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**Structural property of soybean lunasin and development of a
method to quantify lunasin in plasma using an optimized
immunoassay protocol**

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HOJA DE APROBACION DE TESIS

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Reconocimientos

Este trabajo de investigación fue realizado en el laboratorio Edward R. Madigan de la Universidad de Illinois en Urbana-Champaign y fue publicado en la revista estadounidense *Food Chemistry* en Mayo de 2013. Es por esta razón por la que se presenta este documento en su idioma original, el inglés.

Se reconocen a los coautores del trabajo, Vermont P. Dia, Sarah Frankland-Searby, Guadalupe García y Elvira Gonzalez de Mejía, y se les agradece inmensamente por trabajar junto conmigo para la realización de este trabajo.

RESUMEN

Lunasin es un péptido quimiopreventivo de 43-amino ácidos que existe naturalmente con propiedades anticancerígenas y antiinflamatorias demostradas. Los objetivos de este estudio fueron determinar el efecto de la temperatura en la estructura secundaria de lunasin, desarrollar un método para aislar lunasin del plasma humano utilizando una columna microgiratoria de intercambio iónico y cuantificar la cantidad de lunasin utilizando ensayo por inmunoabsorción ligado a enzimas optimizado (ELISA por sus siglas en inglés). Lunasin fue purificada utilizando una combinación de cromatografía por intercambio iónico, ultrafiltración y cromatografía por filtración en gel. Dicroísmo circular demostró que el incremento de temperatura de 25 a 100 °C resultó en cambios en la estructura secundaria de lunasin y en su capacidad para interactuar con anticuerpos policlonales de conejo. ELISA demostró que anticuerpos policlonales de conejo para lunasin tienen una titulación de 250 y una actividad específica de 0.05 ml/μg. Se detectó una respuesta lineal entre 16 a 48 ng de lunasin por mL ($y = 0.03x - 0.38$, $R^2 = 0.96$). El uso de la columna microgiratoria de dietilaminoetil (DEAE) para aislar el plasma con lunasin agregada demostró que la mayor cantidad de lunasin (37.8-46.5%) se unió a la columna eluida con el amortiguador Tris-HCl, pH 7.5 con un rendimiento de hasta 76.6%. En conclusión, lunasin puede ser aislada del plasma humano con una técnica simple utilizando una columna microgiratoria de DEAE y puede ser cuantificada utilizando un procedimiento validado de inmunoensayo optimizado. Este método puede ser usado directamente para cuantificar lunasin en plasma en diferentes estudios humanos y animales apuntando a determinar su biodisponibilidad.

ABSTRACT

Lunasin is a 43-amino acid naturally occurring chemopreventive peptide with demonstrated anti-cancer and anti-inflammatory properties. The objectives of this study were to determine the effect of temperature on the secondary structure of lunasin, to develop a method of isolating lunasin from human plasma using an ion-exchange microspin column and to quantify the amount of lunasin using an optimized enzyme-linked immunosorbent assay. Lunasin was purified using a combination of ion-exchange chromatography, ultrafiltration and gel filtration chromatography. Circular dichroism showed that increased in temperature from 25 to 100 °C resulted in changes on the secondary structure of lunasin and its capability to interact with rabbit polyclonal antibody. Enzyme linked immunosorbent assay showed that lunasin rabbit polyclonal antibody has a titer of 250 and a specific activity of 0.05 mL/ μ g. A linear response was detected between 16 to 48 ng lunasin per mL ($y = 0.03x + 0.38$, $R^2 = 0.96$). The use of diethylaminoethyl microspin column to isolate spiked lunasin in human plasma showed that most lunasin (37.8–46.5%) bound to the column eluted with Tris–HCl buffer, pH 7.5 with a yield up to 76.6%. In conclusion, lunasin can be isolated from human plasma by a simple DEAE microspin column technique and can be quantified using a validated and optimized immunoassay procedure. This method can be used directly to quantify lunasin from plasma in different human and animal studies aiming to determine its bioavailability.

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INTRODUCTION

Lunasin is a 43-amino acid naturally occurring peptide with demonstrated chemopreventive and therapeutic properties (Dia & Gonzalez de Mejia, 2011; Galvez, Chen, Macasieb, & de Lumen, 2001). It was originally isolated in soybean (Galvez & de Lumen, 1999) and has been found in other plant species including amaranth (Maldonado-Cervantes et al., 2011; Silva-Sanchez et al., 2008), Solanum (Jeong, Jeong, Kim et al., 2007, Jeong, Jeong, Park et al., 2007), barley (Jeong, Lam, & de Lumen, 2002), and wheat (Jeong, Jeong, Kim et al., 2007, Jeong, Jeong, Park et al., 2007). Its biological properties are attributed to the presence of a cell adhesion motif composed of arginine, glycine and aspartic acid residues as well as to the nine aspartic acid residues located on its carboxylic acid end. The reported chemopreventive properties of lunasin included prevention of chemically-induced carcinogens (Hsieh, Hernandez-Ledesma, & de Lumen, 2011), inhibition of lipopolysaccharide-induced inflammation in macrophages (de Mejia & Dia, 2009; Dia, Wang, Oh, de Lumen, & de Mejia, 2009a; Hernandez-Ledesma, Hsieh, & de Lumen, 2009a, 2009b; Liu & Pan, 2010) as well as induction of apoptosis in different human cancer cell lines (de Mejia, Wang, & Dia, 2010; Dia & de Mejia, 2010; Hsieh, Hernandez-Ledesma, & de Lumen, 2010b). Animal models also showed the capability of lunasin to prevent or inhibit the process of carcinogenesis such as prevention of mammary carcinogenesis in a xenograft model of breast cancer (Hsieh, Hernandez-Ledesma, & de Lumen, 2010a), prevention of chemically-induced breast carcinogenesis (Hsieh, Hernandez-Ledesma, & de Lumen, 2010c) and inhibition of colon cancer metastasis and

potentiation of the chemotherapeutic effect of oxaliplatin in an experimental model of colon cancer metastasis (Dia & Gonzalez de Mejia, 2011).

One of the most important characteristics of any dietary compound with demonstrated biological properties is its capability to remain intact and bioactive after absorption, distribution and metabolism termed as bioavailability. Bioavailability refers to the fraction of the ingested compound that reaches circulation. In our previous study in human fed with 50 g of soy protein for 5 days, lunasin was found in plasma after 30 min and 1 h of soy protein ingestion (Dia, Torres, de Lumen, Erdman, & de Mejia, 2009b). Another study also demonstrated the bioavailability of soy lunasin in an animal model and was found in a variety of organs and tissues including blood, urine, brain, colon and feces (Hsieh et al., 2010c). Moreover, lunasin from different sources such as barley, rye and wheat was also reported to be bioavailable (Jeong, Jeong, Hsieh, Hernandez-Ledesma, & de Lumen, 2010; Jeong, Jeong, Kim et al., 2007, Jeong, Jeong, Park et al., 2007; Jeong et al., 2009). The method used in these studies were either an expensive kit or isotopic-labelling of the molecule prior to feeding and quantification of the labelled element after animal euthanasia making the process of measuring bioavailability time-consuming and costly.

The objectives of this study were to determine the effect of temperature on structure–activity of lunasin as well as to develop a method of isolating lunasin in human plasma using an ion-exchange microspin column and to quantify the amount of lunasin using an optimized enzyme-linked immunosorbent assay (ELISA). We report here the effect of temperature on the structure–activity property of lunasin purified from defatted soybean flour. Also, we show that lunasin

spiked in human plasma can be isolated and quantified using diethylaminoethyl (DEAE) anion exchange chromatography and an optimized ELISA protocol.

MATERIALS AND METHODS

Materials

Microspin columns and DEAE resin were purchased from GE Healthcare Life Sciences (Piscataway, NJ, USA). Lunasin rabbit polyclonal antibody was a kind gift from Dr. Ben O. de Lumen, University of California at Berkeley. All other chemicals were purchased from Sigma–Aldrich unless otherwise stated.

Purification of lunasin from defatted soybean flour

Lunasin from defatted soybean [Glycine max (L.) Merrill] flour was purified using a previously reported protocol with modifications (Dia et al., 2009a). Briefly, 200 g of defatted soybean flour obtained from National Soybean Research Laboratory, University of Illinois Urbana-Champaign was resuspended in 1-L of deionized water and mixed overnight at 4 °C. The suspension was centrifuged 12,000g for 10 min, supernatant was pooled and filtered. Two-hundred and fifty millilitres of supernatant was loaded in pre-equilibrated XK 50/30 column packed with DEAE anion exchange resin. Separation was carried out using 20 mM Tris–HCl pH 7.5 (buffer A) and buffer B containing 2 M NaCl pH 7.5. Bound protein was eluted with increasing concentration of buffer B in a step gradient mode from 5% (25 min), 10% (75 min), 20% (100 min) and 100% (25 min) at a flow rate of 10 mL/min. Fractions containing high concentration of lunasin were desalted using an Amicon ultrafiltration vessel with YM-1000 membrane (Millipore, molecular weight cut-off of 1 kDa) under 20 psi helium gas until the volume of the retentate was approximately 10% of the original volume. Fifteen millilitre of the retentate was then loaded on a Superdex 75 Prep Grade size exclusion XK 26/70 pre-

equilibrated with 20 mM Tris–HCl containing 0.15 M NaCl pH 7.5. Proteins were eluted with 20 mM Tris–HCl containing 0.15 M NaCl, pH 7.5, at a flow rate of 4 mL/min and fractions were collected every minute for 30 min after one void volume. Fractions with high concentrations of lunasin were pooled, concentrated and further purified. Five hundred microlitres of the retentate were loaded on a pre-equilibrated Superdex 30 Prep Grade size exclusion with 20 mM Tris–HCl containing 0.15 M NaCl, pH 7.5, connected to an AKTA primePlus chromatographic system (GE Healthcare Life Sciences, Piscataway, NJ, USA) and lunasin was eluted with 20 mM Tris–HCl containing 0.15 M NaCl, pH 7.5, at a flow rate of 1 mL/min. Fractions were collected every 2 min and a total of 30 fractions were collected after one void volume. Lunasin was measured and confirmed by ELISA, SDS–PAGE and Western blot procedures.

Circular dichroism (CD)

Lunasin at a concentration of 0.075 mg/mL in PBS (pH 7.5) was heated at 25, 37, 63, 72, 90 and 100 °C in succession, and the solution was maintained for 3 min at specified temperature. CD spectra were recorded from 190 to 260 nm in a 1 mm circular quartz cell at a scan rate of 100 nm/s with a 1-nm wavelength step and with 5 accumulations using the JASCO-720 spectropolarimeter (JASCO, Welltech Enterprises, Inc., Tokyo, Japan). Raw data files were analysed onto the DICHROWEB online server (<http://dichroweb.cryst.bbk.ac.uk/html/process.shtml>) using CDSSTR algorithm with reference set 4, which was optimized for the analysis of data recorded in the range from 190 to 240 nm (Lobley, Whitmore, & Wallace, 2002; Sreerama & Woody, 2000; Whitmore & Wallace, 2004).

Titer and specific activity determination of lunasin rabbit polyclonal antibody

Lunasin purified from defatted soybean flour was dissolved in Tris buffered saline (TBS) at 5 µg/mL. One hundred microlitres of lunasin solution was plated in a 96-well plate and incubated overnight at 4 °C. After incubation, the plate was washed with phosphate buffered saline containing Tween 20 (PBS-T) using a BioTek plate washer (Winooski, VT, USA). The plate was blocked with 5% BSA in TBS containing 1% Tween 20 (TBST-1%) for 1 h at room temperature (RT) and washed again. One hundred microlitres of a 1:5 dilution of lunasin rabbit primary polyclonal antibody was added in the first row of the 96-well plate and a serial 2-fold dilution of this antibody concentration was made in the succeeding wells plated with 50 µL of 3% BSA in TBST-1%. Fifty microlitres of anti-rabbit secondary antibody conjugated with alkaline phosphatase (1:1000) was added to each well and incubated for 1 h at RT. After washing, the colour was developed using p-nitrophenyl phosphate (PNPP) substrate. The absorbance was read at 405 nm using ELX BioTek plate reader. A plot of absorbance versus the logarithm of the dilution factor was prepared and the dilution that resulted in half-maximum binding was reported as the lunasin rabbit primary polyclonal antibody titer. Specific activity of the antibody was calculated by dividing the titer to the protein concentration of the antibody and reported as mL/µg.

Soluble protein determination

Total soluble protein concentration was quantified using Bio-Rad Protein DC assay following manufacturer's instructions. Briefly, 5 µL of sample was plated in a clear 96-well plate, 25 µL of Reagent A was added and 200 µL of Reagent B

thereafter. The colour was allowed to develop for 15 min at RT and the absorbance was measured at 630 nm using ELX Bio-Tek plate reader (Winooski, VT, USA). Protein concentration was calculated using bovine serum albumin (BSA) standard curve.

Optimization of ELISA procedure to quantify lunasin

One hundred microlitres of 8–100 ng/mL lunasin solution was plated in a 96-well plate and incubated overnight at 4 °C. After washing, plate was blocked with 5% BSA in TBST-1% for 1 h at RT. After blocking and washing, 50 µL of the different dilutions of lunasin primary rabbit polyclonal antibody (1:200, 1:500, 1:1000, 1:2000) was added and the plate was incubated for 1 h at RT. After washing, 50 µL of the 1:1000 dilution of anti-rabbit polyclonal secondary antibody was added and incubated for 1 h at RT. After washing, the colour was developed with colour reagent PNPP and the absorbance was read at 405 nm using ELX Bio-Tek plate reader (Winooski, VT, USA). The reaction was stopped by adding 100 µL of 3 N NaOH at 25 min and read again at 35 min. Lunasin concentration was quantified using a standard curve from different concentrations of purified lunasin. Primary antibody was diluted with 3% BSA, 1% Tween 20 and 0.05 M TBS buffer. All washings were done with 300 µL of washing solution, 6 times per well at the lowest dispensing rate (150 µL/well/s) and aspiration rate (5 mm/s) to avoid protein detachment.

Blood sample collection and preparation

The research protocol was approved by the Institutional Review Board of the University of Illinois at Urbana-Champaign. Healthy Caucasian males aged

18–25 y/o were recruited for the study. Exclusion criteria included smoking, vegetarianism and taking dietary supplements. Prior to blood collection, participants were asked to avoid consumption of soy products for one week. After one week of not consuming soy products, blood samples were collected from all participants. Blood samples were transported from the University clinic to the laboratory in a leak-proof container. Blood was centrifuged at 3000g for 15 min at 4 °C to separate plasma from other blood cells. Plasma samples were stored at –80 °C until further analysis.

Method for eluting and quantifying lunasin in human plasma

DEAE resin was successively washed with deionized water until the 20% ethanol storage solution was removed. DEAE resin was resuspended in water in 1:1 (v/v) ratio. Six hundred microlitres of the resuspended DEAE resin was pipetted out using a cut (45° angle) 1000- μ L blue tip and added to a microspin column. The water was eluted by centrifugation at 1000 rpm for 30 s. Prepared human plasma was spiked with different amounts of lunasin ranging from 10 ng to 2 μ L. After spiking, human plasma-lunasin mixture was added to the packed DEAE resin and lunasin was allowed to bind to the resin for 2–3 h at RT by rotating the microspin column in an end-over-end rotator. After binding, unbound fraction was eluted and collected by centrifugation at 1000 rpm for 30 s. The elution profile of lunasin spiked in human plasma was studied by eluting lunasin using 20 mM Tris–HCl buffer pH 7.5 with different concentrations of NaCl from 0 to 2 M. One hundred microlitres of eluate was plated in duplicate in a 96-well plate for quantifying lunasin using ELISA as described above. The total soluble protein concentration of each eluate was also quantified using the Bio-Rad Protein DC

assay as described above. Lunasin and protein elution profiles were plotted versus NaCl concentration. A plasma sample with no-spiked lunasin was also included to identify potential non-specific binding proteins. Yield for lunasin elution was calculated by taking the summation of all lunasin concentrations from each eluate and dividing the sum to the amount of lunasin used for spiking.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western Blot

Eluate from human plasma spiked with 10 µg lunasin was mixed with Laemmli buffer containing β-mercaptoethanol and boiled for 5 min. Samples were loaded in a 4–20% Tris–HCl gel (Bio-Rad, Hercules CA) and proteins were separated at 200 V for 30 min. After electrophoresis separation, one gel was fixed with fixing buffer (acetic acid:methanol:water, 10:40:50 v/v/v) for 15 min, stained with Coomassie Brilliant Blue overnight and destained with 10% acetic acid for 20 min. After destaining, gel was placed in deionized water and image was photographed using Kodak CF440 Imager (New Haven, CT). Another gel was equilibrated in blotting buffer (20% methanol in SDS–PAGE running buffer) for 15 min at RT and proteins were transblotted in PVDF membrane at 110 V for 60 min in the cold room. After transblotting, membrane was blocked with 5% nonfat dry milk (NFDM) for 1 h at 4 °C and washed with TBS containing 0.1% Tween 20 (TBST-0.1%) 3 times 5 min each. After washing, membrane was incubated with lunasin primary rabbit polyclonal antibody (1:1000 in 1% NFDM) overnight at 4 °C. After primary antibody incubation and repeated washings with TBST-0.1%, membrane was incubated with anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:1000 in 1% NFDM) for 2–3 h at RT. After washing, the

membrane was imaged using a chemiluminescence reagent (GE Healthcare Life Sciences) and Kodak CF440 Imager (New Haven, CT, USA).

Statistical analysis

All experiments were done in at least two independent replicates. Data are reported as mean \pm standard deviation. Data were analyzed using SAS software version 9.2 and means separated by Tukey grouping at $P < 0.05$.

RESULTS AND DISCUSSION

Purification of lunasin

We report here a simple method of purification of lunasin from defatted soybean flour. Fig. 1A presents lunasin and protein concentrations of different fractions obtained from Superdex 30 Prep Grade gel filtration chromatography collected after one void volume. Earlier fractions showed that lunasin co-eluted with other high molecular mass proteins as evidenced by high lunasin concentration in these fractions, as determined by ELISA. In addition, a considerable amount of lunasin is observed in fractions 7, 8 and 9. Western blot profile showed that lunasin is present in these fractions (Fig. 1B). Moreover, SDS-PAGE Coomassie staining of these fractions showed high purity of lunasin preparation. Fig. 1C shows the electrophoresis profiles of different fractions during lunasin purification. As can be seen from the SDS-PAGE Coomassie staining, a purified lunasin extract was prepared after a successive DEAE ion-exchange chromatography (lanes 1 and 4), Superdex 75 gel filtration chromatography (lanes 2 and 5) and Superdex 30 gel filtration chromatography (lanes 3 and 6). This is further confirmed by the immunoreactivity of the 5 kDa band against the lunasin rabbit polyclonal antibody corresponding to lanes 4, 5 and 6. This purification method presented several advantages as the previously described method of lunasin preparation (Cavazos, Morales, Dia, & Gonzalez de Mejia, 2012; de Mejia & Dia, 2009; Dia et al., 2009a) including high purity of lunasin preparation and high reproducibility which might be attributed to the use of an automated gel filtration chromatographic systems. In addition, less cost was involved as ultracentrifugation filters (Cavazos et al., 2012) were not used.

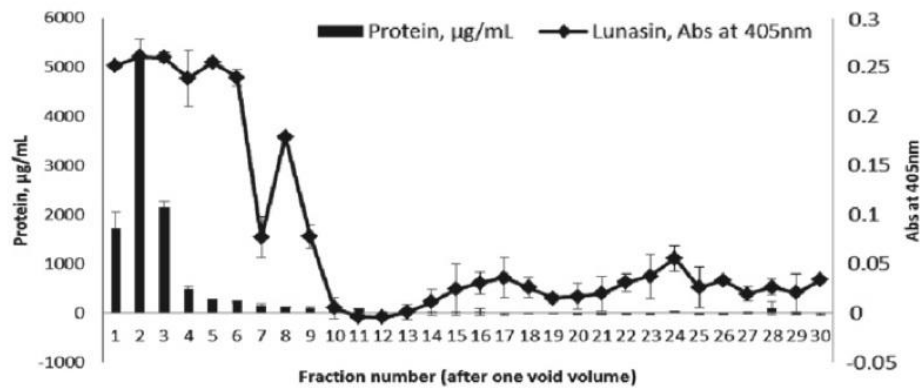
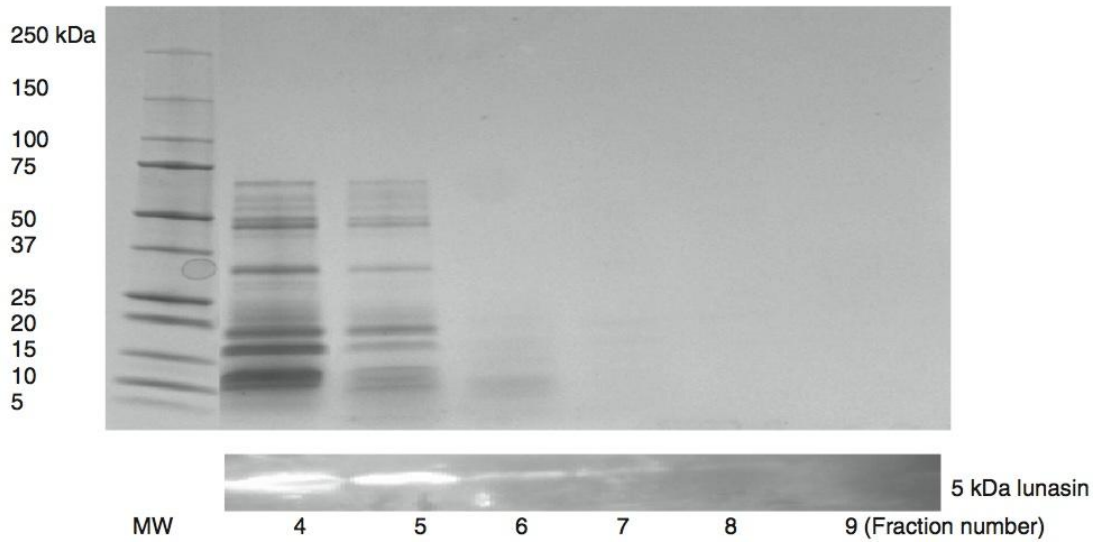
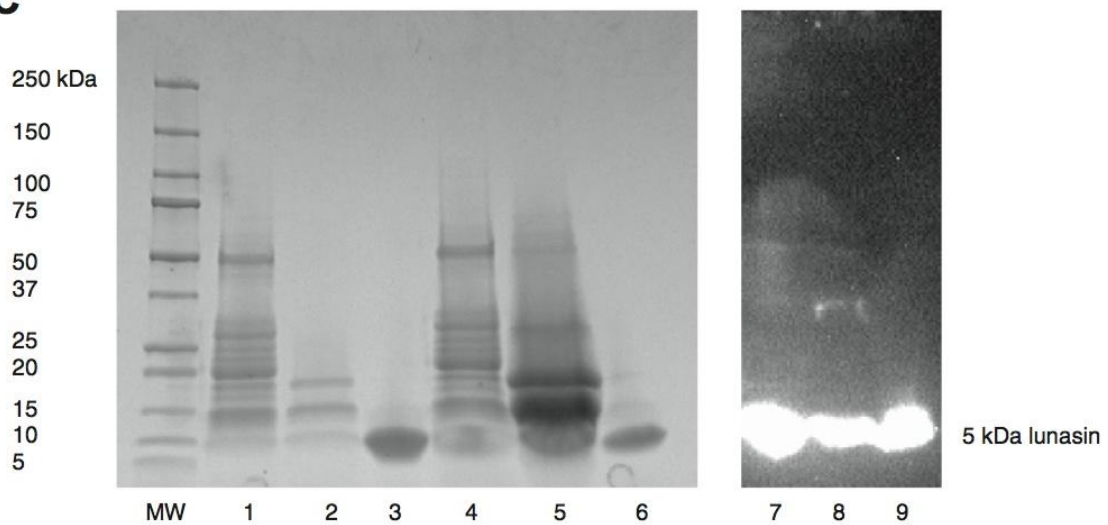
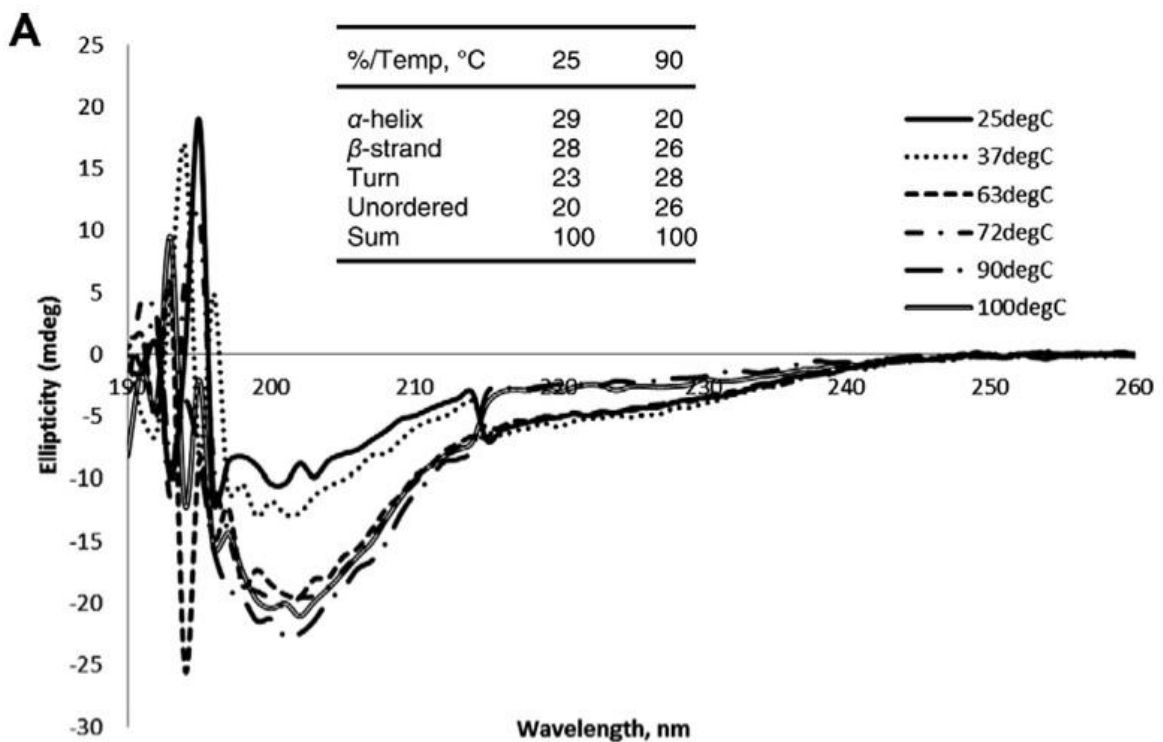
A**B****C**

Fig. 1. Lunasin and protein concentrations of Superdex 30 Prep Grade gel filtration fractions collected after one void volume (A), Coomassie staining and Western blot profiles of Superdex 30 Prepgrade gel filtration fractions containing high concentrations of lunasin as previously measured by ELISA (B) and Coomassie staining and Western blot profiles of lunasin extract from different stages of purification (C). MW: molecular mass standard; lanes 1 and 4: desalted ion-exchange fraction containing high concentration of lunasin from two independent preparations; lanes 2 and 5: desalted gel filtration fraction from Superdex 75 gel filtration chromatography containing high concentration of lunasin from two independent preparations; lanes 3 and 6: desalted and purified lunasin from gel filtration fraction using Superdex 30 from two independent preparations; lanes 7, 8 and 9 represent the Western blot profiles of lanes 4, 5 and 6, respectively.

Structural property of lunasin as measured by CD

Fig. 2 presents the data deconvolution of the ellipticity of lunasin solution at 25 °C using CDSSTR algorithm; lunasin has 29% α -helix, 28% β -strands, 23% turns and 20% unordered. The effect of temperature on the structural changes of lunasin was monitored from the appearance of a minimum peak at 205 nm and a weak shoulder at 222 nm as shown in Fig. 2A. No significant change from the CD spectra at 222 nm was observed until reaching 72 °C while a significant change was observed starting at 90 °C which reflected the α -helix structure of the folded protein. Consistent with this change, the increase in the minimum peak observed at 205 nm when the temperature was increased from 25 to 100 °C indicated increased randomness in the structure. Plotting ellipticity versus heating temperature, allows the determination of the thermal unfolding and denaturation of lunasin (Fig. 2B); lunasin unfolding did not start until 90 °C. The capability of the heated lunasin solution to interact with the rabbit polyclonal antibody produced against lunasin epitope was also determined. There was a 24.4% reduction in the affinity towards the antibody of the heated lunasin (100 °C) when compared to the unheated lunasin solution as evidenced by a reduction of slope of the standard curve prepared from heated lunasin solution ($y = 0.019x - 0.039$, $R^2 = 0.99$) and

unheated lunasin solution ($y = 0.025x + 0.176$, $R^2 = 0.98$). This observation is the first to report the effect of heating on the capability of lunasin to interact with the rabbit polyclonal antibody. Lunasin concentrations in heated soy products such as soymilk and tofu have been reported in previous publications (Cavazos et al., 2012; Gonzalez de Mejia, Vasconez, de Lumen, & Nelson, 2004; Hernandez-Ledesma et al., 2009a, 2009b). The reduction in the affinity of heated lunasin with the antibody can be explained by the changes in the structural features of the lunasin molecule as supported by the CD (inserted table in Fig. 2A).



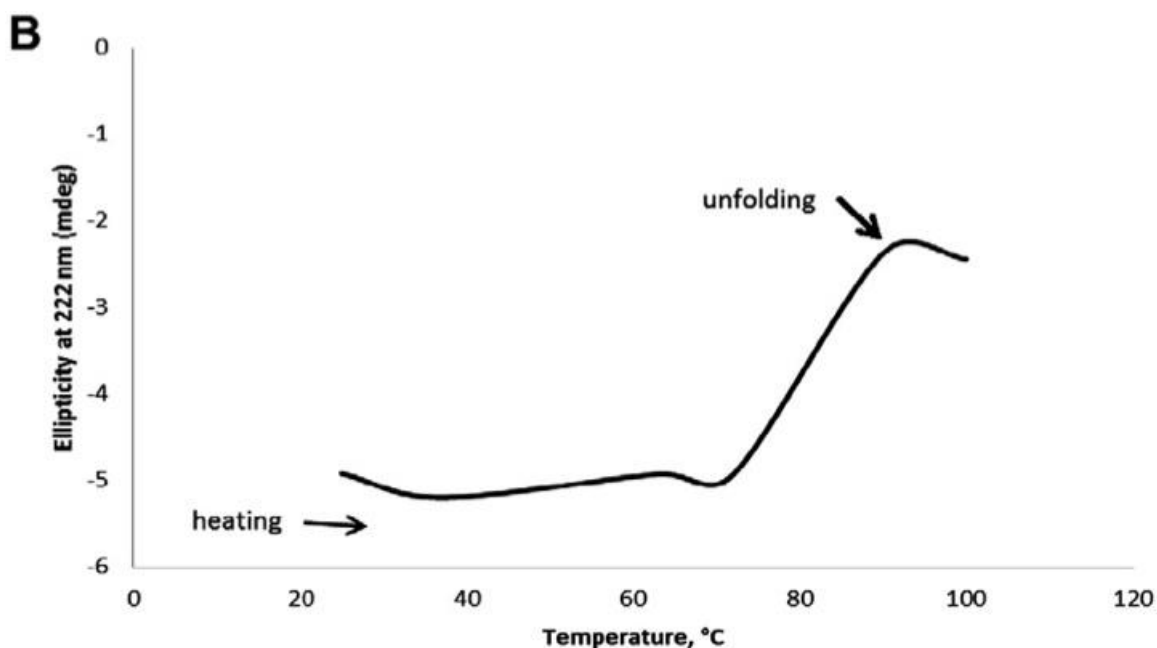


Fig. 2. Circular dichroism spectra of lunasin as affected by different heating temperatures. The secondary structure of lunasin was calculated using the DICHROWEB tool using CDSSTR algorithm (A). Thermal unfolding of lunasin as monitored by CD at 222 nm (B). Protein concentration was 0.075 mg/mL. CD values are presented in ellipticity, mdeg, obtained from 190 to 260 nm in a 1 mm circular quartz cell at a scan rate of 100 nm/s with a 1-nm wavelength step and with 5 accumulations using a JASCO-720 spectropolarimeter. The inserted table presents the percentage of each component of the secondary structure conformation obtained from the CD spectra at 25 and 90 °C.

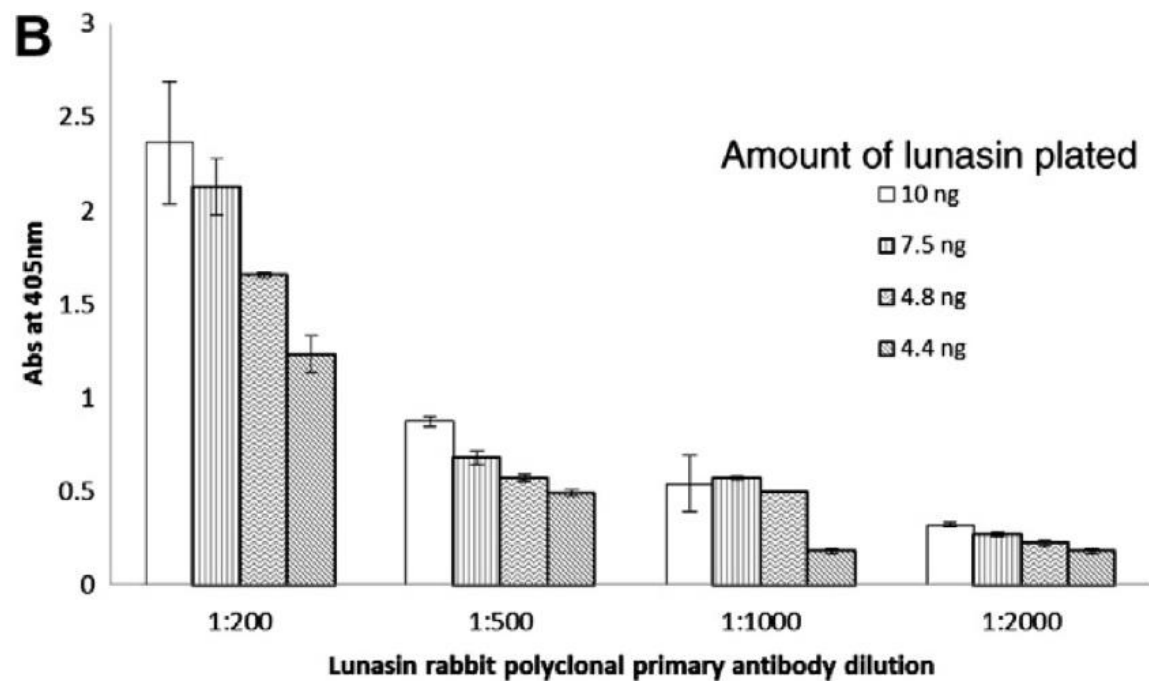
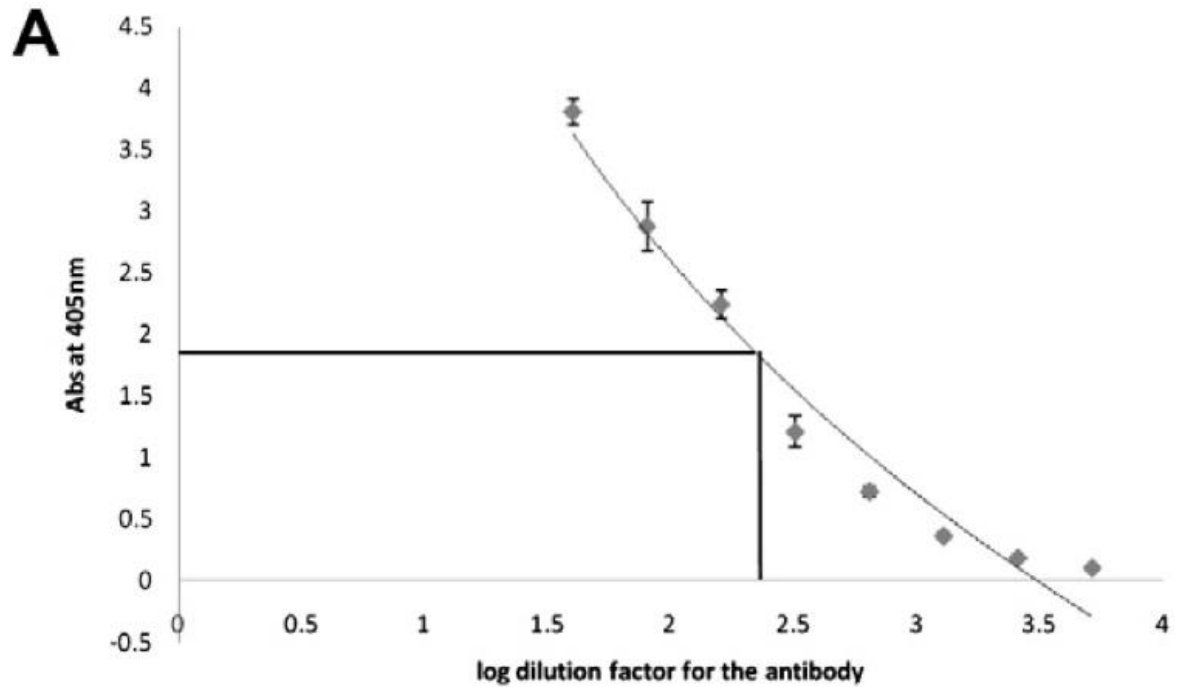
Characteristic of the lunasin rabbit primary polyclonal antibody and optimization of antibody concentrations for ELISA

Determination of the titer of an antibody is a common method of characterising an antibody for an immunoassay protocol in order to identify the optimum dilution or concentration of antibody that will give the best antigen–antibody interaction with minimum background and non-specific binding. Fig. 3A shows the effect of antibody dilution on the capability of lunasin to interact with the antibody resulting in the determination of antibody titer. As shown in Fig. 3A, maximum binding occurred at 1:40 antibody dilution with an average absorbance at 405 nm of 3.8 while minimum response was seen at the lowest dilution of

1:5120 with an absorbance of 0.10. The titer for the antibody, defined as the dilution that resulted in half-maximum binding, was 1.9 absorbance units; the logarithm of the dilution factor was 2.4 which corresponded to a titer (dilution of the antibody) of 250. This means that at a dilution of 1:250, lunasin rabbit polyclonal antibody will give the best antibody-antigen interaction with minimal background and nonspecific binding. The specific activity of the antibody, obtained by dividing the titer (250) to the protein concentration (5187.5 $\mu\text{g}/\text{mL}$) of the antibody, was 0.05 $\text{mL}/\mu\text{g}$.

To further optimize the ELISA procedure for measuring lunasin concentration, independent experiments using different dilutions of the primary antibody and testing different concentrations of lunasin were performed. Fig. 3B shows the response of different primary antibody dilutions to different concentrations of lunasin. A linear response to lunasin concentrations was observed at primary antibody dilution of 1:200 and 1:500. It was decided to use the 1:200 antibody dilution as this dilution was closer to the antibody titer found initially in this study and the response was similar to our previously reported study using mouse monoclonal antibody (Dia, Torres, de Lumen, Erdman, & de Mejia, 2009b). It was also found that at this combination of antibody dilution (1:200 for lunasin primary rabbit polyclonal antibody and 1:1000 for the anti-rabbit secondary antibody), the assay had a linear range between 16 and 48 ng lunasin/mL with a R^2 of 0.96 (Fig. 3C). When the highest concentration was 44ng lunasin/mL, the linearity was improved ($y = 0.028x - 0.298$, R^2 of 0.98). This is the first report on the characteristics of lunasin primary rabbit polyclonal antibody. The linear range obtained in the present study is almost similar to previously reported method using

mouse monoclonal antibody (Gonzalez de Mejia et al., 2004) of 24–72 ng lunasin/mL with very similar R^2 of 0.96 (Fig. 3C).



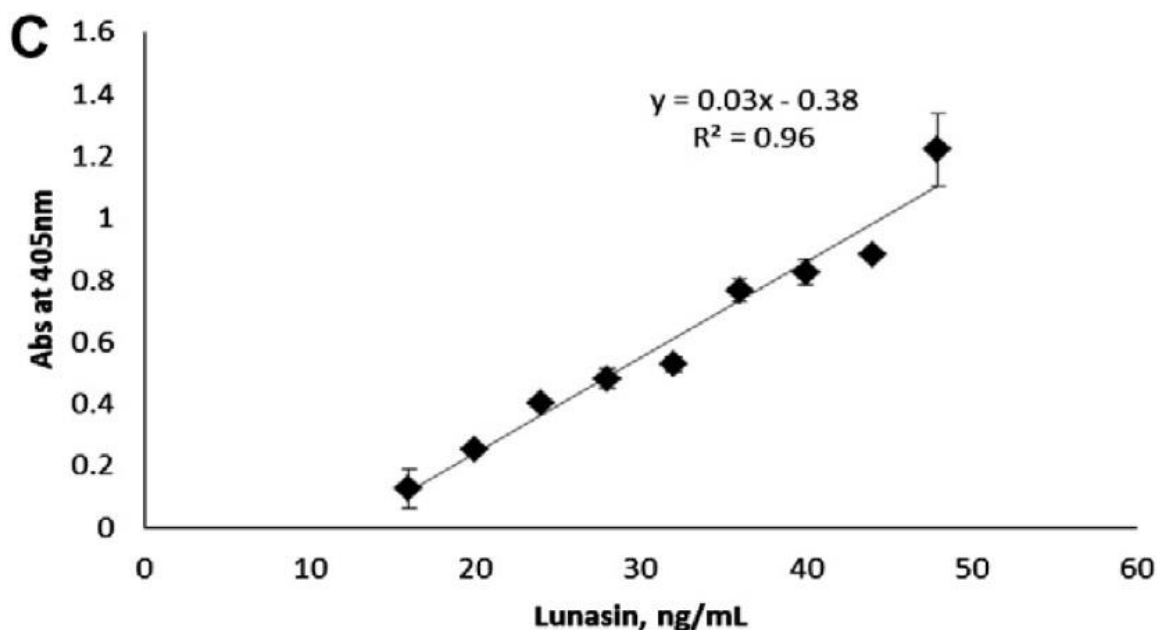


Fig. 3. Determination of titer for lunasin primary rabbit polyclonal antibody. One hundred microlitres of 5 µg/mL lunasin solution was plated in a 96-well plate and incubated overnight at 4 °C, the secondary anti-rabbit antibody conjugated with alkaline phosphatase was diluted 1000 \times with 3% BSA in 1% TBST (A). Effect of primary lunasin antibody dilutions and lunasin concentration on the determination of lunasin in plasma by ELISA. Constant anti-rabbit secondary antibody conjugated with alkaline phosphatase (1:1000) with varying primary lunasin antibody dilutions (B). Standard curve for lunasin using 1:200 and 1:1000 dilutions for primary and secondary antibodies, respectively showing linear range from 16 to 48 ng lunasin/mL (C).

Diethylaminoethyl (DEAE) microspin anion exchange column as a method for quantifying lunasin in plasma

An important characteristic of any bioactive compound is its ability to resist gastrointestinal digestion and be absorbed to the circulation thereby can reach target tissues and organs in order to exert its biological activity. We report here a simple method of quantifying lunasin in human plasma. Fig. 4A presents the lunasin profile of different eluates from the DEAE microspin column eluted with different concentrations of NaCl in Tris–HCl buffer, pH 7.5. A small percentage of lunasin was not able to bind to the resin as measured by lunasin concentration in the unbound eluate. The amount of lunasin in the unbound eluate corresponds to

5.1– 8.2% of the total amount of lunasin eluted from the DEAE microspin resin. On the other hand, most lunasin eluted from the DEAE microspin column at the Tris–HCl buffer with no NaCl added. The amount of lunasin from this eluate ranged from 28.6% (plasma spiked with 100 ng lunasin) to 46.5% (plasma spiked with 2 µg lunasin) of the total amount of lunasin eluted. The amount of spiked lunasin correlated well to the amount of lunasin eluted by Tris–HCl buffer ($R^2 = 0.82$). The remainder of the lunasin was then eluted with Tris–HCl buffer containing different concentrations of NaCl from 0.1 to 2 M. The result showed that the spiked lunasin had a weak binding to the DEAE resin used as it eluted from the resin at a buffer with no need of NaCl. At a pH of 7.5, we expected that most lunasin will be bound to the resin as it will have a strong net negative charge attributed to its nine aspartic acid residues on its carboxylic acid end. Our previous study on the isolation of lunasin from defatted soybean flour showed that most lunasin eluted from the DEAE resin at 0.4 M NaCl (Dia et al., 2009b). Moreover, isolation of lunasin from plasma of men fed with 50 g soy protein showed that lunasin from human plasma eluted at 0.15–0.20 M NaCl (Dia et al., 2009a). The differences were on the pH of the buffer used (20 mM Triethanolamine, pH 8.0 vs 20 mM Tris–HCl, pH 7.5), the resin used to bind lunasin (strong anionic magnetic beads vs. DEAE), as well as the effect of the human plasma protein matrix on the binding capability of the spiked lunasin. In comparing the elution profile of spiked lunasin from lunasin in defatted soybean flour, both procedures used a DEAE resin; however, the spiked lunasin in plasma eluted without the need of NaCl in contrast to the 0.4 M NaCl needed to elute lunasin from defatted soybean flour. This can be explained by the effect of other proteins present in the human plasma, the matrix, which affected the binding affinity of lunasin to DEAE resin. The yield of lunasin

from the DEAE microspin column ranged from 50.7 to 76.6% (Table 1) indicating that there is still a considerable amount of lunasin that was retained in the DEAE microspin column that might be eluted with extended washes of Tris–HCl buffer with higher NaCl concentration. On the other hand, the protein concentrations of each eluate from plasma spiked with different concentrations of lunasin vary slightly with each other (Fig. 4B).

Table 1

Yield of lunasin from the DEAE* microspin column.

Amount of lunasin spiked to plasma, μg	Yield, %**
2.0	$50.7 \pm 2.3^{\text{b}}$
1.0	$76.6 \pm 6.1^{\text{a}}$
0.5	$56.2 \pm 3.1^{\text{b}}$
0.1	$68.6 \pm 8.5^{\text{a}}$
0.05	ND***
0.01	ND

* Diethylaminoethyl anion exchange resin

** Calculated based on the cumulative amount of lunasin eluted from the DEAE microspin column and divided by the amount of lunasin spiked to plasma. Means followed by different letter are significantly different from each other, $P < 0.05$.

*** ND – not determined

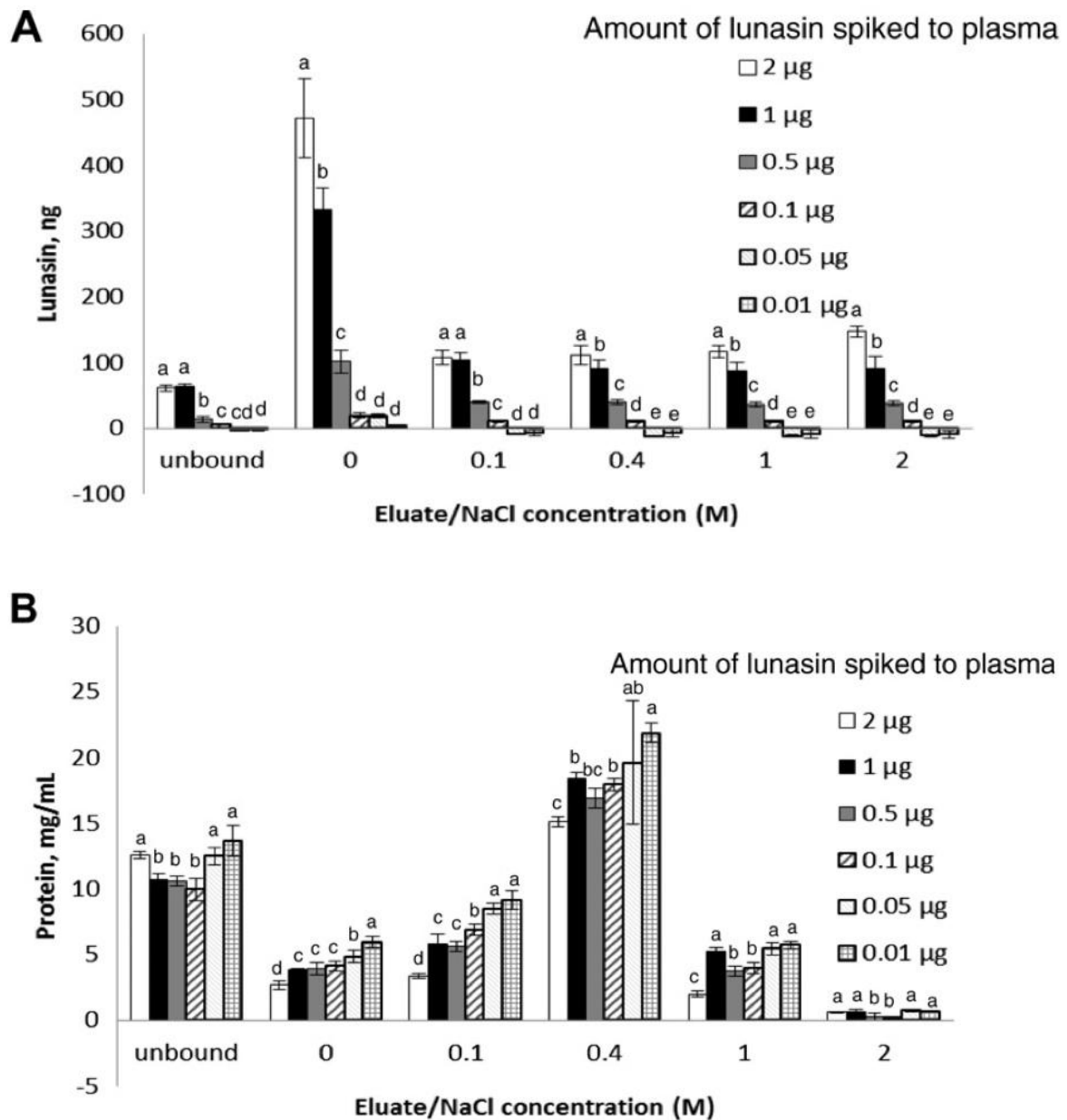


Fig. 4. Lunasin (A) and protein (B) concentrations of eluates from DEAE microspin column derived from human plasma spiked with different amounts of lunasin. Lunasin was eluted from the plasma using 20 mM Tris-HCl buffer pH 7.5 with different NaCl concentrations. PBS-spiked plasma showed no presence of detectable lunasin in the eluates from different concentrations of NaCl used for elution. Means within the same eluate followed by different letters are statistically different from each other ($P < 0.05$).

To further validate the elution profile of lunasin from plasma, the plasma sample was spiked with 10 µg lunasin and eluted with buffer pH 7.5 and different concentrations of NaCl ranging from 0 to 1 M. The protein profile of the eluate was

determined by SDS-PAGE and the identity of lunasin was confirmed by Western blot. Fig. 5 shows the SDS-PAGE profile and the Western blot for these eluates. As can be seen from the gel, the protein profile of the eluate differs from each other based on the NaCl concentration present in the buffer which can be explained by the different proteins present in human plasma. The identity of lunasin eluted from the plasma was further confirmed by a strong chemiluminescence at 5 kDa band, molecular mass of lunasin based on its reported amino acid composition.

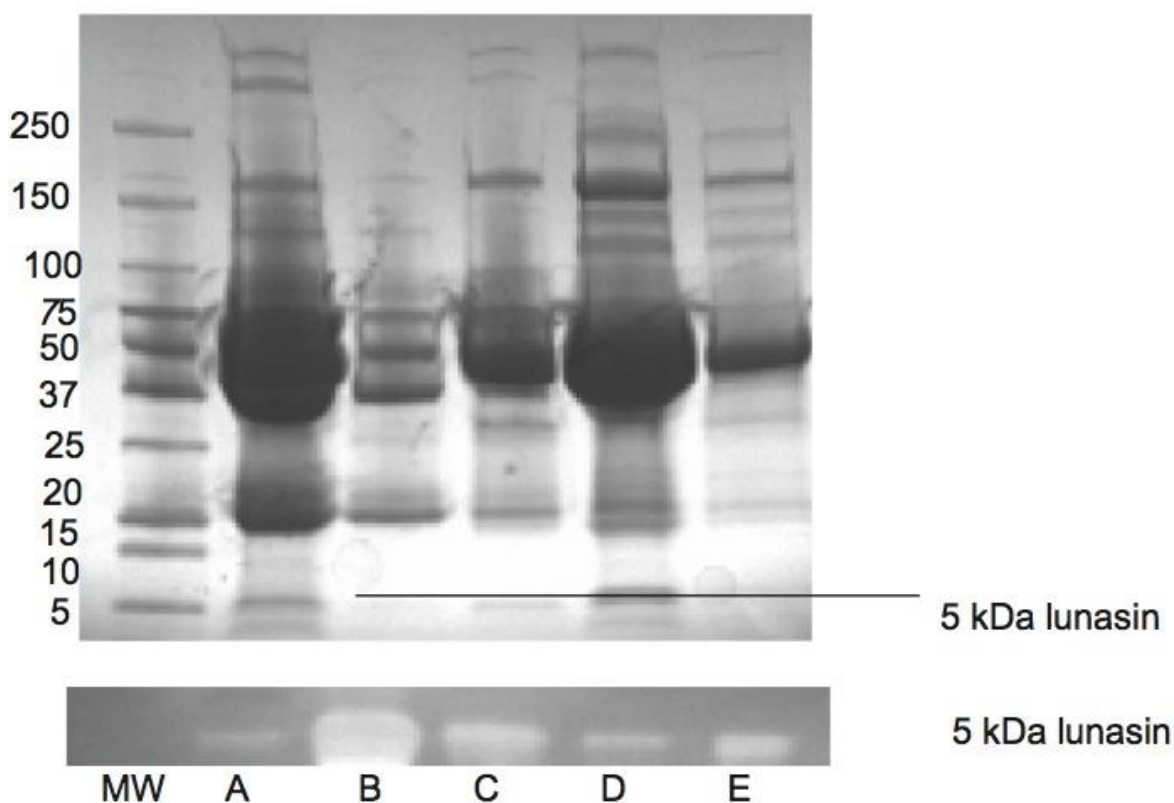


Fig. 5. SDS-PAGE (A) and Western blot (B) profiles of different eluates from DEAE microspin column derived from human plasma spiked with 10 μ g lunasin. MW: molecular mass standard, A: unbound eluate, B: Tris-HCl eluate, C: Tris-HCl with 0.1 M NaCl eluate, D: Tris-HCl with 0.4 M NaCl eluate, E: Tris-HCl with 1 M NaCl eluate.

Previous studies have shown the bioavailability of lunasin in human as well as in animals. For instance, the bioavailability study in humans used an expensive commercially available strong anionic bead to capture lunasin by means of magnetic property of the beads from the plasma and eluted it with buffer with different salt concentrations (Dia et al., 2009b). Bioavailability study in animals used laborious and time-consuming purification protocol to detect and measure lunasin. The study of Hsieh et al. (2010a) used an isotope-labelled ^3H -lunasin and the concentration of lunasin from different tissues and organs were determined by scintillation counting. Other studies conducted a combination of ion-exchange chromatography and HPLC in order to purify lunasin in freeze dried organs showing bioavailability of lunasin (Jeong, Jeong, Kim et al., 2007, Jeong, Jeong, Park et al., 2007; Jeong et al., 2009). The method presented in this study has several advantages such as shorter procedure time, no use of sophisticated equipment or reagents, and less expensive. One disadvantage though is the incomplete elution of the spiked lunasin which can be resolved by adding more wash elution steps with buffer containing concentration of NaCl higher than 2 M.

CONCLUSIONS

For the first time the structural property of lunasin and the effect of temperature on its structure and capability to interact with rabbit polyclonal antibody was demonstrated. In addition, different characteristics of lunasin primary rabbit polyclonal antibody for quantifying lunasin in human plasma using an optimized ELISA protocol are reported. Lunasin from human plasma can be isolated by a simple DEAE microspin column technique and can be quantified using a validated and optimized immunoassay procedure.

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