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Isolation of low-molecular albumins of 2S fraction from soybean (*Glycine max* (L.) Merrill)

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Numerous studies have shown that consumption of soybean products decrease the risk of cancers in humans. Experiments at the molecular level have demonstrated that in most cases proteins and peptides are responsible for the anticancer properties of soybeen. Special attention should be paid to lunasin — a peptide described for the first time 16 years ago. Due to its structure it causes i.a., inhibition of cancer cell proliferation. A novel procedure for the isolation and purification of low-molecular-mass 2S soybean albumin protein is described in the present paper. A fraction of four peptides one of them corresponding to molecular mass and isoelectric point characteristic for lunasin. The obtained peptides decreased on the rate of HeLa cell proliferation.

Key words: soybean proteins, cancer, lunasin

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INTRODUCTION

Soybean proteins include metabolic and storage proteins of fractions 2S, 7S, 11S and 15S. The 2S albumin fraction which is the subject of this study is relatively less allergenic (Lin *et al*., 2006) and contains mainly storage and enzymatic proteins, including protease inhibitors belonging to the Bowman Birk and Kunitz families.

Protein purification procedures are based on several methods using aqueous, ethanol (L'Hocine *et al*., 2006) or Tris buffer extraction (Seber *et al*, 2012). The choice of the method can determine the selectivity of the desired protein fractions and the possibility of scaling scale-up the procedure of isolation.

In the 1990s, Galvez and Lumen discovered an active peptide being a smaller subunit of 2S Gm2-S1 albumin — lunasin (Galvez & Lumen, 1999, Hernández-Ledesma & Lumen, 2008, Hsieh *et al*., 20011). It appeared that transfection and constitutive expression of the lunasin gene in mammalian cells interrupted mitosis and induced fragmentation of chromosomes and apoptosis. Intense studies aimed at recognition of the mechanism of lunasin activity on cells and chromatin are being currently conducted. It is known that the antimitotic properties of lunasin involve binding of aspartic acid residues with regions of hypoacetylated chromatin. As a result, the centromere kinetochore complex is not formed properly and microtubules do not bind to the kinetochore. This leads to an inhibition of the mitotic cycle and as a consequence to apoptosis (Hernández-Ledesma & Lumen, 2008). Studies conducted recently have demonstrated that lunasin decreases proliferation of cancer cells in the presence of a carcinogen. Stearns *et al*. (2007) proposed an epigenetic mechanism explaining the influence of lunasin on cancer cell division. The study conducted *in vitro* proved that lunasin binds to deacetylated histones H3 and H4 thereby inhibiting their acetylation. This mechanism is based on the "E1A-Rb-HDAC" model which explains interactions in the cell nucleus responsible for protection against an excessive histone acetylation characteristic for the action of carcinogenic factors. Rb protein, a role of cancer suppressor, interacts with E2F promoter and forms a complex with histone deacetylase HDAC. Therefore, chromatin is protected against the activity of histone acetyltransferase HAT and thus stays transcriptionally inactive. The activity of some carcinogens is based on the reaction of the oncoprotein E1A with the Rb-HDAC complex causing its dissociation and enabling HAT attachment to chromatin and its acetylation. Lunasin binds deacytelated histones and interacts with histone deacetylase thus transcription preventing (Gonzalez, 2004). The content of this peptide in soybean seeds depends on numerous factors, like genotype, variety or cultivation conditions (Liu, 1997). Due to its high thermal stability, lunasin does not lose its biological activity even during 10 minute cooking (Gonzalez, 2004). Therefore, lunasin is observed in numerous processed food products derived from soybean albumins (Czarnecka & Koziołkiewicz, 2007). The aim of the study was to elaborate an efficient and selective method of isolation and purification of low-molecular proteins from soybean seeds which could also be used for isolation on a larger scale. The second phase of the study was to determine the effect of lunasin on proliferation of HeLa cells (ATCC CCL-2).

MATERIALS AND METHODS

Materials. Proteins of albumin fraction 2S were obtained from seeds of conventional soybean of Augusta (Polish variety, no GM, through the courtesy of Prof. Nawracała, Department of Genetics and Plant Breeding University of Life Sciences Poznań). This cultivar was obtained by crossing the Swedish Fiskeby variety V with line 11 from the *Glycine max* (L.), entered in the Register of Varieties in 2002. The protein content in seeds is approximately 35% of DM, the fat content is approximately 21%. Augusta is resistant to fungal and bacterial diseases — does not require chemical protection.

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Abbreviations: Gm2-S1 albumin, *cotyledon-specific 2S albumin; HDAC,* histone deacetylase; HAT histone acetyltransferase; MWCO, Molecular Weight Cut Off

The effect of a 5 kDa a peptide on cell proliferation was examined on *in vitro* cultures of an established human HeLa cancer cell line (ATCC CCL-2).

Reagents. Cyclohexane, tannin, DEAE Sepharose FF, proliferation of the cells was examined using an MTT kit manufactured by Roche company.

Isolation of protein extract containing 2S fraction. In most studies, protein preparations are obtained by shaking of defatted soybean meal in deionized water (Galvez *et al.*, 2001). In order to optimize the isolation of low-molecular-mass proteins of 2S fraction, three methods of protein extraction from meal previously defatted in cyclohexane were analyzed: shaking in deionized water, in 50 mM Tris/HCl buffer pH 7.2, and in ethanol. For that purpose, 20 g of defatted soybean meal was shaken for one hour in 7× solvent volume at of 37°C. Then, the suspension (3 500 rpm) was centrifuged and sediment was rejected. The yield of the three methods of protein isolation was compared by spectrophotometric examination of protein concentration using tannin method. This method is insensitive to the amino acids composition of proteins, and therefore is suitable for protein mixtures of unknown or variable composition. BSA was used as a standard. Protein solution was precipitated with acetone. Raw extracts were separated on 12% SDS/PAGE gel.

Protein chromatography. Chromatographic separation was performed on DEAE Sepharose Fast Flow ion-exchange column (in an FPLC LKB system) in pH 8 buffers: A (50 mM Tris/HCl) and B (50 mM Tris/HCl 1M NaCl), at 4°C. Ethanolic extract was centrifuged in a Sorval centrifuge at 3500 rpm for 15 minutes at 4°C. Based on the trials performed, the separation was conducted using a linear gradient of B in A. Supernatant (4 ml) was applied on the column, protein fractions were eluted with encreasing concentration of buffer B (20, 40, 60, 80% of buffer B in A), at a flow rate of 2 ml min^{-1}. Fractions of 3 ml were collected and protein concentration was determined using the tannin method. Analysis of the fraction was performed *via* electrophoresis in 21% SDS/PAGE gel in non-reducing and reducing conditions (denaturation in the presence of β-mercaptoethanol). Fractions enriched in peptides of a molecular mass close to 5-kDa, corresponding to lunasin, were combined. The next stage of the fraction analysis was by two-directional gel electrophoresis. Before the separation, samples were additionally subjected to ultrafiltration in a 30-kDa MWCO centrifuge tubes (Millipore). The influence of the peptide obtained on cancer cell proliferation MTT assay after 24 hours of culturing. Growth and morphology of HeLa cells was assessed using Zeiss Axiovert 200 reverted microscope. Confluent cultures were aimed at inoculation on 96-well culture plate. The cells were counted after trypsinization and 20 µl of the obtained cell suspension was mixed with 20 µl of 0.4% trypan blue solution in physiological saline, and then 8 µl of the suspension was counted in Bürker chamber for live cells. Next, the culture was diluted with DMEM to a final concentration of 1×10^6 cells ml^{-1} in 2.5 ml. Similar procedure was applied using a culture medium with an addition of purified low-molecular-mass proteins isolated from soybean seeds. Concentrated filtrate obtained after membrane filtration (30 µl) was added to 2 ml of DMEM medium. The plates were placed in an incubator (37°C, 5% CO_2). After one hour, 100 µl of MTT solution was added to the wells of the control plate, and after 4 hours 200 µl of MTT solvent was added in order to dissolve the formazan formed. Absorbance was read after 24 hours in an ELISA reader at 650 nm. The experimental plate was cultured for 24 hours in the presence of the peptide fraction and the above procedure was then conducted.

Table 1. Effect of extraction method on protein concentration in the extract

Sample	Absorbance 650 nm	Crude extract dilution	Concentration µg ml^{-1} after dilution
40% ethanol extract	0.152	100	4637
Water extract	0.187	100	5150
Tris/HCl extract	0.287	100	7500

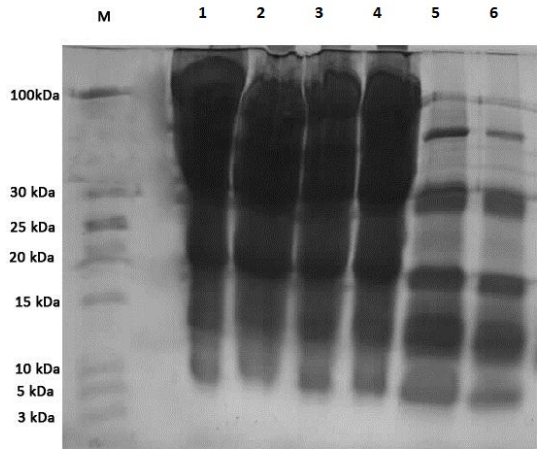


Figure 1. 15% SDS/PAGE electrophoresis of proteins extracted by three different methods.
M, Biorad protein weight marker; 1–2, aqueous extract; 3–4, Tris buffer extract; 5–6, 40% ethanol extract.

RESULTS

In this study, soybean meal protein (fraction 2S) was extracted with three different methods. The yield was determined by measuring protein concentration using the tannin method (Table 1).

After analysis of an electropherogram (Fig. 1), extraction with 40% ethyl alcohol was selected for the isolation of the 2S fraction (due to the purity of the protein extract). Chromatographic separation on DEAE-Sepharose FF ion-exchange column and then electrophoresis in 21% PAGE/SDS gel in non-reducing and reducing conditions allowed us to separate fraction eluted at of 300 mM NaCl containing the highest concentration of a 5-kDa peptide (Fig. 2). The peptide was visible in the case of sample reduction.

An analysis by two-dimensional electrophoresis (Fig. 3–4) confirmed that the isoelectric point of the peptide of ca. 5-kDa is around pH = 5.5, consistent with literature data (Galvez, 2010).

The results of MTT tests showed little effect of the purified fraction of low-molecular-mass soybean proteins on the proliferation rate of HeLa cells (Fig. 5). However, some tendency for a decrease of the proliferation rate following addition of the low-molecular-mass soybean proteins to the was observed.

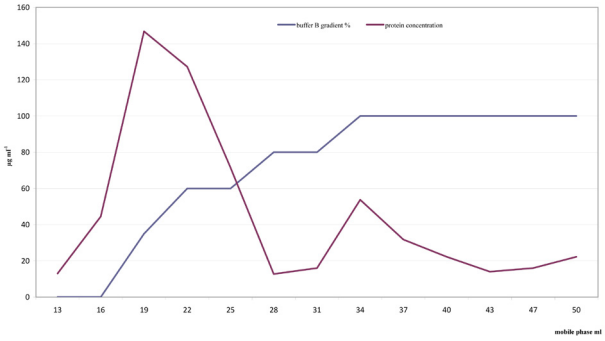
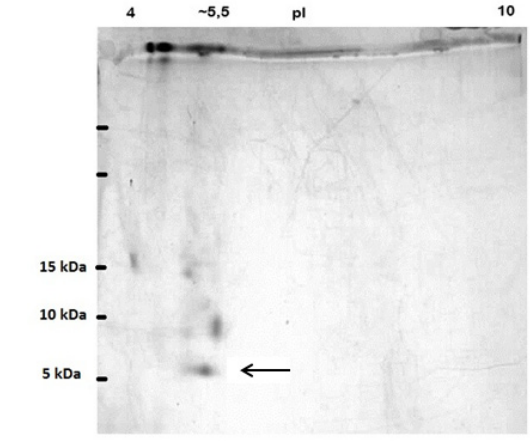


Figure 2. DEAE-Sepharose Fast Flow ion exchange chromatography of ethanol extracted proteins.
Protein concentration determined using tannin method and percentages of buffer B gradient. Range of buffer B gradient 0–1000 mM NaCl. The highest concentration of protein was 146.75 µg ml^{-1}.



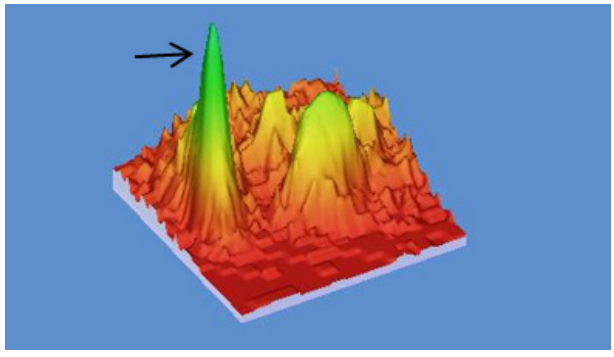


Figure 4. Three-dimensional visualization of proteins separated two-dimensional electrophoresis.
Arrow points to peptide corresponding to lunasin with its molecular mass and isoelectric point.

DISCUSSION

Soybean is a widely studied plant, providing healthy and cheap food. During the last two decades researchers have paid increasing attention to another characteristic of food proteins, which concerns biological activity of peptides. *In vitro* and *in vivo* studies demonstrated that these bioactive peptides play some beneficial functions in organisms, such as: prevention of circulatory system diseases, diabetes, protection against cancer, lowering of cholesterol level, prevention of obesity, and immunomodulating functions (Wang & Mcjia, 2005). Having above reasons in mind it is important to possess precise

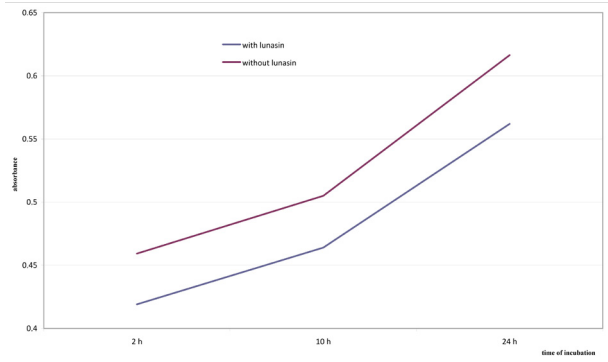


Figure 5. Effect of low-molecular-mass soybean protein on HeLa cell proliferation.

and detailed methods of purification and characteristics of these peptides. The most often examined soybean peptide is lunasin described for the first time 16 years ago. Purification and analysis of a low-molecular-mass peptide of 5-kDa from the 2S fraction of soybean meal is presented in this study. The first important stage of protein purification is its isolation. Selection of a protein extraction method of high yield is often connected with a decrease in the extraction selectivity, and thus balast proteins need to be removed at further stages of purification. Most publications present an application of distilled water for extraction, or water buffers containing Tris or phosphate (Amnuaycheewa & Gonzalez de Mejia, 2010). Three methods of protein isolation were used in this study for comparative purposes. Isolation with distilled water, Tris/HCl buffer and 40% ethanol were compared. Distilled water and Tris/HCl buffer gave higher yield with respect to the overall protein concentration compared to 40% ethanol. Further quantitative - qualitative analysis with SDS PAGE technique demonstrated that the content of low-molecular-mass proteins in the alcohol extract is close to that in the water extract and the Tris/HCL buffer one. Howeever some differences in proteins of higher molecular weighs, especially those above 30-kDa, were noted. Higher amount of these proteins in water solution and Tris/HCl buffer is undesirable during further purification stages. Fractions containing proteins of a weight close to that of lunasin weight were selected based on chromatogram analysis. In order to separate proteins based on their molecular masses we applied ultrafiltration, thus rejecting larger proteins. Two-dimensional electrophoresis was used to assess purity of isolated fraction. Analysis of the gel using the MELANIE software for three-dimensional visualization enabled us to determine the presence of a peptide whose molecular mass and isoelectric point correspond to lunasin. Concentrated fraction after ultrafiltration, of a concentration of 100 µg ml^{-1}, was selected for MTT proliferation test. The results obtained demonstrate a tendency for inhibition of proliferation of cancer cells cultured in the presence of the low-molecular-mass proteins isolated from soybean.

CONCLUSIONS

Diverse isolation methods have been used for protein purification by various authors. Application of 40% alcohol offers good selectivity of isolation of low-molecular-mass proteins and peptides corresponding of 5-kDa, and separation of soybean proteins on DEAE Sepharose FF column enabled us to obtain initially purified fractions

containing low-molecular-mass proteins and peptides of a weight of 5-kDa. Disruption of disulfide bridges (in the presence of β-mercaptoethanol) in the proteins led to the appearance of a smaller subunit in filtrate through a 30 kDa MWCO membrane. Some tendency for inhibition of the rate of HeLa cancer cell proliferation was observed after addition of the fraction containing peptide of 5-kDa corresponding to lunasin.

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