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Analytical Methods

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

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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/μg. 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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1. 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

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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.

2. Materials and methods

2.1. 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.

2.2. Purification of lunasin from defatted soybean flour

Lunasin from defatted soybean [*Glycine max* (L.) Merill] 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 12,000*g* 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 preequilibrated 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.

2.3. 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).

2.4. 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.

2.5. 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.

2.6. 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 luna-

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