutrition Research. 2014 Jul 8:435-442

(6) Muanda F, Kone´ D, Dicko A, Soulimani R,Younos C, Phytochemical composition and antioxidant capacityof three Malian medicinal plant parts. Evidence -Based C omplementary Medicine 2011 July 2011 :1–8

(7) Hanna JN , McBride WJ , Brookes DL, Shield J, Taylor CT, Smith IL, Craig SB, Smith GA. Nuchanart R, Som kid S, Luksamee W, Chulabhorn M, Mathuros R, Jutamaad S, Evaluation of free radical scavenging an dantityrosinaseactiviti es of standardized longan fruit extract.Food and Chemical Toxicology. 2006 Aug 45:328–336

(8) Samaranayaka AGP, Li-Chan ECY, Food derived peptidic antioxidants: A review of their production, assessment, and potential applications. Journal of Functional Foods 2011, 3:229 -254.

(9) Zheng SR, Wang Y P, Yang YQ,Zhang J, Expression and identificationof a small recombinant beefy meaty peptide secreted by the methylotrophic yeast *Pichia pastoris*. African Journal of Microbiology Research . 2010 Dec 4: 2754-2762.

(10) Sambrook J,Fritsch EF, Maniatis T, Molecular cloning : a laboratory manual, 2nd ed. ColdSpring Harbor Laboratory, US; 1989.