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BIOACTIVE ACTIVITY OF A RECOMBINANT LONGAN (*Dimocarpus longan* LOUR.) SEED PEPTIDE

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ABSTRACT

Background: *Consumption of antioxidants has been evident to prevent diseases caused by free radicals damage. Antioxidants can be found in the form of peptide in various natural sources. From our previous study, to overcome obstacles of direct longan seed hydrolysate extraction, the recombinant Longan1 peptide which contains 4 repeats of ISYVVPVYIAEITPKTFRGGF linked by D was produced from Escherichia coli. The in vitro bioactive properties of this recombinant peptide were characterized.*
Methods: *The recombinant and chemically synthesized Longan1 peptides were tested for bioactive activity including, DPPH, ABTS, and nitric oxide radical scavenging assays, the ability to protect plasmid DNA from hydroxyl radicals, anti-proliferative activity to several cancer cell lines, and anti-inflammatory effects in cell culture level.*
Results: *The recombinant peptide revealed antioxidative activities, including DPPH, ABTS, and nitric oxide radical scavenging activity, which are similar to the chemically-synthesized one. However, the recombinant peptide exhibited higher in vitro ability to protect DNA from hydroxyl radicals. The IC_{50} value of the recombinant Longan1 peptide could only be calculated through the assay of anti-proliferation of stomach KATO-III cancer cell line, while IC_{50} value from the chemically synthesized peptide could not be calculated in any tested cell lines. Finally, the anti-inflammatory effect determined by the inhibition of nitric oxide production from macrophages RAW 264.7 activated by LPS revealed that the recombinant Longan1 peptide could inhibit nitric oxide production from macrophage cells, whereas the chemically-synthesized one could not.*
Conclusion: *With all these properties, the recombinant Longan1 peptide seems to have bioactivity that can possibly be a candidate for further medical application or supplementary products.*

Keywords: *Antioxidant, anti-proliferation, cancer, free radicals, inflammation, longan, peptide*

INTRODUCTION

Free radicals are by-products of aerobic metabolism which are very reactive and can damage biomolecules such as DNA and proteins. This damage could result in pathological consequences such as Alzheimer's disease, high blood pressure, and aging [1]. Mechanisms in human body to eliminate these harmful free radicals may be insufficient due to rapid accumulation caused by numbers of factors such as pollution and inappropriate cooking methods. Therefore, additional consumption of antioxidants could provide added protection against oxidative damage [2]. Antioxidants have been found in various natural sources [3,4] and have gained interest due to their safety over chemically synthesized ones [5,6]. Antioxidative peptides from plants have also gained attraction [7-9] and have

potentially been applied into various products [10]. However, direct extraction of antioxidative peptides from natural sources usually encounters particular problems, for example, the requirement of large amounts of samples and their storage area, low yields of product, and relatively difficult extraction process. To solve these problems, the recombinant Longan1 peptide, which is originally found in longan seed protein hydrolysate, was produced from the genetically engineered *E. coli* MG1655 cells [11]. This recombinant peptide contains 4 repeats of the Longan1 peptide (ISYVVPVYIAEITPKTFRGGF), and each repeat is linked by single aspartic acid residues (D). The recombinant peptide with repetitive repeats was reported previously as being used for synthesizing small recombinant peptides [12]. This characteristic is expected to overcome the limitation of genetic manipulation and to gain greater numbers of activities as they are observed from other recombinant peptides [13].

In this study, we focused on recombinant Longan1 peptide's properties including the ability to scavenge DPPH, ABTS and nitric oxide radicals. Moreover, ability to protect DNA from damage induced by hydroxyl radicals was also studied. The relationship between cancer and the presence of free radical has been reported [14,15], therefore, anti-proliferation activity of recombinant Longan1 peptide against several cancer cell lines was tested. Due to the recombinant peptide's abilities to eliminate nitric oxide radicals, the anti-inflammatory effect of the recombinant Longan1 peptide was performed. Although inflammation is a protective mechanism to remove harmful stimuli, this unspecific reaction can cause tissue injury, and chronic inflammation has a significant role in various diseases such as diabetes, cardiovascular and autoimmune diseases [16]. Many previous studies suggests the relationship between nitric oxide generation and pathophysiological consequences of inflammation [17]. Therefore, the ability of recombinant Longan1 peptide to inhibit nitric oxide generation from macrophage cells induced by LPS is determined. This study is a fundamental step for further possible application of the recombinant Longan1 peptide such as the medication of free radical-related diseases, cancer, and chronic inflammation.

METHODS

The recombinant Longan1 peptide was expressed from *E. coli* TWLG1 stain and purified by Protino® Ni-IDA packed column (MACHEREY-NAGEL, Düren, Germany) as already described in our previous report [11]. Although the columns were specific to his-tagged proteins, there were some unspecific proteins in the elution. Therefore, the recombinant Longan1 peptide is purified further by cutting the target 13 kDa bands into pieces and soaking this gel fragments in DI water at 4 °C with gentle shakes overnight. The recovered eluted protein was analysed by 18% SDS-PAGE. The Bradford assay was utilized to determine the concentration of protein. The chemically- synthesized Longan1 peptide was synthesized by First BASE Laboratories, Sdn Bhd, Malaysia.

The *in vitro* antioxidative activities of recombinant and chemically-synthesized Longan1 peptides were characterized using DPPH, ABTS and nitric oxide as free radical scavenging tests [18-20]. Ten different concentrations of each peptide were tested. Ascorbic acid was used to standardize DPPH and ABTS, while Curcumin was used to test nitric oxide tests. The protocol of *in vitro* protective ability against hydroxyl radical induced DNA damage was followed the previous report [21] by using pBR322 plasmid (New England Bilolab, Ipswich, MA, USA) as the DNA template, and hydroxyl radicals is generated from the Fenton reaction.

The anti-proliferation properties of recombinant and chemically-synthesized Longan1 peptides were tested by *in vitro* cytotoxicity of each peptide to five different human malignant cell lines including BT474 (breast), HEP-G2 (hepatoma), CHAGO (lung), SW620 (colon), and KATO-3 (gastric) [22]. The Wi-38 human lung fibroblasts were also tested as normal cells. The anti-inflammatory effects of the recombinant and chemically-synthesized Longan1 peptide (0 -400 µg/ml) were determined by the ability to inhibit nitric oxide generation from macrophage cells RAW 264.7 activated by LPS (lipopolysaccharide) [23]. MTT test was also used to determine cell viability.

All tests were performed in triplicate, and the results are reported as mean values ± standard errors of the measurement. The IC_{50} values were calculated by GraphPad Prism (Version 5.00, GraphPad Software Inc., La Jolla, CA, USA) for Windows.

RESULTS

Both recombinant and chemically-synthesized Longan1 peptides revealed similar ability to scavenge DPPH, ABTS and nitric oxide radical (Table 1).

To determine the ability of the recombinant Longan1 peptide to protect DNA damage from hydroxyl radicals, we tested plasmid pBR322 migration in agarose gel after being exposed to Fenton reaction. Most undamaged plasmid DNA is in supercoil form and migrates faster in the agarose gel than other DNA forms. However, if plasmid DNA is damaged, it could be transformed into circular and linear forms which migrate slower than the supercoil form [21]. Without any peptides, the plasmid was damaged by hydroxyl radicals and most were seen in open circular form (Figure 1A). With the peptides, DNA was protected from hydroxyl radical inducing damage. The recombinant Longan1 peptide could protect the DNA at lower concentrations suggesting the higher protective effect (Figure 1B).

Sample	IC_{50} (μg/ml)		
	DPPH assay	ABTS assay	NO assay
Recombinant Longan1	52.31 ± 1.36^{a}	42.11 ± 2.47^{c}	18.46 ± 1.45^{e}
Chemically-synthesized Longan1	56.85 ± 1.95^{a}	46.64 ± 2.04^{c}	22.94 ± 1.32^{e}
Control	101.48 ± 0.86^{b}	89.45 ± 1.22^{d}	73.06 ± 0.20^{f}

Table 1. Antioxidant activity of the recombinant and chemically-synthesized Longan1 peptides

Statistics analysis was determined by SPSS variance (ANOVA) with post hoc comparison (one-way) using Duncan's Multiple Range Test (DMRT). The same superscripts are not significantly different from each other ($p < 0.05$).

The purple crystal of formazan can be observed in cancer cell culture in MTT test due to a great number of living cells from unlimited proliferation. However, if our peptides are toxic to these cancer cells, less purple crystal will be formed. The IC_{50} values from this experiment could not be calculated due to more than 50% of cancer cells could survive in most peptide concentrations; except for the recombinant Longan1 peptide inhibiting KATO-III stomach cancer cell line with IC_{50} values measured 425.96 ± 23.61 μg/ml. However, when the percentage of cytotoxicity at 0.2 mg/ml of each peptide was observed, it was discovered that the capability of recombinant and chemically-synthesized peptides to inhibit cancer cell proliferation were significantly different (Figure 2).

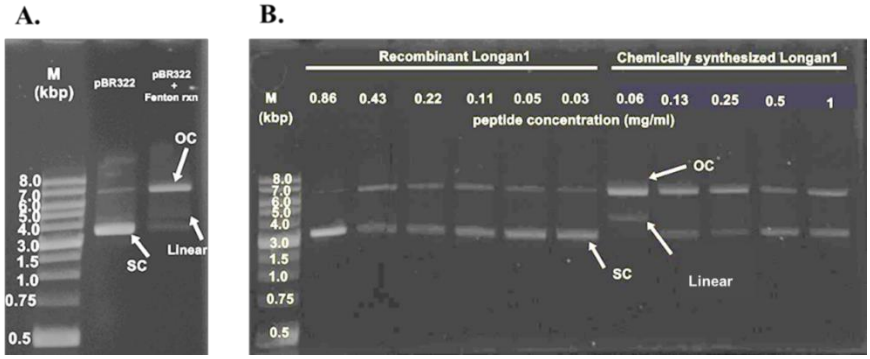

Figure 1. The *in vitro* DNA protective effect against damage induced by hydroxyl radicals. (A) Empty pBR322 plasmid without and with hydroxyl radicals (B) The hydroxyl radicals inducing plasmid DNA damage treated with recombinant or chemically-synthesized Longan1 peptides with the concentrations as indicated. (OC = open circular, Linear= double strand break DNA, and SC = supercoiled DNA).

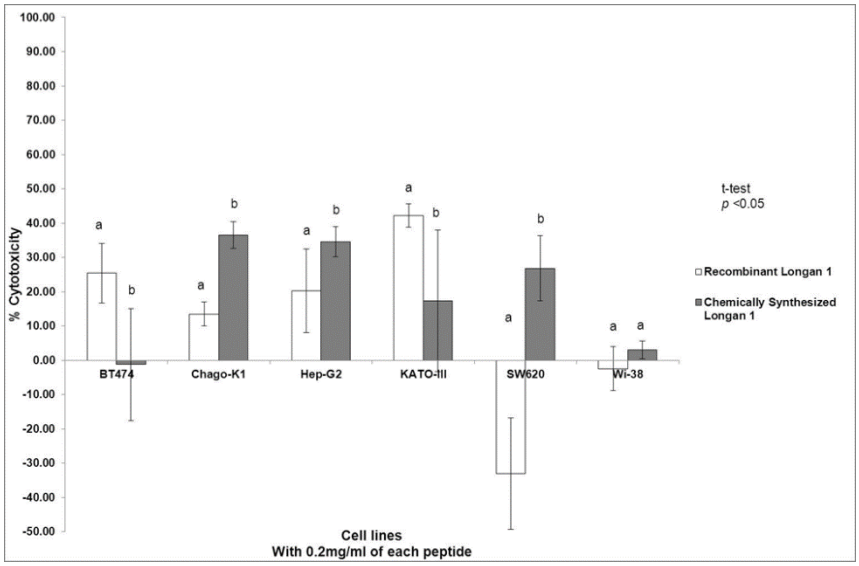

Figure 2. Percentage of cytotoxicity against breast (BT474), lung (Chago-K1), liver (Hep-G2), stomach (KATO-III) and colon (SW620) cancer cell lines, and normal Wi-38 cell lines (Fibroblast-like fetal lung) when 0.2 mg/ml of the recombinant or chemically-synthesized Longan1 peptides was added. Statistics analysis was determined by SPSS variance (Paired Samples t-test). The same superscripts are not significantly different from each other ($p < 0.05$).

From Table 1, both recombinant and chemically-synthesized Longan1 peptides yield high nitric oxide radical scavenging abilities. Therefore, the anti-inflammatory effects of these peptides were observed because of the relevancy between inflammation and nitric oxide generation [16]. The recombinant Longan1 peptide could inhibit nitric oxide generation from murine macrophages activated by LPS. (Figure 3) with the IC_{50} value calculated 4.86 µg/ml while the chemically-synthesized Longan1 peptide could not, on the other hand, induce the production of nitric oxide (data not shown). Then, cell viability after reaction was tested to confirm that the inhibition that was observed did not come from cell death. At high concentration (50-400 µg/ml), the recombinant Longan1 peptide seemed to be toxic to macrophage cells induced by LPS (Figure 4A). However, for the reaction without LPS (Figure 4B), the cell survival rate was still higher than 50% at the highest concentration of the recombinant peptide (400 µg/ml). This suggested that recombinant Longan1 peptide solely was not highly harmful to macrophages.

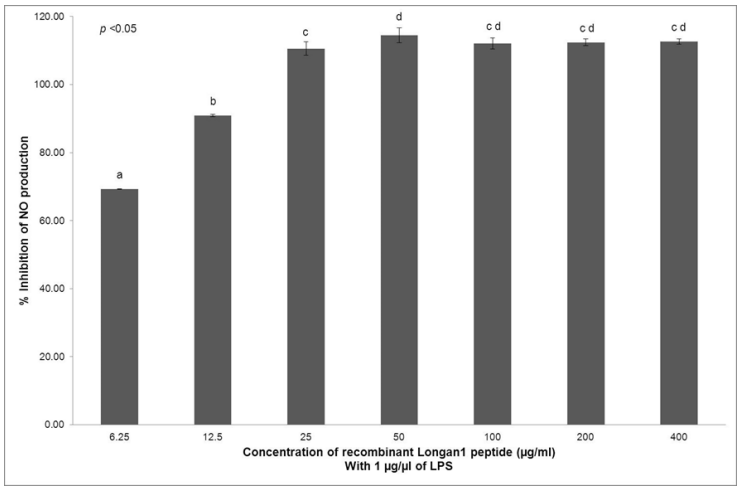

Figure 3. Percentage of inhibition of nitric oxide generation from murine macrophage RAW 264.7 induced by 1 µg/µl of LPS at different concentrations of recombinant Longan1 peptide. Statistics analysis was determined by SPSS variance (ANOVA) with post hoc comparison (one-way) using Duncan's Multiple Range Test (DMRT). The same superscripts are not significantly different from each other ($p < 0.05$).

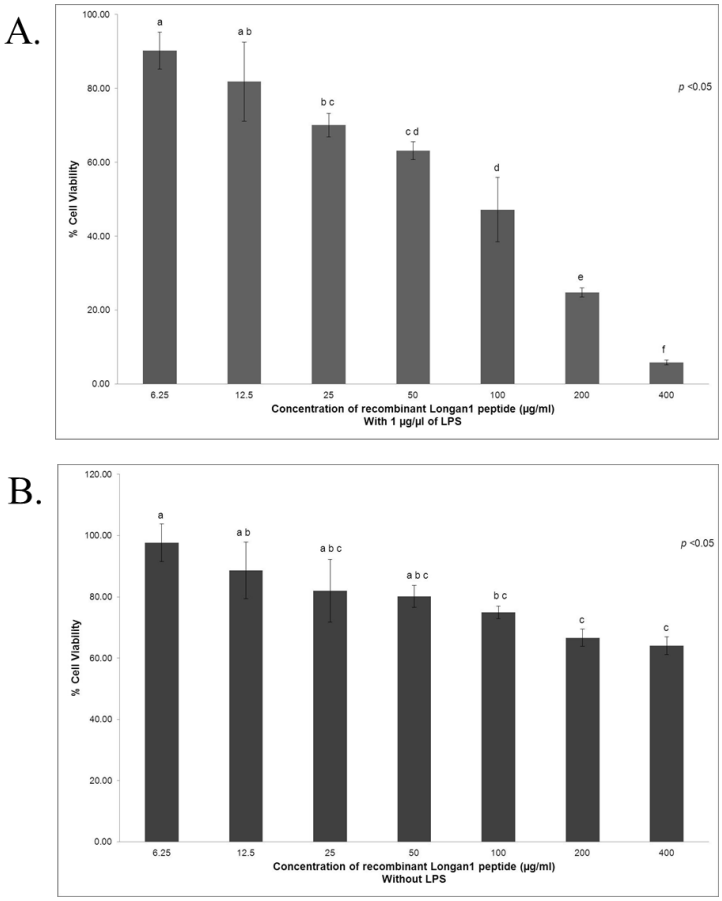

Figure 4. Percentage of cell viability at different concentrations of recombinant Longan1 peptide in the reaction of inhibition of nitric oxide from murine macrophage RAW 264.7 (A.) with 1 µg/µl of LPS (B.) without LPS.

DISCUSSION

In this study, the bioactive activity of the recombinant Longan1 peptide, originally found in longan seed hydrolysate, comparing to that of the chemically-synthesized one was conducted. We expected higher bioactive activity in the recombinant Longan1 peptide due to its greater copies. However, high antioxidative activity was found from both recombinant and chemically-synthesized Longan1 peptides without significant difference. It is possible that the four repeats of ISYVVPVYIAEITPKTFRGGF be insufficient to exhibit greater antioxidative activity while the hairy basil seed waste peptide that previously reported higher activity containing more copies [13]. However, the higher repeats of the recombinant Longan1 peptide may result in the higher protective effect against DNA damage caused by hydroxyl radical.

The damage of biomolecules and tissues by free radicals can result in cancer. Thus, the anti-proliferative property against several cancer cell lines was tested and then, suggested the possibility of specific inhibition from recombinant Longan1 peptide to stomach cancer cell proliferation. When the concentration was increased to 0.4 mg/ml, it tended to express higher capability to inhibit other cancer cell proliferation, but toxic to normal fibroblast cells, Wi-38 (data not shown). Therefore, this suggested us concerning the future study. However, the chemically-synthesized one, even it slightly inhibited proliferation of certain kinds of cancer cell lines (Figure 2), it was found that the increment of peptide concentration did not increase cytotoxicity (data not shown). In addition, this experiment also suggested the different mechanisms in cell culture level from the different peptide structure.

Furthermore, the difference in peptide structure can affect the inhibition of nitric oxide generation. There have been evidences confirming the relevance between inflammation and nitric oxide production [17]. LPS is a composition of bacterial cell wall capable of inducing macrophage to produce nitric oxide as a defence mechanism. Nitric oxide dissolved in cell medium in nitrite form can react with Griess reagent resulting in a pink compound, then concentration of nitric oxide from this colorimetric reaction can be determined by comparison of the standard graph of $NaNO_2$. The recombinant Longan1 peptide could inhibit nitric oxide production from murine macrophage activated by LPS (Figure 3), while the chemically-synthesized one could not but, instead, could induce the production of nitric oxide. It is possible that the macrophage is capable of recognizing the small synthesized Longan1 peptide as a foreign body and forms defensing mechanisms by producing nitric oxide. However, the recombinant Longan1 peptide seemed to be toxic to macrophage cell induced by LPS at high peptide concentration (Figure 4), it would be essential if further studies on anti-inflammatory activity of the recombinant Longan1 peptide be focused on the concentration of recombinant peptide at lower than 25 µg/ml. In addition, optimization of recombinant Longan1 peptide production and appropriate purification methods for larger scale are also required.

CONCLUSION

Both recombinant and chemically-synthesized Longan1 peptide exhibited high antioxidative ability. However, the recombinant Longan1 peptide seemed to have higher *in vitro* capacity of protecting DNA from damage caused by hydroxyl radicals. In cell culture level, it seemed that the recombinant Longan1 peptide specifically inhibited stomach KATO-III cancer cell line. Furthermore, it was revealed that the real inhibition of nitric oxide generation from macrophage RAW 264.7 induced by LPS, not from cell death, was found when low concentration of recombinant Longan1 peptide was applied. This study suggests that the recombinant Longan1 peptide is a promising treatment development for diseases related to free radicals, stomach cancer and inflammation.

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DECLARATION OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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