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# LUNASIN DETECTION IN COLOURED WHEAT GENOTYPE

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## ABSTRACT

Lunasin is a biologically active protein, composed of 43 amino acid residues. There has been proven many health-promoting effects of lunasin peptide. The most important health benefits include: anti-hypertension, antioxidant activity, cancer prevention or therapy. It was also demonstrated anti-inflammation, hypocholesterolemic activity, anti-obesity and immunomodulation. The focus of our research is to summarize the discovery, characterization and biological activities of lunasin, which will provide a reference for the future development and utilization of lunasin, and a basis for exploring the underlying mechanisms of these health-beneficial functions. Lunasin was first isolated in 1987 at Niigata University School of Medicine in Japan, during the screening of protease inhibitors from soybean seeds. It was subsequently found in other beans, grains and herbal plants, including wheat, barley, rye, triticale. Concentration of lunasin is ranging from 0.013 to 70.5 mg protein lunasin/g of protein. Big step forward in the understanding of the lunasine operating mechanism in the fight against cancer has arisen after study on cloning of the soybean lunasin gene and subsequent transfection into mammalian cells which led to the discovery that the lunasin gene can disrupt mitosis and induce chromosome breakage, ultimately leading to cell apoptosis. The main goal of our work was to evaluate collection of wheat with unusual grain colour for presence of lunasin gene. DNA was extracted by commercial kit and lunasin gene was detected by PCR reaction. Our results showed presence of lunasin gene detected by 3 combinations of 2 sets of primer pair and indicated lunasin peptide presence in cereal grains. These findings are necessary to confirmed by proteome analysis.

**Keywords**: cancer; coloured wheat; gene detection; lunasin; PCR

## INTRODUCTION

Civilization diseases are one of the most worldwide problems of mankind. Cancer is the largest and the most widespread illness. Surgical treatment was the most effective, in past, but there are a lot of less invasive methods of cancer healing, in presence. New substances originated from plants or animals, which show chemopreventive effects, are shown by ongoing studies **(Hernández-Ledesma et al., 2009)**.

Carcinogenesis is a process which consists of combination of multiple heritable and environmental factors. Epidemiological studies shown, that cancer appearance and mortality significantly varied across the world. Cancer remains the main cause of mortality in western world. These parts of world where is diet centered on plant foods tending to have a lower rate of cancer, but prevalence of cancer is rising rapidly in one generation after their emigration to the western countries. This indicates that genetic factors are not the primary factors that cause cancer and modification of nutritional habits and lifestyle, as well as, consumption of foods containing bioactive components can offer a significant protection against carcinogenesis **(Hernándes-Ledesma et al. 2009)**.

Lunasin is one of these substances, which produce not only a lot of positive effects on human organism, but also anticancer activity. Lunasin is biological active peptid, which consist of 43 amino acids. There has been confirmed, that lunasin protected cells against chemical transformation induced by chemical carcinogens and virus and ras oncogenes. Mechanism of lunasin action is based on balance influence between acetylation and deacetylation of histones. This mechanism may cause cell death, because in this case lunasin acts as a tumour suppressor which is tightly bounded on deacetylated histones in cell nucleus and have ability to influence cancer cells apoptotically and cytotoxic **(Chang et al., 2014)**.

In vitro studies, animal treatment and epidemiologic researches showed that soy consumption is in connection with decreasing of some cancer types **(Hernández-Ledesma et al., 2009)**.

**Hsieh et al., (2010)** reported, that the first animal model confirmed preventive properties against chemical carcinogen-induced skin cancer in mice. Lunasin also play role as an active cancer preventive agent in treatment of human breast cancer. Lunasin in combination with aspirin arrest the cell cycle in the S- and G-phases, respectively, acting synergistically to induce apoptosis which was achieved by modulating the expression of genes encoding G1 and S-phase regulatory proteins.

Lunasin is a soybean derived peptide with a MW of 5.5 kDa and contains 9 aspartic acid residues on C domains, cell adhesion motifs consit of 3 amino acids residues (arginin – glycine – aspartic acid) and predicted helix with structural homology to a conserved region of chromatin binding

proteins. Lunasin is not fully digested in gastrointestinal system, but is absorbed intact, reaching target tissues. The biological activity of lunasin depends on cultivar, environmental factors and processing conditions, which in turn affected its concentration **(Wang et al., 2008)**.

Lunasin has been discovered in most of soybean varieties and its concentration ranged from 4.4 to 70.5 mg lunasin in one gram of protein **(Hernándes-Ledesma et al., 2009)**.

**Jeong et al., (2007)** detected lunasin in wheat using mass spectrometry. They determined 14 amino acids fragment (KQLQGVNLTPCEKH) with m/z 656, 8640 Da. This fragment corresponded to 12-25 amino acids fragment of soy albumin subunits, which was identified as a lunasin peptide.

The main goal of our research was to detected lunasin gene in collection of coloured wheat grain.

## MATERIAL AND METHODOLOGY

There was analysed collection of 8 genotypes of wheat grain (Table 1) with unusual grain colour.

DNA isolation was performed from wheat grain by commercial kit GeneJET TM (Fermentas). Isolation protocol was modified for isolation DNA not only from fresh tissue, but also from grain. Modification contains supplementation of Lysis buffer A with 2% (w/v) polyvinylpyrrolidone. Wheat grains of each cultivar (up to 100 mg) were grinded in liquid nitrogen using a mortar and pestle. Then were grounded plant tissue powder transferred into the tubes with the prealiquoted Lysis buffer A (with PVP). The rest of extraction steps were held according standard procedure with usage of Lysis buffer B, RNase A. Purificatrion of extracted DNA were realized in spin column tubes with utilization of Plant g-DNA binding solution which anchored extracted DNA on spin column membrane. Wash buffer I and Wash buffer II purified anchored DNA. Elution of DNA from membrane to solution was realized by Elution buffer. The quantity and quality of puried DNA were measured by nanophotometer and visualisated by 1.5% horizontal agarose electorphoresis.

GoTaq® Green Master Mix from Promega Company was used for Polymerase chain reaction (PCR) DNA amplification. GoTaq® Green Master Mix is a premixed ready-to-use solution containing bacterially derived Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. PCR amplification protocol of DNA fragments were realized using primer pairs (Table 2) and their combination (Table 3) according to **Dinelli et al., (2014)**. 25 µL of reaction mix was prepared on ice and contained 12.5 µL of GoTaq® Green Master Mix, 2.5 µL of 10 µM upstream primer, 2.5 µL of 10 µM downstream primer, 2.5 µL of nuclease-free water and 5 µL of 0.025 $ng.\mu L^{-1}$ DNA template. Standard PCR procedure consists of 2 min initial denaturation step at 95 °C for activation of reaction mix. Product amplification contained 3 subsequent steps. 20 s denaturation of DNA at 95 °C, 30 s anelation of primers at 54 °C and 30 s polymerization of DNA fragmets at 72 °C. These 3 steps repeat 50 times. The last step of PCR procedure is 5 min final extension at 72 °C. Quality of amplyfied product were confirmed by 1.5% horizontal agarose electrophoresis.

DNA fragments were separated in 8% vertical polyacrylamide gel electrophoresis for 360 min at 500V in Hoeffer SE 660 electrophoretic system and visualised by silver staining.

**Table 1** List of analysed genotypes.

| | Name | Species | Originated | Colour |
|---|---|---|---|---|
| 1 | Barevná 25 – modrá | *Triticum aestivum* L. | CZ | **Blue** |
| 2 | Trojzrnka | *Triticum aestivum* L. | CZ | **Red** |
| 3 | Mnohokvietková | *Triticum aestivum* L. | CHINA | **Red** |
| 4 | Tr.Etiopicum Jakubz | *Triticum aestivum* L. | Ethiopia | **Purple** |
| 5 | Tr. Etiopicum araratica-červená | *Triticum aestivum* L. | Ethiopia | **Purple** |
| 6 | UC 66094 | *Triticum aestivum* L. | USA | **Blue** |
| 7 | Koniny-červená | *Triticum aestivum* L. | CZ | **Red** |
| 8 | Modré zrno | *Triticum aestivum* L. | CZ | Blue |

**Table 2** Nukleotid sequences of used primers.

| Name | Primer type | Sequence |
|---|---|---|
| **Lun1** | forward | **AAATGGCANCACCAGNA** |
| | revers | **CGTCATCATCATNATCGTNA** |
| **Lun2** | forward | **GATANCTGCCNCAAGCA** |
| | revers | **TCTTNTCCATNATGTGCTTCTC** |

**Table 3** Primer pairs combinations.

| Number | Primer pair combination |
|---|---|
| 1 | Lun1 F x Lun1 R |
| 2 | Lun1 F x Lun2 R |
| 3 | Lun2 F x Lun1 R |
| 4 | Lun2 F x Lun2 R |

Electrophoretic separation and visualisation of DNA fragments were performed according **Bassam et al., (1991)**.

Visualised DNA fragments were captured by UVP digital imagine system and detected by Doc-IT LS software from UVP Jena, Germany.

## RESULTS AND DISCUSSION

Lunasin is a novel, cancer-preventive peptide whose efficacy against chemical carcinogens and oncogenes has been demonstrated in mammalian cells and in a skin cancer mouse model. Isolated and characterized in soy, lunsin peptide is also documented in barley, wheat, tritikale, rye and oat **(Lumen et al., 2005)**.

The characterisation of cDNAs encoding lunasin shows that it corresponds to the small subunit of the soybean 2S albumin. The biological activity of lunasin has led to searches for related peptides in other plant species, including reported isolation from *Solanum*, amaranthus seeds, Brazil nut, sunflower and cereal seeds.

The identity of the peptides in wheat was confirmed by partial sequences which match exactly to the soybean sequence over stretches of 14 amino acids **(Mitchell et al., 2013)**.

We therefore decided to search for lunasin gene sequence aroud colour wheat genotype by utilization of 2 sets of primers developed by **Dinelli et al., (2014)**.

Our results show that utilization of primer pair Lun1 forward and Lun1 revers showed presence of 121 bp length DNA fragment. This primers pair combination seems to be suitable for lunasin gene detection, because we were able to detect lunasin gene in all of genotypes (Figure 1 and Figure 2).

Primer pair combination Lun1 forward and Lun2 revers not provide any fragment with desirable length 85 bp Using of this primer pair combination is contradictory and has to be tested in future.

Primer pair combination Lun2 forward and Lun1 revers was used for detection of DNA fragment with length 103 bp. There were obtained presence of desired DNA fragment in each genotype of evaluated wheat collection (Figure 3 and 4).

Application of primer pair combination Lun2 forward and

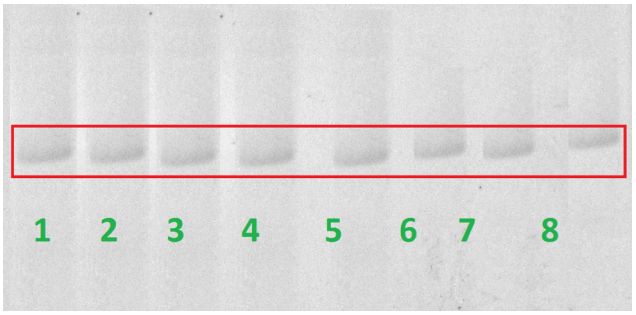


**Figure 3** Lunasin gene detection with primer pair combination F2R1 – 103 bp.

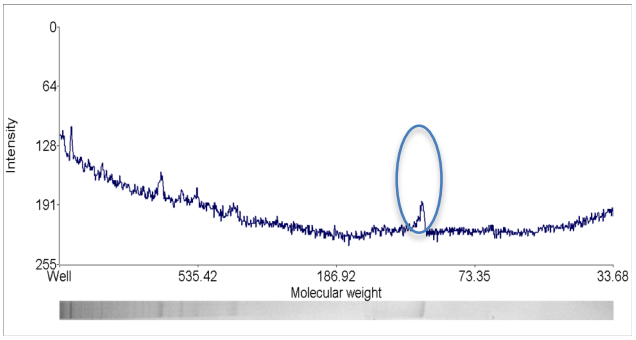


**Figure 4** Barevná 25 – F2R1.

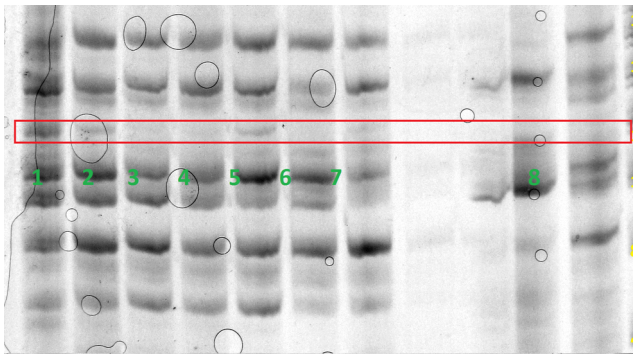


**Figure 1** Lunasin gene detection with primer pair combination F1R1 – 121 bp.

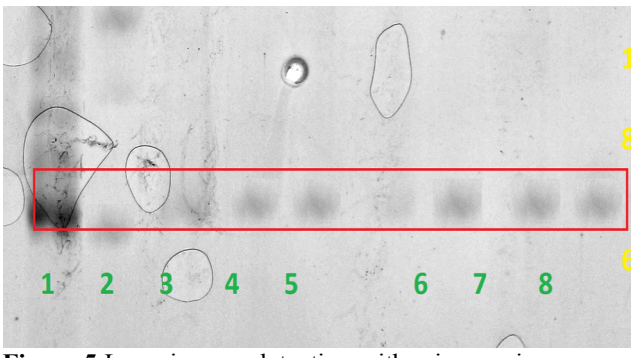


**Figure 5** Lunasin gene detection with primer pair combination F2R2 – 67 bp.

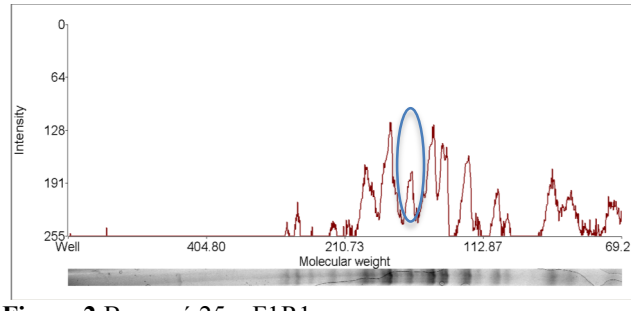


**Figure 2** Barevná 25 – F1R1.

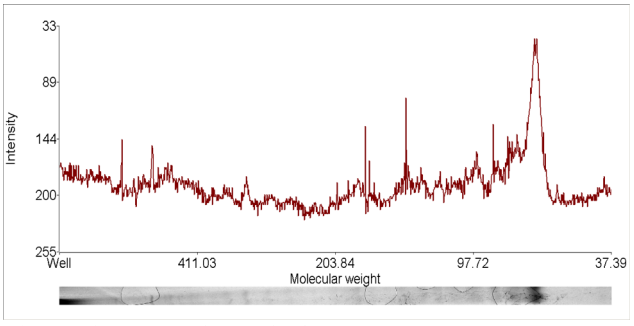


**Figure 6** Barevná 25 – F2R2.

Lun2 revers provide detection of lunasin gene fragment with length 67 bp. This primer pair combination was suitable for detection of lunasin gene fragment in all genotype (Figure 5 and Figure 6).

We focused on confirmation of **Dinelli et al., (2014)** results in our research. **Dinelli et al., (2014)** monitored, that although there was positive presence of lunasin peptide in wheat proteome analysis, no gene coding this peptide was detected. However, **Jeong et al., (2009)** and **Maldonado-Cervantes et al., (2010)** observed lunasin peptide in proteome analysis and postulated also presence of gene coding this peptide.

Our results showed presence of gene coding lunasin peptide, but are in controversy with **Dinelli et al., (2014)** results. These lunasin gene detection findings require analysis of wheat proteome for detection of lunasin peptide to confirm expression of monitored DNA fragment.

**Nakurte et al., (2012)** and **Mitchell et al., (2013)** observed presence of lunasin peptide in cereals and their results indicated importance of mass spectrometry in cereal proteome analysis to confirm lunasin gene detection.

Presence of lunasin in triticale (X *Triticosecale* Wittmack) confirmed by **Nakurte et al., (2012)** indicated, that triticale is the most lunasin-rich cereal. The greatest lunasin content was 6.46 $mg.g^{-1}$ in the grain of triticale genotype 0002-26. In comparison, the highest lunasin content in rye variety Dankovske Diament was 1.5 $mg.g^{-1}$ of grain and the highest lunasin content in the winter wheat variety Fredis was 0.23 $mg.g^{-1}$ of grain. They conclude that triticale can play significant role as functional food, with great potential for the use of triticale products in human and animal diets.

Results of **Nakurte et al., (2012)**, which detected lunasin peptide in wheat and triticale corresponds to our observation about presence of lunasin gene in colour wheat genotype.

**Jeong et al., (2009)** focused their research on identification of lunasin peptide in rye (*Secale cereale* L.) cultivars. Lunasin was present in 15 out of 21 cultivars of analyzed rye cultivars. Lunasin present in rye crude protein preparation was stable to pepsin and pancreatin in *in vitro* digestion. They concluded that lunasin in rye is bioavailable and that consumption of rye may play an important role of cancer prevention in rye consuming population. Wheat is close relative to rye and therefore is possibility of wheat utilization in cancer prevention. Our results indicate presence of lunasin gene in wheat. Although lunasin peptide presence in wheat is contradictory, observation obtained by **Nakurte et al., (2012)** and **Jeong et al., (2007)** are in agreement with our observations.

Lunasin peptide detection in oat genotypes (*Avena sativa* L.) was performed by **Nakurte et al., (2013)**. Lunasin was detected using LC-MS/MS analysis. They observed genotype-related fluctuations in the lunasin content. The highest lunasin level was 0.197 $mg.g^{-1}$ of grain. There was also no correlation between lunasin and protein content, but genotype-dependent variations of the lunasin content was demonstrated during different years. Therefore, is very important to study influence of farming system, crop management and climate conditions on lunasin content in cereals as well as clarifying if consumption of lunasin-containing foods plays as important role in cancer and cardiovascular disease prevention.

**Jeong et al., (2010)** also elucidated role of cereals in cancer prevention. They reported the prevalence; bioavailability and bioactivity of lunasin from barley.

The liver and kidney of rats were fed with lunasin-enriched barley and inhibits the activities of histone acetyl transferases.

These findins and our results indicated that lunasin is prevalent in cereals and is bioavailable and bioactive. Consuption of cereals could play an important role of cancer prevention in cereal-consuming populations.

Recombinant production of the therapeutic peptide lunasin was widely studied by **Kyle at al., (2012)**. They used a pET28 vector to express cellulose binding domain (CBD)-lunasin fusion with a hexahistidine tag and Tobacco Etch Virus protease site, to allow protease-mediated release of native lunasin. The use of CBD as a fusion partner gave high protein yields by autoinduction, with lunasin release by TEV protease cleavage. This approach could provide a potentially valuable route for production of this therapeutic peptide.

## CONCLUSION

Utilization of 2 sets of primer pair in 4 combinations showed suitability of F1R1, F2R1 and F2R2 primer pair combination for detection of lunasin gene. Identification of lunasin gene may be used for chromosome site identification and genetic manipulation with promotor to enhance gene activity and production of desirable level of peptide.

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