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RESEARCH ARTICLE

Investigating the Antioxidant Capacity of Lunasin Expressed in *Aspergillus oryzae*

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ABSTRACT

Objective: Lunasin is a bioactive protein that possesses anti-carcinogenic, anti-inflammatory, and antioxidant properties. Traditional isolation methods are resource-intensive, and chemical synthesis faces cost and environmental issues. This study aims to achieve cost-effective lunasin expression in *Aspergillus oryzae* with a focus on exploring its antioxidant properties *in vitro*.

Materials and Methods: The expression vector carrying four lunasin sequences fused with amylase and an 8xHis-tag was introduced into *pyrG* auxotrophic *A. oryzae.* Subsequently, the recombinant protein was purified using metal affinity chromatography. The study uses sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), western blot analyses, and size-exclusion chromatography to evaluate the composition and purity of the protein, a linoleic acid assay to demonstrate the inhibitory effect on lipid peroxidation, and the 2,2'-azinobis-[3-ethylbenzothiazoline-6-sulfonic acid] ABTS) assay to evaluate the radical scavenging activity.

Results: SDS-PAGE and western blot analyses confirmed sustained lunasin expression in *A. oryzae*, appearing in both fusion and non-fusion forms. Yields were 5.8 mg/L for non-fusion and 4 mg/L for fusion lunasin expression. Moreover, 0.1 µM non-fusion lunasin surpassed α-tocopherol and butylated hydroxyanisole (BHA; $p < 0.05$) in reducing lipid peroxidation at 4 and 72 h. Unlike the fusion lunasin, the non-fusion lunasin displayed concentration- and time-independent inhibitory effects on linoleic acid peroxidation as well as significant ABTS scavenging activity ($p < 0.05$).

Conclusion: The study has shown for the first time *A. oryzae* to efficiently express and secrete both fusion and non-fusion lunasin proteins in a soluble form, with the non-fusion lunasin exhibiting superior antioxidant effectiveness compared to the fusion lunasin. The findings underscore *A. oryzae*'s potential as a promising host for producing functional lunasin with antioxidant properties, opening avenues for broader applications in biotechnology and bioactive peptides.

Keywords: Lunasin, *Aspergillus oryzae,* Antioxidant, Biotechnology

INTRODUCTION

Lunasin is a bioactive peptide predominantly found in soybeans and various natural plant products such as quinoa and wheat and has garnered considerable research attention. It features a cell adhesion motif facilitating binding to the extracellular membrane and a negatively charged carboxyl region that interacts with nuclear histone molecules, inhibiting their acetylation.[1,2] These characteristics make lunasin a remarkable bioactive protein with anti-carcinogenic, anti-inflammatory, cholesterol-lowering, and antioxidant properties.[3-10]

Dietary components are increasingly being recognized for their potential as health-promoting substances in chronic diseases, cancer included.[9] The World Health Organization (WHO) reported over two million new cancer cases in 2024, with projections indicating a surge to 29.4 million annual cases by 2040.[11] Lunasin demonstrates anti-carcinogenic effects on multiple cancers, including lung, colon, leukemia, melanoma, and breast cancer. Its mechanisms involve inhibiting histone acetylation, blocking integrin signaling, inducing cell cycle arrest, and promoting apoptosis. In leukemia, lunasin acts as a chemoprotective by interrupting the cell cycle at the G2 level, increasing caspase enzymes, and triggering apoptosis.[12] Lunasin exhibits antimitotic properties akin to widely-used anticancer drugs such as paclitaxel by binding to chromatin and hindering the formation of kinetochore complexes, thereby inducing mitotic disruption and subsequent cell lysis. Additionally, lunasin prevents tumor cell metastasis by adhering to the extracellular matrix.[1,13] Lunasin also acts as an anti-inflammatory agent in rheumatoid arthritis by reducing synovial cell proliferation and cytokine levels through the inhibition of interleukin-6, interleukin-8, and matrix metalloproteinase-3, along with

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suppressing nuclear factor kappa B (NF-κB) activity.[14] As a cholesterol-lowering agent, lunasin directly inhibits 3-hydroxy-3-methyl-glutaryl-CoA reductase, thus lowering low-density lipoprotein and cholesterol levels.[15] As an antioxidant, lunasin additionally inhibits apoptosis induced by free radicals, regulates apoptotic Bax and Bcl-2 molecules, and stimulates dendritic and natural killer immune cells.[16]

Lunasin is emerging as a notable bioactive peptide with biological activities, making it a promising candidate for addressing various health concerns, including cancer, obesity, cardiovascular, and immune-related disorders. Moreover, several nutritional supplements containing lunasin (e.g., LunaRichX®, LunasinXP®, Carefast FSP100, LunaCell™) have been developed to harness its beneficial properties.[9] However, obtaining adequate quantities of lunasin from natural sources presents obstacles, highlighting the necessity for developing reliable production methodologies in order to meet the demand for research and biotechnological applications.

Lunasin is a 43-amino acid peptide and was initially isolated from soybeans in 1987.[17] Traditional methods for isolating lunasin from soybeans pose challenges that require substantial raw materials and intricate time-consuming processes while only yielding variable results. Similarly, while chemical synthesis methods are an alternative, these face rising costs, present complexities for large-scale production, and raise environmental concerns related to chemical usage.[2,5,9]

This study aims to achieve a cost-effective and high-yield expression of lunasin in the *Aspergillus oryzae (A. oryzae)* microorganism along with conducting *in vitro* investigations into its antioxidant properties. *A. oryzae* is a filamentous fungus that has been approved by the Food and Drug Administration (FDA) with the status of being Generally Recognized as Safe.[18,19] *A. oryzae* is known for its robust secretion mechanisms attributed to its larger genome size compared to other *Aspergillus* species, primarily due to its genes encoding secretory hydrolases. This secretion system enables large-scale production of heterologous proteins. Widely utilized in industrial-scale protein production, this expression system relies on auxotrophic nutritional markers such as pyrG, high-yield promoter genes, and efficient transformation. Furthermore, *A. oryzae* offers advantages such as cost-effective media and resistance to diverse environmental conditions.[18] With its pivotal role in fermentation technology, *A. oryzae* has emerged as an optimal choice for lunasin production.

MATERIALS AND METHODS

Strains, Materials and Reagents

Competent *Escherichia coli (E. coli)* TOP10 cells (#C404010, Thermo Fisher Scientific, MA, USA) were employed for gene subcloning and plasmid replication prior to expression of the proteins in *pyrG* auxotrophic *A. oryzae. E. coli* cells carrying plasmids were cultured in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) with 100 g/mL of ampicillin. The *pyrG* auxotrophic *A. oryzae* used in this study was derived from the *A. oryzae* RIB40 strain (#42149, American Type Culture Collection, VA, USA) in a previous study.[20] A DPY medium containing 2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4, and 0.05% $MgSO_4.7H_2O$ (pH 5.5) was used to cultivate *A. oryzae.* The *pyrG* auxotrophic *A. oryzae* was cultured in the DPY medium enhanced with 20 mM uridine and 0.2% uracil. Chemicals, reagents, and the ingredients of the cultivation media for *E. coli* and *A. oryzae* were purchased from Sigma (MO, USA) and Biofroxx (Germany).

The plasmid isolation kit (12143) was purchased from Qiagen (VLC, CA). Restriction enzymes were purchased from New England Biolabs (MA, USA), while Yatalase (T017) was purchased from Takara Bio Inc. (Japan). The nickel resin (HisPur™ Ni-NTA Resin) and the dialysis membrane (68035, 3.5k MWCO) were purchased from Thermo Fisher Scientific (MA, USA). The protein ladder (24052) was purchased from Intron Biotechnology (South Korea). The nitrocellulose membrane (Amersham™ Protran® Premium, 10600003) and Superdex 75 Increase 10/300 GL column were purchased from GE Healthcare (IL, USA). The HRP-conjugated anti-6xHis-tag® antibody (ab1269) was purchased from Abcam (Cambridge, UK). The chemiluminescence detection kit (Western BrightTM Sirius, K-12043-D10) was purchased from Advansta (CA, USA). 96-well enzyme-linked immunosorbent assay (ELISA) plates (514201) were purchased from NEST Biotechnology Co., Ltd. (China). Linoleic acid (90150) was purchased from Cayman Chemical (MI, USA). Butylated hydroxyanisole (BHA; B1253), α-tocopherol (T3251), 2,2-azinobis-[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS) diammonium salt, and 6-hydroxy-2,578-tetramethylchroman-2-carboxylic acid (Trolox; 238813) was purchased from Sigma (MO, USA).

Designing the Lunasin Expression Vectors

The lunasin protein sequence (amino acids 22-64) was derived from the 2S seed storage albumin protein (UniProtKB-P19594). The expression vector was constructed using the *pUC57* commercial vector, which includes the amylase promoter, signal sequence, terminator, and *pyrG* gene. The expression vector containing four repetitive lunasin sequences fused with amylase (UniprotKB-P0C1B3) was designed to produce lunasin (Figure 1), with the sequences separated by GGGS linker sites. The tobacco etch virus (TEV) recognition site (ENLYFQS) distinguished fungal amylase from the initial lunasin sequence, and an 8xHis-tag was added to the C-terminus for efficient detection and purification of the recombinant lunasin protein. The designed construct was optimized for *A. oryzae*, and its nucleotide sequence was synthesized by GenScript Biotech PTE. LTD. (NJ, USA).

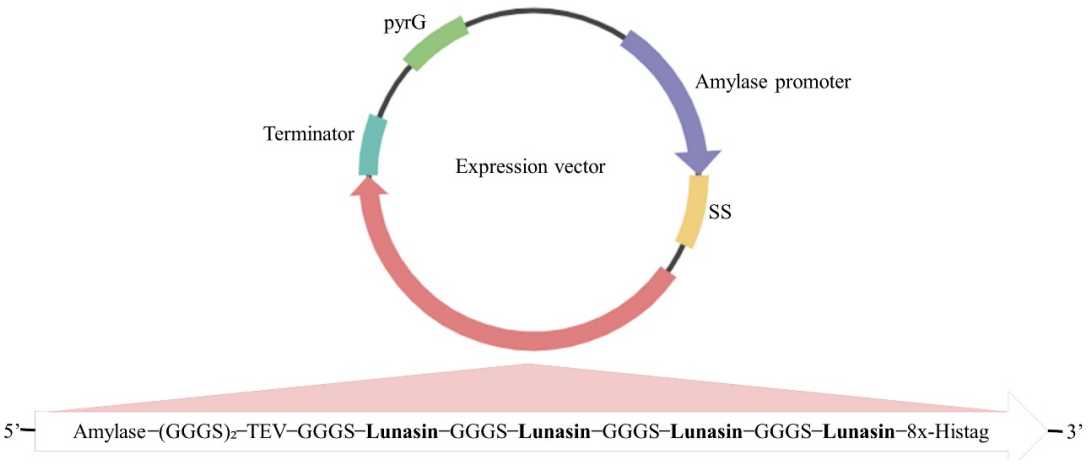

Figure 1. Illustration of expression vector containing the amylase promoter, signal sequence (SS), amylase-encoding gene, TEV recognition site, 4 tandem repetitive lunasin sequences, terminator, and *pyrG* gene. GGGS sequences served as the linker.

Transformation and Expression of Lunasin in *A. oryzae*

The expression vector was amplified in *E. coli*, purified, and digested using *EcoRI* and *HindIII* restriction enzymes. The linearized vector was then transformed into *pyrG* auxotrophic *A. oryzae* via protoplast-mediated transformation.[21] In brief, *pyrG* auxotrophic *A. oryzae* was cultivated on the DPY medium containing uracil and uridine at 30°C and 180 rpm. After an overnight incubation, the mycelia were lysed for 4 h at 30°C and 80 rpm using a lysis solution (50 mM malate buffer, 0.6 M $(NH_4)_2SO_4$, pH 5.5) with 1% Yatalase. Spheroplasts were washed with a solution containing 50 mM $CaCl_2$ and 1.2 M sorbitol. Following the combination of the linearized expression vector with the resulting spheroplasts and polyethylene glycol 4000 (PEG4000), the mixture was spread on minimal agar media and incubated at 30°C for 5-7 days. The control group received no insert DNA treatment.

To identify the recombinant colonies capable of expressing lunasin, multiple randomly selected transformant colonies were inoculated into 15 mL of the DPY medium. Following an overnight incubation at 30°C and 180 rpm, each preculture was diluted (1:10) into 75 mL of a DPY medium containing 4% dextrin. The incubation was then carried out at 30°C and 180 rpm for five days. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed on the culture samples obtained on the third and fifth days of expression.

Purification of Lunasin Proteins

The aqueous phase of the expression culture was collected by filtering using Whatman filter paper to eliminate fungal cells after five days of incubation at 30 °C and 180 rpm. For purification, the resulting sample was loaded onto a nickel-nitrilotriacetic acid (Ni-NTA) affinity column utilizing HisPur™ Ni-NTA resin. After washing the column with the washing solution (50 mM NaH_2PO_4 and 500 mM NaCl (pH 7.4)), the 8xHis-tagged recombinant proteins were eluted with the elution solution (50 mM NaH_2PO_4, 300 mM NaCl, and 200 mM imidazole (pH 7.4)). Imidazole was removed by dialyzing the eluted protein solutions against a phosphate-buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4 [pH 7.4]). SDS-PAGE (12%) was used to evaluate the purity of the protein samples obtained from the purification processes. A Bradford assay was employed to determine the concentration of pure recombinant proteins at 595 nm, with bovine serum albumin serving as the protein standard.

Size-exclusion chromatography was performed to assess the composition and purity of the dialyzed recombinant proteins. The samples were fed into a Superdex 75 Increase 10/300 GL column at room temperature via the ÄKTA pure chromatography system. The column was pre-equilibrated using PBS (pH 7.4) prepared in accordance with manufacturer's instructions. The PBS buffer (pH 7.4) was employed as a wash and elution buffer, with the flow rate maintained at 0.5 mL/min.

Western Blot

Expression and purification protein samples were separated using 12% SDS-PAGE and then transferred to a nitrocellulose membrane. 24 mM Trizma® base, 192 mM glycine, and 20% methanol were used as the transfer solution. 10 mM Tris-HCl (pH 7.4), 0.9% NaCl, and 0.2% Tween® 20 Detergent were used as the wash solution. Following overnight blocking using the wash solution supplemented with 5% nonfat milk powder, the membrane was washed three times. The membrane was then blotted with HRP-conjugated anti-6xHis-tag® antibody (1:5,000) for 2 h at room temperature. The 8xHis-tag lunasin proteins were coupled with the HRP-anti-His antibody and detected using a chemiluminescence western blotting detection kit in accordance with manufacturer's instructions.

Linoleic Acid Peroxidation Assay

A linoleic acid assay[22] was employed to demonstrate the *in vitro* inhibitory effect of purified lunasin proteins on lipid peroxidation. 50 mM linoleic acid was prepared in 100% ethanol. In a glass tube, 1 mL of 0.1 M sodium phosphate buffer (pH 7.0), 1 mL of 50 mM linoleic acid, and 0.5 mL of dH_2O were mixed. After adding the dialyzed lunasin protein sample, the mixture was kept for 72 h at 40 °C. The tubes were closed tightly and kept away from light. BHA (10 µM) and α-tocopherol (1 µM) served as control groups. In all control and test groups, samples to be tested were added to the mixture at equal total volumes. For the measurements, samples from the mixture in the glass tubes were withdrawn at 4, 24, 48, and 72 h and combined with 2.35 mL of 75% ethanol, 50 µL of 30% ammonium thiocyanate, and 50 µL of 20 mM $FeCl_2$ (freshly prepared in 3.5% HCl). The absorbance of the samples was measured at 500 nm after exactly 3 min. The control group contained only linoleic acid with no additional compound.

ABTS Radical Scavenging Activity Assay

To evaluate the radical scavenging activity of purified lunasin proteins, a modified ABTS assay was employed.[22] A 7 mM ABTS stock solution was prepared in dH_2O and mixed with 2.45 mM potassium persulfate at a total volume of 10 mL. The reaction was kept at room temperature in the dark for 16 h to obtain ABTS radicals. Following the incubation, the ABTS radical solution was diluted in a 5 mM PBS buffer (pH 7.4) until the absorbance was 0.7 at 734 nm. 10 µL of the purified lunasin protein to be tested were loaded alongside the standard samples into wells on the ELISA plate, and 190 µL of diluted ABTS radical solutions were added to the samples. The absorbance of colored mixtures was measured every min for 10 min. The percentage of decolorization based on the observed absorbance after 7 min was determined as follows:

$$\%\text{decolarization} = \frac{(Control\ absorbance - Sample\ absorbance)}{(Control\ absorbance)} \times 100 \quad (1)$$

Trolox (standard) was prepared at a concentration of 2 mM, and its repeated dilutions in the 5 mM PBS buffer (pH 7.4) were performed to generate the standard curve. The Trolox equivalent antioxidant capacity (TEAC) was calculated by dividing the gradient of the concentration-dependent absorbance plot for lunasin by the gradient of the Trolox plot.

Statistical Analysis

The results were evaluated using the program SPSS Statistics (ver. 28.0.0.0, IBM). Statistical analyses of the acquired data were conducted for the linoleic acid peroxidation assay using the Kruskal-Wallis H and Mann Whitney U tests, with $p < 0.05$ being considered statistically significant. Multiple comparisons were performed using the Kruskal-Wallis H test. The data acquired for the ABTS radical scavenging activity assay were statistically evaluated using Student's t-tests, again with $p < 0.05$ being considered statistically significant.

RESULTS

Expression of Lunasin in *A. oryzae*

The study has employed an expression vector carrying the *pyrG* gene to successfully incorporate the lunasin gene into the *pyrG* auxotrophic *A. oryzae* genome via gene replacement. Following transformation, several colonies were randomly selected for expression tests to determine the colonies expressing the recombinant protein in small flask volumes. Following a 5-day incubation period, the SDS-PAGE analysis conclusively demonstrated the sustained expression of the recombinant protein in *A. oryzae* (Figure 2). *A. oryzae* was observed in the SDS-PAGE analyses to express the lunasin protein in two distinct manners: an amylase fusion (shown with a band measuring approximately 72 kDa in Figure 2A) and without being fused with amylase (shown with a band measuring approximately 14 kDa in Figure 2B). Non-fusion lunasin was released into the culture medium following its separation from the amylase as a result of self-cleavage.

Purification of Recombinant Lunasin Proteins

A. oryzae efficiently expressed and secreted both fusion and non-fusion lunasin proteins in soluble form alongside the fungal amylase as a product of *A. oryzae* secretion into the culture medium. After filtration of the expression culture medium, recombinant lunasin proteins were purified under native conditions. The expressed 8xHis-tagged recombinant lunasin proteins were effectively separated from the host proteins using a metal affinity chromatography column. After purification, the analyzed eluted samples consistently displayed protein bands with a molecular weight of approximately 72 kDa for the fusion lunasin and approximately 14 kDa for the non-fusion lunasin in SDS-PAGE, as shown in Figures 3A and 4A. After the metal affinity chromatography, the fractions harboring lunasin proteins were concentrated, and the purity of Lunasin proteins was validated through western blot analysis using the HRP-conjugated anti-6xHis-tag® antibody for the detection. This confirmed the successful expression and purification of lunasin proteins by *A. oryzae*, as shown in Figures 3 and 4.

Size exclusion chromatography was employed to achieve a high level of purity and to evaluate structural stability of the purified lunasin proteins. Both recombinant lunasin variants eluted in a single peak on the chromatograms, as shown in Figures 3 and 4, indicating their stability. The purified fusion lunasin demonstrated a yield of 4 mg per liter of culture medium,

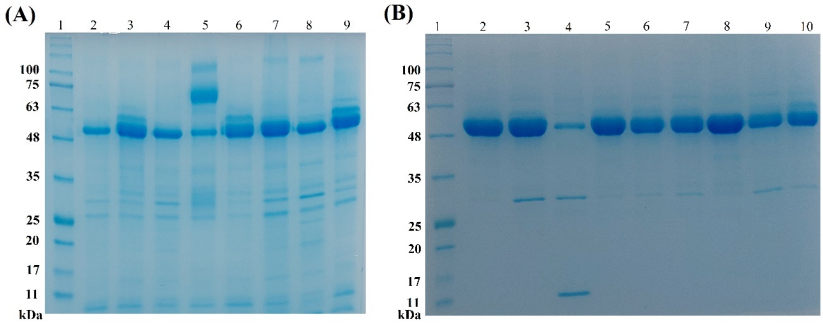

Figure 2. Protein expression tests on a small scale using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) examination of multiple transformed colonies. **(A)** SDS-PAGE analysis of fusion lunasin. Lane 1: Gangnam-Stain Protein Ladder. Lane 2–4, 6–9: the colonies not expressing recombinant protein. Lane 5: the colony expressing fusion lunasin (~72 kDa). **(B)** SDS-PAGE analysis of non-fusion lunasin. Lane 1: Gangnam-Stain Protein Ladder. Lanes 2, 3, 5–10: the colonies not expressing recombinant protein. Lane 4: the colony expressing recombinant non-fusion lunasin protein (~14 kDa).

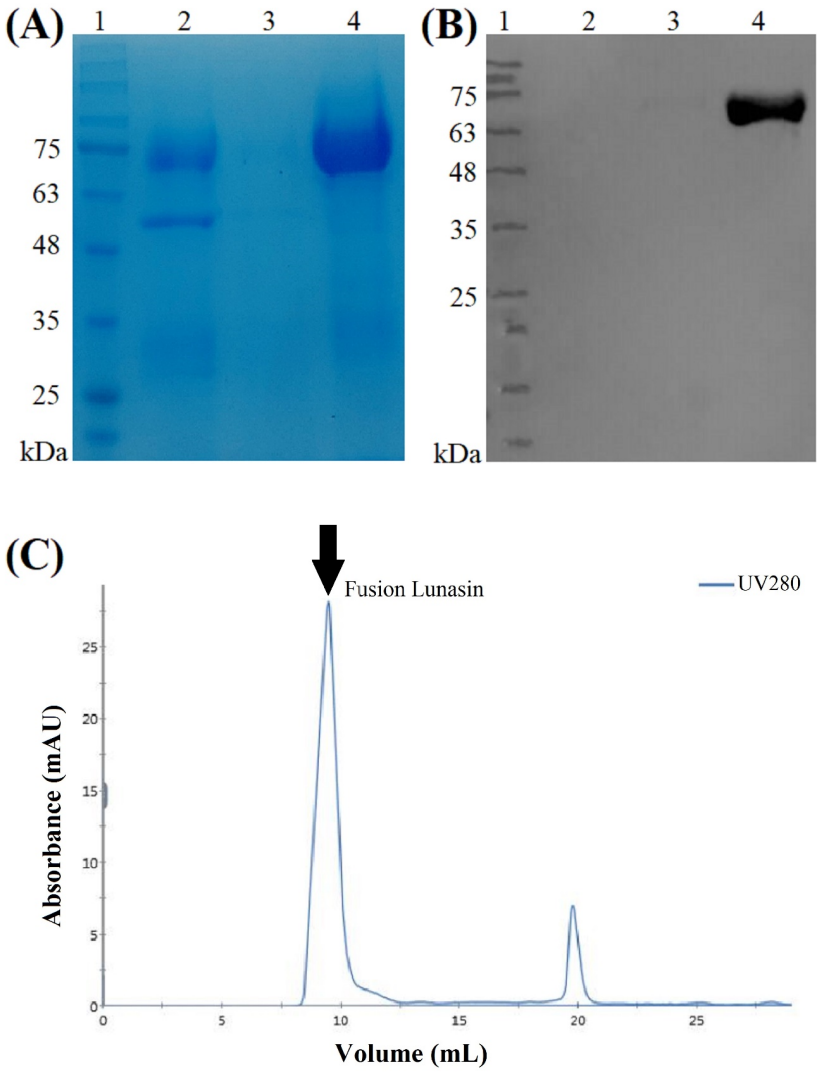

Figure 3. SDS-PAGE examination **(A)** and Western blot analysis **(B)** of purification samples of His-tagged fusion lunasin with the Ni-NTA column. Lanes 1: Gangnam-Stain Protein Ladder. Lanes 2: the culture medium of the colony expressing the fusion lunasin. Lanes 3: the wash solutions collected from the Ni-NTA column. Lanes 4: the eluted sample containing fusion lunasin. **(C)** Size exclusion chromatography purification chromatogram on a Superdex 75 Increase 10/300 GL column. The peak indicates the purified fusion lunasin.

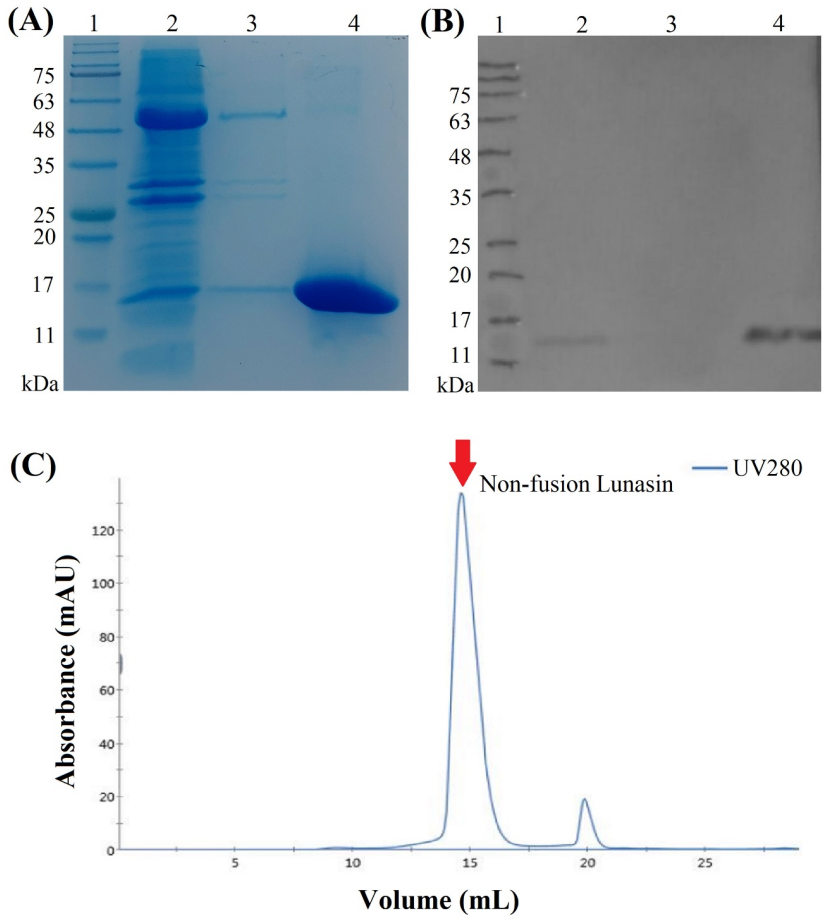

Figure 4. SDS-PAGE examination **(A)** and Western blot analysis **(B)** of purification samples of His-tagged non-fusion lunasin with the Ni-NTA column. Lanes 1: Gangnam-Stain Protein Ladder. Lanes 2: the culture medium of the colony expressing the non-fusion lunasin. Lanes 3: the wash solutions collected from the Ni-NTA column. Lanes 4: the eluted sample containing non-fusion lunasin. **(C)** Size exclusion chromatography purification chromatogram on a Superdex 75 Increase 10/300 GL column. The peak indicates the purified non-fusion lunasin.

whereas the non-fusion lunasin exhibited a yield of 5.8 mg per liter of culture medium.

Inhibitory Inhibition of Linoleic Acid Peroxidation

The linoleic acid inhibition assay measures the oxidation of ferrous ions into ferric ions, resulting in the formation of a colored complex. Therefore, the study investigated the *in vitro* peroxidation levels triggered by ferrous ions in the presence of ferrous chloride.

The antioxidant capacity of the purified fusion and non-fusion lunasin proteins was evaluated through their inhibition of lipid peroxidation in linoleic acid over a 72-hour period. Inhibition activities were assessed at different concentrations (0.1, 1, and 10 µM) and compared to standard compounds known for their significant antioxidant capacity.

Figure 5A illustrates the time course of linoleic acid oxidation in the presence and absence of non-fusion lunasin at varying concentrations. At 10 µM, non-fusion lunasin showed no noticeable inhibitory effect. However, 1 µM of non-fusion lunasin induced a slight, albeit statistically insignificant, decrease in peroxidation. Notably, 0.1 µM non-fusion lunasin exhibited a significant inhibition of lipid peroxidation at both the 4 h and 72 h time points after being introduced into the linoleic acid reaction mixture ($p < 0.05$).

Figure 5B shows the assessment of the inhibitory effect of 0.1 µM non-fusion lunasin in comparison with standard compounds at the time points of 4 h, 24 h, 48 h, and 72 h. At a concentration of 0.1 µM, the non-fusion lunasin exhibited a substantial reduction in absorbance at both the 4 h and 72 h time points, signifying a superior inhibitory effect when compared with -tocopherol and BHA ($p < 0.05$).

ABTS Radical Scavenging Activity of Lunasin

The antioxidant capacity of lunasin proteins was also evaluated utilizing the ABTS assay, which involves the chemical generation of ABTS radicals through the reaction between ABTS and

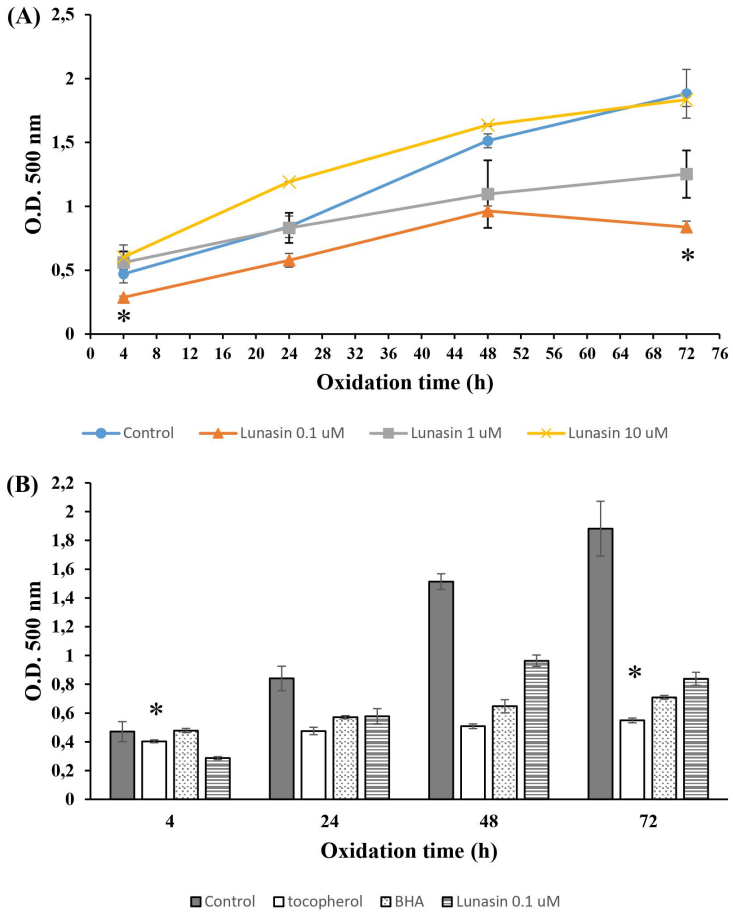

Figure 5. Inhibitory effect of the purified non-fusion lunasin on lipid peroxidation. **(A)** Lipid peroxidation inhibitory activity of non-fusion lunasin at concentrations of 0.1 µM, 1 µM, and 10 µM for the periods of 4, 24, 48, and 72 h. (**B**) Inhibitory effect of 0.1 µM non-fusion lunasin compared to standards (1 µM α-tocopherol and 10 µM BHA). Each value displays the mean and standard deviation in error bars. * Statistically different ($P < 0.05$).

potassium persulfate. This assay quantifies a substance's antioxidant capability for scavenging ABTS radicals, leading to a reduction in absorbance as a result of hydrogen atom donation. Consequently, the concentration of the antioxidant is inversely proportional to the measured absorbance.

The percentage inhibition of ABTS radicals (% decolorization) was determined as a function of concentration and time, normalized against the reactivity of Trolox as a standard. A calibration curve was constructed based on the % decolorization of Trolox against varying Trolox concentrations at 7 min. The TEAC value for non-fusion lunasin was calculated, representing its antioxidant capacity in comparison to Trolox's at 7 min. The unit of activity, known as TEAC, signifies the concentration of one unit of Trolox that matches the antioxidant capacity of one unit of the tested substance at a specific time point. The non-fusion lunasin demonstrated significant ABTS scavenging activity ($p < 0.05$), while the fusion lunasin did not exhibit a comparable effect. Additionally, the ABTS assay parameter exhibited a robust level of reliability, as indicated in Table 1.

DISCUSSION

Lunasin is currently produced through natural sources, chemical synthesis, and recombinant methods. The yields for lunasin from plant sources vary based on origin and methods used.[23] Cost-effective industrial production is essential due to high production costs. Lunasin has been the subject of 25 worldwide patents since its discovery. Interestingly, three of these patents address its recombinant expression in different microorganisms, namely *E. coli* (CN103819546A), *Pichia pastoris (P. pastoris)* (CN105647960A), and CHO-S mammalian cells (CN107574172).[9] These studies showed lunasin being expressed in fusion form in order to protect it from proteolytic degradation and increase its solubility. The present

Table 1. Formula and R^2 values of curves representing % decolorization values after 7 min incubation. The TEAC value of non-fusion lunasin was determined in comparison with Trolox. n + SD =3, each performed in triplicate.

Sample	Formula	R^2 value	TEAC value (µM)[α]
Trolox	y = 0.0734x + 0.2554	0.9993	-
Non-fusion lunasin	y = 0.333x + 9.3123	0.9943	4.450±0.093

[α] n + SD =3, each performed in triplicate.

study has for the first time successfully produced lunasin in two forms in *A. oryzae*, with final yields of 4 mg/L for fusion lunasin and 5.8 mg/L for non-fusion lunasin. The study stands apart from others by demonstrating for the first time the production of functional lunasin as a non-fusion compound utilizing a microorganism suitable for industrial-scale production.

Liu et al.[24] achieved soluble expression of His-tagged lunasin in *E. coli*, yielding biologically active lunasin at 4.73 mg/L. Kyle et al.[25] overexpressed His-tagged lunasin in *E. coli* using the Clostridium thermocellum cipB domain as a fusion partner, achieving a final yield of 210 mg/L. Setrerrahmane et al.[26] explored a cost-effective strategy in *E. coli* to obtain a purified protein yield of 75 mg/L. Tian et al.[27] used a fusion tag strategy with the TEV protease cleavage mechanism in *E. coli*, achieving an approximate yield of 86 mg/L after cleavage. Zhu et al.[28] expressed tandem-repeated lunasin units in *P. pastoris*, obtaining 0.24 mg/mL of purified proteins. The separated lunasin analogs showed dose-dependent reduction in inflammatory response in lipopolysaccharide-stimulated RAW 246.7 cells, which suggests an industrial strategy for lunasin use.[28]

Lunasin has been proven to ameliorate vascular endothelial cell damage by regulating the apoptotic pathway induced by free radicals.[16] Jeong et al.'s[29] study discovered lunasin to protect DNA from oxidation by chelating ferrous ions. Additionally, they noted lunasin to decrease the formation of intermediate products in lipid peroxidation, thereby reducing the free radical burden.[29] Another study conducted by Hernandez-Ledesma et al.[22] demonstrated lunasin to inhibit the oxidation of linoleic acid, leading to a substantial reduction in reactive oxygen species production in macrophages in a dose-dependent manner. The present study has examined the inhibitory effect of non-fusion and fusion lunasin expressed in *A. oryzae* with regard to lipid peroxidation as a measure of antioxidant capacity under stable environmental conditions. Remarkably, the results suggest the substantial inhibitory activity of non-fusion lunasin on linoleic acid peroxidation to be independent of both concentration and time. This result is believed to be attributed to the redox state of non-fusion lunasin expressed in *A. oryzae* at the tested time points.

Lunasin is known for its histone-binding ability at the C-terminal end and shares homology with conserved regions in chromatin binding proteins.[1,13,30] Similar to other chromatin binding proteins, lunasin exhibits a dynamic equilibrium between oxidized and reduced secondary structural forms. Aleksis et al.'s[30] study utilized NMR techniques on lunasin and its truncated N-terminal variant expressed in *E. coli* and revealed the oxidized form to exhibit enhanced stability compared to the reduced form. Depending on environmental conditions, lunasin can exist in a reduced form with free cysteine thiols or in an oxidized form featuring an intramolecular disulfide bond.[30]

Unlike the non-fusion lunasin in the current study, the fusion lunasin exhibited no significant inhibitory effect on linoleic acid peroxidation. The fusion of lunasin with amylase is believed to be able to potentially result in the modification of its redox properties or the weakening of its interaction with lipid molecules by affecting flexibility. This could be attributed to the structural complexity introduced by the fusion protein itself. As a result, the antioxidant efficacy of fusion lunasin has been determined to be less robust compared to its non-fused lunasin. The results of the antioxidant effects on lipid peroxidation for both the fusion lunasin and non-fusion lunasin are supported by the outcomes of the free radical scavenging activity. Alves de Souza et al.[31] investigated conformational differences between the oxidized and reduced forms of lunasin, highlighting the structural heterogeneity that imparts flexibility in binding to various partners. Consequently, the redox properties of lunasin, particularly its cysteine bond, play a crucial role in its interaction with other molecules and antioxidative activity. Furthermore, the time- and environment-dependent nature of the disulfide bond formation affects the stabilization of the redox state and consequently the antioxidative activity due to the ab-

sence of a stable tertiary structure. Additionally, the abundance of hydrophilic regions, combined with the presence of an aspartic acid end, not only enhances flexibility but also induces structural instability through electrostatic attractions.[31]

CONCLUSION

In conclusion, the study has successfully demonstrated for the first time the efficient expression and secretion of lunasin proteins in a soluble form using *A. oryzae*. Self-cleavage led to the release of non-fusion lunasin into the culture medium after separation from the amylase. The outcomes derived from the assays measuring linoleic acid peroxidation and radical scavenging activity distinctly emphasize the enhanced antioxidant efficacy of the non-fusion lunasin when compared to the fusion lunasin. This innovative approach has not only achieved a cost-effective and high-yield expression of lunasin but has also provided valuable insights into its antioxidant properties through in vitro investigations. The findings underscore the potential *A. oryzae* has as a promising host organism for producing functional lunasin with antioxidant capabilities, thus paving the way for further applications in the fields of biotechnology and bioactive peptides.

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