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# Tandem repeated expression of lunasin gene in *Pichia pastoris* and its anti-inflammatory activity *in vitro*

Yingying Zhu,[1,2,‡] Everaert Nadia,[2,‡] Yang Yao,[1,‡] Zhenxing Shi,[1,3] and Guixing Ren[1,*]

*Institute of Crop Science, Chinese Academy of Agricultural Sciences (CAAS), 80 South Xueyuan Road, Haidian, Beijing 100081, People's Republic of China,[1] Precision Livestock and Nutrition Unit, Gembloux Agro-Bio Tech, TERRA Teaching and Research Centre, University of Liège, Passage des Déportés 2, 5030 Gembloux, Belgium,[2] and Laboratory of Biomass and Green Technologies, Gembloux Agro-Bio Tech, University of Liege, Passage des Déportés 2, 5030 Gembloux, Belgium[3]*

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**Lunasin is a novel promising health-beneficial peptide derived from soybean. However, the application of lunasin is limited by its high cost. In this study, we developed a successful protocol for expression of a dimer formation protein containing 4 tandem repeated lunasin analogs (lunasin-4) in *Pichia pastoris*. The expression level at the optimal condition (initial pH 7.0, 1.0% final methanol concentration and induction for 72 h at 26 °C) was 0.24 mg/mL cell-free broth. Lunasin analog, obtained from purified lunasin-4 protein through enterokinase digestion and ultrafiltration, significantly decreased ($p < 0.05$) the release of nitric oxide (NO), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages in a dose-dependent manner. In addition, intracellular signaling array analysis demonstrated down-regulated levels of phosphorylated Akt, mechanistic target of rapamycin (mTOR) and p70 s6 kinase (p70s6k) and an up-regulated level of glycogen synthase kinase-3β (GSK-3β) after lunasin analog treatment. These results suggest that lunasin analog exerted anti-inflammatory activities in LPS-stimulated RAW264.7 cells partly via inhibiting the activation of Akt/mTOR/p70s6k signaling pathway. In conclusion, this study provides a potential strategy for recombinant production of bioactive lunasin in industry.**



[**Key words:** Lunasin; *Pichia pastoris*; Fermentation conditions; Anti-inflammatory; Akt/mTOR/p70s6k pathway]

Soybean (*Glycine max*), an ancient food crop in East Asia, is generally consumed as a major and excellence source of dietary protein (1). Soy product consumption has been suggested to reduce the risk of various disorders such as different types of cancers (2) and cardiovascular disease (3). These effects are partially attributed to bioactive peptides derived from soybean protein. Among them, increasing interests have been focused on lunasin which is a unique 43-amino acid peptide sequence encoded within the soybean Gm2s-1 gene (1). Both *in vitro* and animal studies have demonstrated that lunasin peptide has anti-cancer, anti-inflammatory, antioxidant and cholesterol-lowing effects (4,5).

Recently, because of the potential application of lunasin to improve human health, how to obtain bioactive lunasin at a low cost has drawn much attention. Different methods including extraction of lunasin from natural plants, synthesis of lunasin through chemical method and expression of recombinant lunasin through genetic engineering have been developed (6). Among these ways, genetic engineering is reported to be a practical and economical method to express target proteins. The yeast *Pichia pastoris* expression system is reported to have several advantages, i.e., high production yield, genetically stable expression strain, the potential to secrete recombinant proteins freely into the medium, and inexpensive culture conditions for production of foreign proteins (7). In our unpublished study, recombinant lunasin with a comparable anti-inflammatory activity as natural lunasin was successfully produced from recombinant *P. pastoris* GS115 strain (Zhu, Y., Nadia, E., Shi, Z., Dun, B., and Ren, G., manuscript submitted). However, the recombinant lunasin has a low expression level (12.13 mg/mL cell free broth) and was difficult to purify. Therefore, it is necessary to put forward a new expression strategy.

In this paper, a formation gene, containing 4 tandem repeat of the lunasin gene was expressed in *P. pastoris*, resulting in the production of a dimer formation protein (lunasin-4). The anti-inflammatory activity of the lunasin analog obtained from lunasin-4 by enterokinase digestion was evaluated in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages.

## MATERIALS AND METHODS

**Strains, plasmids, enzymes, antibody and chemicals** The *Escherichia coli* strain DH5α, methylotrophic yeast *P. pastoris* strain GS115 and plasmid pPIC9K were purchased from Invitrogen Co. (Carlsbad, CA, USA). T4 DNA ligase, EcoRI, NotI and SacI were purchased from Takara Bio, Inc. (Shiga, Japan). Geneticin (G418), enterokinase, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), LPS and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mouse monoclonal antibody against His-tag and goat anti-mouse IgG-HRP were purchased from Santa Cruz Biotech. Inc. (Santa Cruz, CA, USA). Roswell Park Memorial Institute (RPMI)-1640 medium, fetal bovine serum (FBS) and penicillin/streptomycin/glutamine (100×) were purchased from Thermo Fisher Scientific (CA, USA). Other chemicals were of analytical grade.

* Corresponding author. Tel.: +86 10 62115596; fax: +86 10 62156596.
*E-mail address:* renguixing@caas.cn (G. Ren).
‡ The first three authors contribute equally to this work.

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**Gene design for 4 tandem repeated lunasin analogs** A gene fragment encoding 4 tandem repeated lunasin analogs with optimized codon for *P. pastoris* was designed as follows: TCCAAATGGCAGCATCAGCAGGACAGTTGCAGAAAGCAGCT GCAAGGTGTTAATCTTACACCTTGCGAAAAGCACATCATGGAAAAGATACAAGGCAGAGG CGACGACGACGATGATGATGATGACGACCTGGTGCCGCGCGGCAGCTCAAAGTGGCAA-CATCAGCAGGATTCCTGTAGGAAACAGTTACAAGGTGTCAACCTTACTCCCTGCGAAAAA-CACATTATGGAAAAAATACAAGGAAGAGGTGACGACGACGACGATGACGACGACGACCTG GTGCCGCGCGGCAGCAGTAAGTGGCAACATCAACAAGACTCTTGCAGAAAACAGCTA-CAAGGAGTCAATTTAACCCCCTGCGAAAAACATATCATGGAAAAAATACAGGGAAGAGGT GATGATGATGACGACGATGATGACGATCTGGTGCCGCGCGGCAGCAGTAAATGGCAACA TCAGCAAGATAGTTGCAGAAAGCAATTGCAGGGCGTGAATCTAACGCCATGCGAGAAGCA CATCATGGAGAAGATTCAAGGCAGGGGAGACGACGATGATGATGACGACGACGATCTGG TGCCGCGCGGCAGCAGCAGCGGCCACCATCACCATCACCATTAA. The first 4 underlined nucleotides indicate the location of enterokinase digestion. The last underlined nucleotide indicate a tag of six histidine residues (6×His) at its C end. A pUC57 vector containing the lunasin-4 gene fragment was obtained from Beijing Genomics Institute (Beijing, China).

**Construction of recombinant strain and G418 selection** The lunasin-4 gene fragment was amplified from pUC57 with an upstream primer 5′-GGAATTCTCCAAATGGCAGCAC-3′ and a downstream primer 5′-TTGCGGCCGCTTAAT GGTGATGGTGATGGTGATC-3′ (the underlined nucleotides indicate the location of EcoRIand NotIrestriction sites). The PCR products were purified using QIAquick Gel

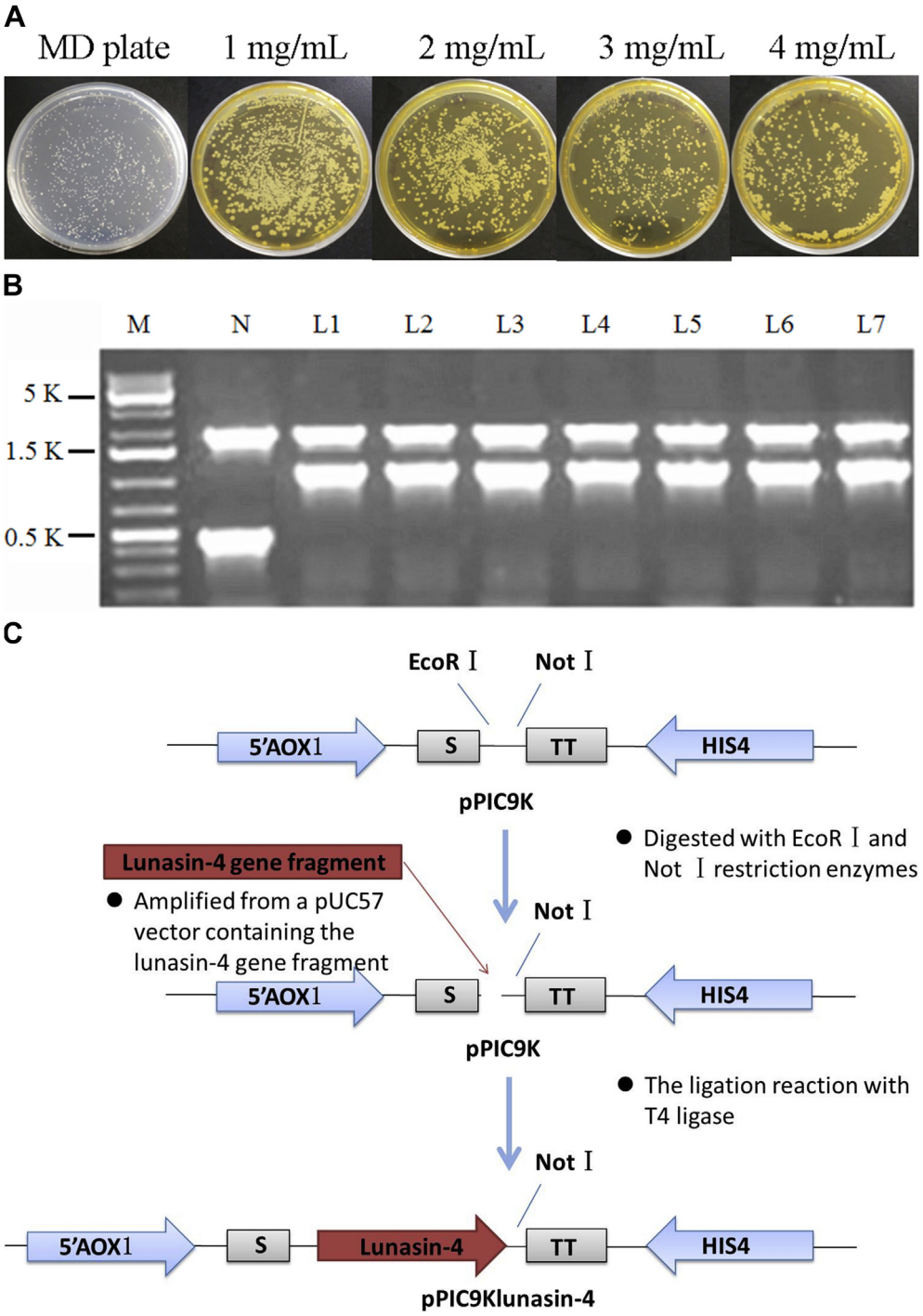


FIG. 1. G418 selection and PCR analysis of *P. pastoris* transformants. (A) Transformant from MD plates was re-plated onto YPD plates containing 1–4 mg/mL G418. (B) Verification of transformants by PCR analysis with 5′- and 3′-AOX1 primers. (C) The schematic diagram showing the lunasin-4 expression within pPIC9K vector. M, DNA marker; N, negative control (GS115 strain transformed with pPIC9K); L1–L7, seven positive transformants from 4 mg/mL G418 YPD plates.

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