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Proceeding Paper

Proteomic and Genetic Approach for Lunasin Peptide and Gene Presence Detection in Various Plants †

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Abstract: Lunasin is a biologically active peptide, with polypeptide chain consisting of 43 amino acids, originally discovered as a 2S albumin protein from the seed of the soybean coded by *GM2S-1* gene. The most significant health benefits include: antioxidant activity, anti-hypertension activity and chemoprevention activity. Lunasin peptide was initially detected in many plant species, but there is controversy about its exact origins, in present. Therefore, we focused on detection of Lunasin gene by polymerase chain reaction and Lunasin peptide by one and two dimensional electrophoresis, in various plants. These results confirmed, that Lunasin peptide as well as Lunasin gene were observed only in soy bean seeds. There were also confirmed presence of Lunasin like peptide in various plants (Spelt wheat, bean and oat), but gene detection was not successful.

Keywords: lunasin; peptide; gene; electrophoresis; polymerase chain reaction

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1. Introduction

Lunasin is one of the most important bioactive peptides [1]. It occurs predominantly in soybeans [2], other types of legumes and cereals [3]. Lunasin demonstrate positive effects on human health and is considered as a chemo preventive agent against cancer because it shows significant bioactivity in inhibiting the formation and growth of cancer cells [4]. The most important biological properties of Lunasin is its bioavailability, mainly capability to remain unchanged and bioactive after oral ingestion. Lunasin after absorption can be distributed to metabolism. In vitro bioavailability studies have shown that isolated Lunasin, is easily digested after only 2 min of incubation with simulated gastric (SGF) and simulated intestinal fluids (SIF) [5]. Lunasin is a heat-stable peptide because remains bioactivity even after boiling for 10 min [6]. The primary structure of Lunasin consist of 43 amino acids. Ser^{1}-Lys-Trp-Gln-His-Gln-Gln-Asp-Ser-Cys^{10}-Arg-Lys-Gln-Leu-Gln-Gly-Val-Asn-Leu-Thr^{20}-Pro-Cys-Glu-Lys-His-Ile-Met-Glu-Lys-Ile^{30}-Gln-Gly-Arg-Gly-Asp-Asp-Asp-Asp-Asp-Asp^{40}-Asp-Asp-Asp [7]. N-terminal amino acid contain neutral side chain unlike C-terminal amino acid which contain polar side chain [8]. The primary sequence of the Lunasin peptide can be cleaved into four fragments. Fragment SKWQHQQDSCRKQLQGVNLTPC has unknown function. Second fragment is structural preserved area of α-helix (KHIMEKIQ), which targets Lunasin to H3-H4 histones. Amino acid motif RGD internalizes Lunasin into cell, is third fragment which also preventing acetylation of deacetylated histones H3 and H4. The last, fourth fragment consist of eight

aspartic acid residues (DDDDDDDD) which directly binds to core histones [9]. Lunasin peptide secondary structure consist of α-helix (29%), β-helix (28%), β-sheet (23%) and disordered regions (20%) [10]. Researches [11] discovered, that disulfide bond creation capability of Lunasin, Cys^{10} and Cys^{22}, enable two forms of Lunasin, oxidized and reduced. The high cost of producing synthetic Lunasin determined requirement of identification and isolation Lunasin from natural plant sources.

The aim of the work was to investigate the proteome of selected varieties of plants with emphasis on the detection of Lunasin peptide and Lunasin peptide gene detection.

2. Materials and Methods

2.1. Biological Material

There were used three genotypes of soy bean (*Glycine max* L.), Zora, Ischigo Wase, Lokus and kidney bean (*Phaseolus vulgaris* L.) Zlatý roh, Dandy, Wiscont, in our research. We also analysed one genotype of buckwheat (*Fagopyrum esculentum* Moench) Mihovo, rye (*Secale cereale* L.) Radošínsky rekord, oat (*Avena sativa* L.) Veles, spelt wheat (*Triticum spelta* L.) Roquin, emmer wheat (*Triticum dicoccum* L.), peas (*Pisum sativum* L.) Jantar and lentil (*Lens esculentum* L.) Svit. Analyzed genotypes were obtained from the Gene Bank of Seed Species of the Slovak Republic NPPC VÚRV in Piešťany.

2.2. Sample Extraction

We focused on analysis of peptide Lunasin presence and detection of genetic information for this peptide in our research. Subsequently, we realized protein extraction and extraction of DNA

2.2.1. Protein Extraction

There were performed system for one dimensional polyacrylamide gel electrophoresis in presence of sodium dodecyl sulphate (1D SDS PAGE) and system for two dimensional electrophoretical separation in our investigation. Accordingly, we preformed two different isolation of protein complex from our samples.

Protein complex for 1D SDS PAGE system was isolated according to standard reference method of International Seed Testing Assotiation (ISTA) [12]. Grain of analyzed samples were mechanically homogenized. Protein complex were extracted by solution consist of 4.25 mL storage solution (12.5 mL 1M Tris-HCl pH 6.8; 20 mL glycerol; 4 g SDS; 20 mg Pyronin G in 100 mL of deionized water), 0.75 mL 2-mercaptoethanol in 10 mL of deionized water. Volume of extraction solution were calculated by ratio 1 mg of sample = 8 μL of extraction solution. Extraction procedure was carried out 30 min, 100 100 °C, 1200 rpm in thermal shaker followed by centrifugation (15,000× *g*, 10 min).

Protein complex for 2D electrophoretical separation were extracted by phenol extraction according to [13]. 0.1 g of sample was mechanically homogenized and then was protein complex extracted by adding of 200 μL extracion solution (30.88 g sucrose, 0.37 g EDTA, 6.65 mL 1M Tris-HCl pH 8.8, 0.4 mL 2-mercaptoethanol in 100 mL of deionize water) and 200 μL Tris-Phenol solution (4,706 g phenol in 100 mL 1M Tris-HCl pH 8.8). Extraction procedure takes 30 min shake on ice and centrifugation 5000× *g* 10 min. 200 μL of supernatant was precipitated by 1 mL of precipitating solution (1.95 g ammonium acetate in 250 mL methanol) overnight in the fridge. Sample was then centrifugated 15,000× *g* 20 min and pelet was resuspended in precipitating solution and chilled −20 °C 20 min. Pelet was obtained by centrifugation 5000× *g* 10 min, washed by 80% acetone and 70% methanol and centrifuged 15,000× *g* 20 min, dried and stored. Pelet was resuspended in 200 μL IEF rehydration buffer before use.

2.2.2. DNA Extraction

DNA was extracted by Thermo Scientific™ GeneJET Plant Genomic DNA Purification Kit (Thermo Fisher Scientific) on the basis of the standard procedure supplied by the manufacturer.

2.3. Analyses of Lunasin Peptide Presence and Detection of Genetic Information for Lunasin

Analyse of Lunasin peptide presence was realized by 1D SDS PAGE and 2D electrophoresis. Identification of Lunasin peptide gene was performed by polymerase chain reaction (PCR) and PCR product was detected by agarose electrophoresis.

2.3.1. Electrophoretical Separation

1D electrophoresis separation was performed in vertical discontinual SDS-PAGE system (HeoferTM SE 600 Chroma—Fisher Scientific) according to standard ISTA method [12]. Separation of protein complex was performed in 8% Tris-HCl polyacrylamide stacking gel and 4% Tris-HCl polyacrylamide running gel. Electrophoretical buffer system was Tris-glycine buffer. Protein complex electrophoretical separation takes 20 h (20 mA) and protein identification was performed by comparison with a Broad Range Protein Molecular Weight Marker (Promega). Staining system was solution of Commasie Brilliant Blue R250 in methanol. Gels were destained in water.

2D electrophoresis separation was performed using a combination of isoelectric focusing and 1D SDS PAGE. Isoelectric focusing was performed using Serva IPG BlueStrips 11 cm pH Gradient 3–10 (Serva Electrophoresis) according to manual of manufacturer on PROTEAN i12 IEF System (Bio-Rad). 1D SDS PAGE was realized by modification of standard ISTA procedure [12] application only stacking gel. Electroperetical separation takes 15 h with constant current 15 mA (HeoferTM SE 600 Chroma—Fisher Scientific).

2.3.2. Polymerase Chain Reaction (PCR)

Primers and reaction conditions of PCR were performed according to Dinelli et al. (2014) method. PCR was realized in GoTaq Green Master Mix (Promega) buffer solution. Dinelli et al. (2014) method was modified by optimalization of reaction conditions PCR according to manual of GoTaq Green Master Mix (Promega) manufacturer. PCR products were separated and vizualized in 2% agarose electrophoresis. Buffer system of agarose electrophoresis was TBE (54 g Tris, 24.5 g boric acid, 20 mL 0.5M EDTA pH 8.0 in 1000 mL of deionized water). Electrophoresis takes 90 min with constant voltage 100 V. Molecular weight were established by comparison with molecular standards.

2.3.3. Detection System

Analysis and evaluation of the results was performed with a ChemiDoc MP imagine system (Bio-Rad) using ImageLab software (Bio-Rad).

3. Results and Discussion

Lunasin was first isolated from soybean seeds in 1987 [8] using the 60% ethanol extraction method, followed by ion exchange chromatography and reverse phase chromatography. Subsequently was Lunasin also isolated from other plants, barley [15], wheat [3], rye [16], oats [17], amaranth [18], triticale [3] and quinoa [19]. Alaswad and Krishnan used an immunological approach to detect the presence or absence of Lunasin peptide in cereals or other plant species. Their research included the use of polyclonal antibodies that responded to the 20-amino acid N-terminal sequence (SKWQHQQDSCRKQLQGVNLT) and 15-amino acid C-terminal sequence (CEKHIMEKIQGRGDD) of peptide Lunasin. The results of this analysis showed no occurrence of peptide Lunasin in the seeds of different plants except for soy bean. Detected peptides were not identical to soy Lunasin, because in some species the same peptide failed to react with the N- and C-terminal peptide antibody [9]. Other researchers have focused on the detection of the lunazine peptide gene in

various plant species. Dinelli et al. [14] used PCR method for identification of lunasin peptide gene, although Mitchell et al. [20] in their work realized in silico analysis failed to identify the gene for Lunasin peptide based on a search of available databases.

Based on the above, we decided to monitor the presence of Lunasin peptide and the gene for Lunasin peptide in various plant species, in our work. We also evaluated the applicability of basic laboratory methods to identify the presence of the gene and its product, in our research. Electrophoresis and polymerase chain reaction are methods which are cheap and quite fast, but their sensitivity may not be accurate to evaluate presence of individual peptide or gene for that peptide.

Our results show, that 1D SDS PAGE (Figure 1) is very fast and very cheap method for separation of whole proteome of individual plant species, but there is no possibility to exact identify individual peptide. Advantage of this method is the fast screening of presence possibility of peptide of interest according molecular weight.

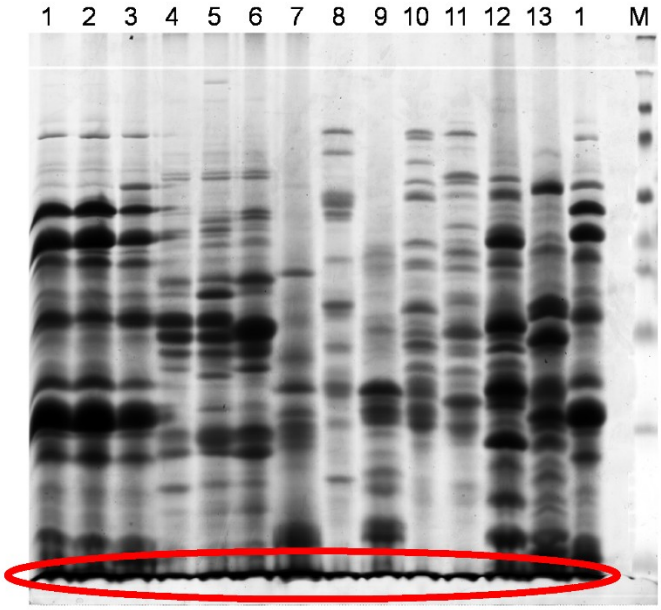

Figure 1. 1D SDS-PAGE. Explanations: 1—Soy bean Zora, 2—Soy bean Iscigo Wase, 3—Soy bean Lokus, 4—Kidney bean Zlatý roh, 5—Kidney bean Dandy, 6—Kidney bean Wiscont, 7—Buckwheat Mihovo, 8—Rye Radošínsky rekord, 9—Oat Veles, 10—Spelt Wheat, 11—Emmer wheat, 12—Bean Jantar, 13—Lentil, M—Multicolor Broad Range Protein Ladder (Promega).

Second used electrophoretic method was two dimensional electrophoresis, which combine utilization of peptide pI and molecular size. First dimension of this separation method is isoelectric focusing which separates peptides in pH gradient according their pI, which enable differentiate peptides which similar molecular weight. Second dimension of this method separate peptides according their molecular weight. Utilization of this method is very wide, because is widely used not only for peptide separation, but also for mass spectrometry peptide identification. We were able to identify several peptide spots with molecular weight very closed to Lunasin peptide (5.5 kDa), but differ in their pI only in soy bean genotypes (Figure 2) (Table 1).

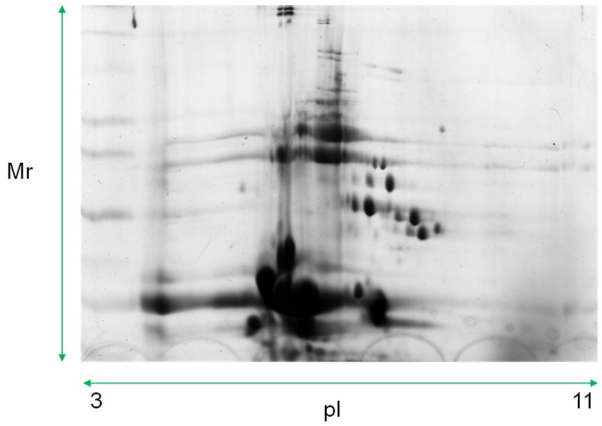

Figure 2. 2D electrophoresis of soy bean genotype Zora. Explanations: pI—isolectric poit, Mr—molecular weight.

Table 1. Lunasin like peptide.

Spot Number	Molecular Weight	Isoelectric Point
3	5.93	3.47
3001	5.99	5.82
4001	5.74	6.11
6002	5.97	7.04
7002	5.87	7.41
7003	5.96	7.67
8001	5.72	9.51
9001	5.89	10.1

Lunasin peptide gene detection was performed by PCR according to Dinelli method [14]. There were used 2 sets of primers which were combined in 4 detection systems. Each detection system should be able to provide product with exact molecular weight. Our results confirmed investigations obtained by 2D electrophoresis, that only soy bean genotypes were able to provide product with exact molecular weight. Whereupon we decided to optimalizated this method according to protocol of used buffer manufacturer (Promega), however obtained results showed the same findings.

4. Conclusions

1D SDS PAGE separation is suitable for fast separation of the protein complex and identification of proteins with molecular weight close to lunazine peptide and because of this may be included mainly for basic screening of proteome. 2D electrophoresis is method which allow exact detection of individual peptide and our results shows, that soy beans genotypes proteom consit of peptides which are very similar to Lunasin peptide. Exact determination of Lunasin peptide presence in plant genotypes requires use of synthetic Lunasin peptide. Polymerase chain reaction is basic method for detection of gene presence. Application of PCR for detection of Lunasin peptide gene confirmed our results obtained by 2D electrophoresis.

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