Research Article

Protein hydrolysates from β-conglycinin enriched soybean genotypes inhibit lipid accumulation and inflammation *in vitro*

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Obesity is a worldwide health concern and a well recognized predictor of premature mortality associated with a state of chronic inflammation. The objective was to evaluate the effect of soy protein hydrolysates (SPH) produced from different soybean genotypes by alcalase (SAH) or simulated gastrointestinal digestion (SGIH) on lipid accumulation in 3T3-L1 adipocytes. The anti-inflammatory effect of SPH produced by alcalase on LPS-induced macrophage RAW 264.7 cell line was also investigated. SAH (100 μM) derived from soybean enriched in β-conglycinin (BC) (up to 47% total protein) decreased lipid accumulation (33 – 37% inhibition) through downregulation of gene expression of lipoprotein lipase (LPL) and fatty acid synthase (FAS). SGIH (100 μM) inhibited lipid accumulation to a lesser extent (8 – 14% inhibition) through inhibition of LPL gene expression. SAH (5 μM) decreased the production of nitric oxide (NO) (18 – 35%) and prostaglandin E_2 (PGE_2) (47 – 71%) and the expression of inducible nitric oxide synthase (iNOS) (31 – 53%) and cycloxygenase-2 (COX-2) (30 – 52%). This is the first investigation showing that soy hydrolysates inhibit LPS-induced iNOS/NO and COX-2/PGE_2 pathways in macrophages. Soybeans enriched in BCs can provide hydrolysates that limit fat accumulation in fat cells and inflammatory pathways *in vitro* and therefore warrant further studies as a healthful food.

Keywords: Biomarkers / β-conglycinin / Inflammation / Lipid metabolism / Soy protein hydrolysates

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1 Introduction

The use of soybean has expanded in the food industry due to its nutritional characteristics, technological properties, and health benefits [1]. Some of these health benefits have been attributed to its content of biologically active peptides and proteins [2, 3]. These active peptides are either naturally occurring or hidden in a latent state within the precursor protein sequence but can be released by enzymatic proteolysis during gastrointestinal (GI) digestion, or during processing by enzymatic hydrolysis or fermentation [2]. Due to the increasing importance of soybeans as a source of protein in the diet, attention is being given to its composition and bioactivities as an ingredient source for healthful foods.

Unhealthy weight gain is a worldwide health concern because it is a well recognized independent predictor of premature mortality [4]. Accumulation of body fat arises from a chronic imbalance between energy acquisition and expenditure that may lead to a pathologic growth of adipocytes, characterized by increased fat cell size and number [5]. It is known that the amount of adipose tissue can be regulated by the inhibition of adipogenesis and fat deposition [6]. Fat deposition in adipose tissue can be reduced by reducing lipid uptake by adipocytes *via* suppressing lipoprotein lipase (LPL) or reducing lipid synthesis through inhibiting fatty acid synthase (FAS) among other mechanisms [7]. FAS inhibitors have been reported to provide a potential pathway to target obesity therapy [8]. For example, a drug suggested for the treatment of obesity, Orlistat, has been found to be a FAS inhibitor [9].

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Abbreviations: ANOVA, analysis of variance; **BC,** β-conglycinin; **COX-2,** cyclooxygenase-2; **FAS,** fatty acid synthase; **FBS,** fetal bovine serum; **GI,** gastrointestinal; **iNOS,** inducible nitric oxide synthase; **LPL,** lipoprotein lipase; **NO,** nitric oxide; **PGE_2,** prostaglandin E_2; **SAH,** soy alcalase hydrolysates; **SGIH,** soy gastrointestinal hydrolysates; **SPH,** soy protein hydrolysates

High body fat levels are also closely associated with a state of chronic low-grade inflammation characterized by abnormal cytokine production and activation of inflammatory signaling pathways in adipose tissue [10]. Recent studies have indicated that adipose tissue macrophages accumulated during diet-induced accumulation of body fat are not only an important source of adipose tissue inflammation but also alter insulin sensitivity and promote atherosclerosis [11, 12]. Macrophages can secrete pro-inflammatory responses such as nitric oxide (NO) and prostaglandin E_2 (PGE_2). NO is produced by inducible NO synthase (iNOS) in activated macrophages and is one of the most important inflammatory mediators [13]. Physiologically, it induces various harmful responses including tissue injury, septic shock, and apoptosis [14], which have been attributed to the iNOS mediated production of NO and the associated generation of potent reactive radicals such as peroxynitrite [15]. Cyclooxygenase-2 (COX-2) catalyzes the production of prostaglandins during the inflammatory process. Therefore, good tasting foods that limit the formation of body fat and pro-inflammatory responses are expected to be especially healthful choices. Ringseis *et al*. [16] reported previously that pepsin/trypsin/chymotrypsin/elastase hydrolysates from casein and soy proteins influenced the release of 6-keto-prostaglandin F1α and NO but did not influence the release of thromboxane B2 and endothelin-1; however, the roles of alcalase, and pepsin-pancreatin hydrolysates from β-conglycinin (BC) enriched soybean flour on markers of inflammation are unknown.

The objective of this research was to evaluate the effect of soy alcalase hydrolysates (SAH) and soy gastrointestinal hydrolysates (SGIH) derived from soybean genotypes with different protein profiles on lipid accumulation, and LPL and FAS gene expression using 3T3-L1 adipocytes as experimental model. In addition, the effect of SAH produced from soybean genotypes on NO and PGE_2 production as well as iNOS and COX-2 expression in LPS-induced macrophage RAW 264.7 cell line was also studied.

2 Materials and methods

2.1 Materials

Eight defatted soybean flour samples (A1 – D2) were provided by the Monsanto Company (St Louis, MO, USA). They were derived from distinct soy genotypes that by selective breeding were designed to have different protein profiles, particularly in the content of BC and glycinin. 3T3-L1 fibroblasts (also designated as ATCC® CCL-92.1™) from Swiss albino mouse, macrophage RAW 264.7 cell line and DMEM were purchased from American Type Culture Collection (Manassas, VA, USA). Fetal bovine serum (FBS) was purchased from Invitrogen (Grand Island, NY, USA). Alcalase from *Bacillus licheniformis* (E.C. 3.4.21.62), pepsin (EC 3.4.23.1, 662 U/mg), pancreatin from porcine pancreas (8 × USP), isobutylmethylxanthine (IBMX), dexamethasone (DEX), insulin, sodium pyruvate solution, penicillin (1000 U/mL), streptomycin (1000 U/mL), sodium nitrite, sulfanilamide, *N*-1-(naphthyl)ethylenediamine-diHCl, and LPS from *Escherichia coli* O55:B5 were purchased from Sigma – Aldrich (St. Louis, MO, USA). Actin mouse mAb epitope mapping at the C-terminus of actin of human origin, COX-2 mouse mAb against amino acids 580 – 598 of human COX-2 and inducible iNOS mouse mAb epitope mapping at the C-terminus of mouse iNOS were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antimouse IgG, horseradish peroxidase conjugate secondary antibody was purchased from GE Healthcare (Buckinghamshire, UK).

2.2 Preparation of soy protein hydrolysates (SPH)

The hydrolysates were prepared as described by Martinez-Villaluenga *et al*. [17]. Briefly, 2 g of defatted soy flour were added to 25 mL of deionized water and brought to 50°C at pH 8.0. Then, 5 mg of alcalase (11 U/mg) were added to hydrolyze the proteins. Hydrolysis was carried out for 3 h at 50°C and pH 8.0 was maintained by adding 0.5 M NaOH. Hydrolysis was stopped by the addition of 75 μL of 0.1 N HCl. Hydrolysates were centrifuged at 14 000 × *g* at 10°C for 30 min. After centrifugation, 10% trichloroacetic acid (TCA) was added in a 1:1 ratio. The hydrolysates were centrifuged again under the same conditions, and the liquid hydrolysates were concentrated using stirred ultra-filtration cell 1 kDa membrane (Millipore). The hydrolysates (SAH) were freeze dried in a FreeZone freeze dry system (Kansas City, MO, USA). Protein DC assay (BioRad) was followed for protein quantification using BSA as standard ($y = 0.0002x - 0.0021$, $R^2 = 0.997$).

Pepsin-pancreatin hydrolysis (SGIH) of defatted soy flours in a way to simulate the *in vivo* enzyme hydrolysis was performed according to Wang *et al*. [18]. All samples were stored at −80°C until analysis.

2.3 SDS-PAGE gel electrophoresis

Defatted soy flours were extracted with 1 × Laemmli SDS buffer pH 6.8 with 0.07 M DTT (~30 mg/mL). For each sample, 10 μg of total protein was run on a 26 lane 12% homogenous Bis-Tris Criterion gel. A broad range prestained SDS-PAGE standard (BioRad Laboratories, Hercules, CA, USA) was used as molecular weight marker. Proteins were reported as the relative percent of the total protein bands in the lane. The theoretical molecular mass of each protein was calculated from the amino acid sequence with ProtParam program (http://ca.expasy.org/tools/protparam.html).

Protein bands of the eight soybean genotypes were identified comparing the experimental molecular masses with the theoretical molecular masses calculated from the amino

acid sequence of the proteins given by ProtParam tool in ExPASY database. Furthermore, the identities of the major protein bands, on the SDS-PAGE gel, were compared with previous publications [18 – 20].

2.4 Isoflavones and saponins analysis in SPH

Isoflavones and saponins concentrations were determined by extraction and quantification by HPLC as described previously in Wang *et al*. [18]. Briefly, samples (typically 0.25 g) were placed in a vial and 3 mL of a DMSO/methanol (1:1) solution was added. The vials were capped and wrapped with sealing tape and incubated in an oven for 72 h at 50°C. Then, the samples were sonicated for 15 min at 50°C and allowed to stand at room temperature for 1 – 2 h. An aliquot was removed from the vial and filtered through a 0.45 μM nylon 66 filter for HPLC analysis for both isoflavones and saponins. The samples were run on a quaternary pump HPLC system with a photodiode array detector and an Inertsil ODS-3 RP C-18 column (5 μM, 250 mm × 4.6 mm). For isoflavone analysis, the initial conditions were 20% methanol and 0.025% TFA in water, at a flow rate of 1 mL/min. The effluent was monitored at 285 nm. After injection (typically 15 μL), the column was held at the initial conditions for 2 min, then developed to 100% methanol and 0.025% TFA in a linear gradient over 53 min. Standard curves based on nanomoles injected were prepared from pure standards of daidzin, glycitin, and genistin prepared in the laboratory. For saponin analysis, the initial conditions were 30% ACN and 0.025% TFA in water, at a flow rate of 1 mL/min. The effluent was monitored at 210 nm. After injection (typically 20 μL), the column was developed to 50% ACN and 0.025% TFA in a linear gradient over 45 min. Standard curves based on nanomoles injected were prepared from a characterized pure soyasaponin I prepared in the laboratory.

2.5 Effect of SPH on lipid metabolism in 3T3-L1 adipocytes

2.5.1 Cell culture and treatments

The 3T3-L1 preadipocytes were seeded at 6×10^3 cells/cm^2 in six-well plates and cultured in DMEM containing 10 mM sodium pyruvate, 100 U/mL penicillin, 100 U/mL streptomycin, and 10% FBS (FBS/DMEM medium). For preadipocytes differentiation, 2 days after reaching 100% confluence, the cells were stimulated with FBS/DMEM medium containing 167 nM insulin, 0.5 mM IBMX, and 1 μM DEX for 2 days. Cells were then maintained in FBS/DMEM medium with 167 nM insulin for another 2 days, followed by culturing with FBS/DMEM medium for an additional 4 days, at which time up to 90% of cells were mature adipocytes with accumulated fat droplets.

Mature adipocytes were treated separately with soy hydrolysates dissolved in water at a concentration of 100 μM. This concentration was selected based on previous dose-response studies on lipid accumulation in 3T3-L1 adipocytes. It corresponds to 1 mg soluble protein *per* milliliter of media and it was calculated using an average molecular weight of 10 kDa determined in this study, and incubated at 37°C in a 5% CO_2 atmosphere for 72 h. Kinetic studies in our laboratory and others have shown that optimum time to detect changes in lipid accumulation is 72 h [21, 22].

2.5.2 3T3-L1 adipocytes viability assay

For the cell viability assay, 3T3-L1 preadipocytes were seeded (1.5×10^4 cells/cm^2) in 96-well flat-bottom cell culture plates, differentiated and further treated as indicated above. The CellTiter 96® AQueous One Solution was used to determine the number of viable cells according to the manufacturer's manual (Promega, Madison, WI, USA). Cell viability was calculated using the following equation:

$$\% \text{ cell viability} = A_{\text{treatment 515 nm}}/A_{\text{control 515 nm}} \times 100 \quad (1)$$

2.5.3 Lipid quantification in 3T3-L1 adipocytes by Oil Red O assay

Treated adipocytes were washed with Dulbecco's PBS (DPBS) and fixed with 10% formalin (in DPBS) in six-well plates for 1 h. Then, cells were washed with 60% isopropanol and let air dried. The Oil Red O stock solution (6:4 v/v with water) was added to lipid droplets for 10 min. After Oil Red O lipid staining, cells were washed with water four times and were air dried. Oil Red O dye was eluted by adding 100% isopropanol after 10 min incubation at room temperature. Absorbance at 510 nm of eluted isopropanol was measured using a spectrophotometer. Inhibition of lipid accumulation in adipocytes was calculated using the following equation:

$$\% \text{ inhibition of lipid accumulation} = (A_{\text{control, 510 nm}} - A_{\text{treatment, 510 nm}})/A_{\text{control, 510 nm}} \times 100 \quad (2)$$

2.5.4 RT-PCR analysis

Total RNA from 3T3-L1 adipocytes was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacture's instructions. RNA quality and concentration were determined by agarose gel electrophoresis and spectrophotometry, respectively. RNA expression of target genes was measured using a real-time quantitative PCR method with SYBR Green fluorescence dye (Applied Biosystems, Foster City, CA, USA). Briefly, 2 μg purified RNA were reverse transcribed into complementary DNA. Published primer sequences were used for LPL [23] and FAS [24]. The following primers sequences were used:

LPL: forward 5′-CTGCTGGCGTAGCAGGAAGT-3′

reverse 5′-GCTGGAAAGTGCCTCCATTG-3′

FAS: forward 5′-TCGGCGAGTCTATGCCACTATT-3′

reverse 5′-ACAGAGAACGGATGAGTTGTTCCT-3′

18S: forward 5′-GATCCATTGGAGGGCAAGTCT-3′

reverse 5′-AACTGCAGCAACTTTAATATACGCTATT-3′

All primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA). Real-time PCR was performed using an ABI Prism 7700 sequence detection system (Applied Biosystems). Amplification efficiency of each primer set was verified before sample analysis. The mRNA abundance relative to 18 S rRNA was determined using comparative critical threshold method according to manufacturer's instructions.

2.6 Effect of SPH on inflammatory markers

2.6.1 Cell culture and cell proliferation assay

Macrophage cell line RAW 264.7 was cultured in growth medium containing DMEM, 1% penicillin/streptomycin, 1% sodium pyruvate, and 10% FBS at 37°C in 5% CO_2/95% air. The cell proliferation assay was conducted using the CellTiter 96 Aqueous One Solution Proliferation assay kit using the novel tetrazolium compound,3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), and an electron coupling reagent, phenazine ethosulfate (PES) (Promega Corporation, Madison, WI, USA). Briefly 5×10^4 cells were seeded in a 96-well plate and the total volume was adjusted to 200 μL with growth medium. The cells were allowed to grow for 24 h at 37°C in 5% CO_2/95% air. After 24 h incubation, they were treated with different concentrations of SAH ranging from 0 to 25 μM for 24 h. After 24 h treatment, the growth medium was replaced by 100 μL fresh growth medium and 20 μL MTS/PES was added to each well. The plate was incubated for 2 h at 37°C and the absorbance read at 515 nm. The percentage of viable cells was calculated with respect to cells treated with PBS as in Eq. (1).

2.6.2 Western blot analysis

COX-2 and iNOS expression were determined in cell lysates. Briefly, treated cells were washed with ice cold DMEM and ice cold PBS before treatment with 200 μL Laemmli buffer (BioRad Laboratories) with 5% β-mercaptoethanol lysing buffer. After lysis, the cell lysates were boiled for 5 min and ~25 μg protein were loaded in 4–20% Tris-HCl ready gels (BioRad Laboratories) for protein separation. The separated proteins were transferred to PVDF membranes and blocked with 5% non-fat dry milk in 0.1% Tris-buffered saline Tween 20 (TBST) for 1 h at 4°C. After blocking, the membrane was washed with 0.1% TBST (five times, 5 min each) and incubated with either COX-2 or iNOS mouse monoclonal antibodies (1:1000) at 4°C overnight. The membrane was washed again and incubated with antimouse IgG horseradish peroxidase conjugate secondary antibody for 3–4 h at room temperature. After incubation and repeated washing, the expression of COX-2 and iNOS was visualized using chemiluminescent reagent (GE Healthcare) following manufacturer's instructions. The membrane picture was taken with a Kodak Image station 440 CF (Eastman Kodak Company, New Haven, CT, USA).

2.6.3 Measurement of NO and PGE_2 production

Approximately 2×10^5 cells were seeded in a six-well plate and allowed to grow to its 80–90% confluency. The cells were treated with 1 μg/mL LPS with or without different concentrations of SPH dissolved in PBS ranging from 5 to 25 μM for 24 h. After 24 h treatment, the spent medium was collected and analyzed for NO and PGE_2. Nitrite accumulation, and indicator of NO synthesis, was measured in the culture medium by Griess reaction [25]. Briefly, 100 μL of cell culture medium were plated in 96-well plate and an equal amount of Griess reagent constituted by 1% w/v sulfanilamide and 0.1% w/v *N*-1-(naphthyl)ethylenediamine-diHCl in 2.5% v/v H_3PO_4, was added. The plate was incubated for 5 min and the absorbance measured at 550 nm. The amount of NO was calculated using a sodium nitrite standard curve ($y = 0.14 + 0.04$, $R^2 = 0.99$). For PGE_2 measurement, PGE_2 ELISA kit monoclonal was used following manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). The amount of PGE_2 was calculated using PGE_2 standard curve ($y = -37.29x + 108.45$, $R^2 = 0.98$).

2.7 Statistical analysis

Data were expressed as means of at least two independent replicates. Results were compared by one-way analysis of variance (ANOVA) using the GLM procedure of SAS (SAS Institute, Cary, NC, USA). Group means were considered to be significantly different at $p < 0.05$, as determined by the technique of protective least-significant differences (LSD) when ANOVA indicated an overall significant treatment effect, $p < 0.05$.

3 Results and discussion

3.1 Protein profile of soybean genotypes and characterization of SPH

The total protein concentration of soy flours derived from genotypes A1–D2 ranged from 45.3 to 50.6%. Table 1 shows the total protein content and the percent contribution of each identified protein to total proteins of defatted soy flour from the different genotype. Considerable variation among soy genotypes was observed for α′ subunit (8.1–20.7% of total protein), α subunit (10.4–20.8% of total protein), and β subunit (4.9–11.3% of total protein) of BC. Similarly, the protein percentage distribution of A3 chain (0.0–5.6% of total protein), A1,2,4 chains (0.0–15.3% of total protein), and basic (0.0–17.5% of total protein) glyci-

Table 1. Total protein content (%, dry basis) and percent contribution of identified proteins to total proteins of defatted soybean flours derived from different genotypes (A1 – D2)

Proteins identified	A1	A2	B1	B2	C1	C2	D1	D2	LSD ($p < 0.05$)
Total protein (%)	49.4	49.0	46.5	46.4	47.8	50.6	45.7	45.3	
				% of total protein					
α′ subunit of BC	8.2^{d}	8.1^{d}	14.1^{c}	13.5^{c}	18.0^{ab}	17.7^{b}	20.7^{a}	20.1^{ab}	2.69
α subunit of BC	11.1^{cd}	10.4^{d}	18.6^{ab}	17.7^{b}	20.8^{a}	20.0^{ab}	13.7^{c}	13.1^{cd}	2.99
β subunit of BC	5.2^{d}	4.9^{d}	8.3^{cd}	11.3^{a}	7.7^{c}	9.2^{b}	8.6^{bc}	7.6^{c}	1.44
Glycinin A3 chain	3.1^{b}	3.1^{b}	5.6^{a}	5.5^{a}	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{c}	0.72
Glycinin A1,2,4 chains	15.3^{a}	14.6^{a}	2.1^{c}	1.8^{c}	2.6^{bc}	3.0^{b}	0.0^{d}	0.0^{d}	0.84
Glycinin basic chains	17.2^{a}	17.5^{a}	7.0^{b}	6.5^{b}	3.5^{c}	3.0^{c}	0.0^{d}	0.0^{d}	2.43
Kunitz trypsin inhibitor	5.5^{b}	5.1^{b}	7.1^{a}	5.9^{b}	5.9^{b}	5.6^{b}	7.5^{a}	7.5^{a}	1.14
Total BC	24.5^{c}	23.4^{c}	40.9^{b}	42.5^{ab}	46.5^{a}	46.9^{a}	43.0^{ab}	40.8^{b}	4.64
Total Glycinin	35.7^{a}	35.3^{a}	14.6^{b}	13.7^{b}	6.1^{c}	6.0^{c}	0.0^{d}	0.0^{d}	3.01

Means with different superscript letters in the same row are significantly different ($p < 0.0001$).

nin subunits varied among soy genotypes. D1 and D2 were completely lacking glycinin subunits. The total BC and total glycinin average content of the eight flours from the different genotypes ranged from 23.4 to 46.9% of total protein and from 0.0 to 35.7% of total protein, respectively. ANOVA analysis divided the soybean flours into two groups, a 43% BC group (B1, B2, C1, C2, D1, D2 with average BC 43.43 ± 2.66% of total extracted protein), and a 24% BC group (A1 and A2 with average BC 23.95 ± 0.78% of total extracted protein). The 43% BC group was a result of a selective breeding program for modified levels of glycinins and BCs, including some lines lacking glycinins (D1 and D2). These changes in the protein profile of soybeans may improve the benefits of ingredients made from them because peptides derived from BC have been proposed to help maintain healthy tissues [2, 3, 18]. The difference in protein profiles of each soy genotype shown in the present work may provide different relative amounts of soy bioactive peptides after enzymatic hydrolysis and, as a result, variations in biological activities may occur.

Based on our previous studies [26], we have shown that SAH is a mixture of hydrolyzed peptides with a strong band at 14 kDa. Also, SGIH contained peptide fragments with a molecular mass < 3 kDa. This is in agreement with previous findings [27].

The presence of other soy bioactive compounds such as isoflavones and saponins in SAH and SGIH was also studied. Regarding SAH, isoflavone, and saponin concentrations ranged between 200 – 750 μM and 8 – 300 μM, respectively. Moreover, aglycone forms of isoflavones, genistein, glycitein, and daidzein were not detected in SAH. Regarding SGIH, a wide variation from 94 to 157 μM and from 24 to 40 μM on isoflavone and saponin concentrations was also observed, respectively; genistein and glycitein were not detected while daidzein was found at a range from 3.78 to 8.3 μM. Isoflavones and saponins losses occurred during the preparation of soy protein hydrolysates. Centrifugation and the final ultrafiltration step using 1000 Da of MWCO membranes, led to significant reductions in the concentration of these compounds.

3.2 Effect of SPH on lipid accumulation in 3T3-L1 adipocytes

Table 2 presents the percentage of inhibition of lipid accumulation in 3T3-L1 adipocytes after 72 h of treatment with 100 μM SAH or SGIH (A1 – D2) compared to their controls. Treatment of 100 μM SAH decreased lipid accumulation in 3T3-L1 adipocytes from 29 to 37% compared to negative control (untreated cells). Furthermore, SAH derived from soybean genotypes with an average of 45.5% BC (C1, C2, D1) showed a significant higher inhibitory effect compared to SAH derived from 24% BC (A1 and A2) ($p < 0.05$). To confirm this finding a linear correlation analysis was performed between total BC concentration and inhibition on lipid accumulation. Higher total BC soy varieties correlated ($R^2 = 0.85$) with higher percentage of inhibition on lipid accumulation (Table 2). SGIH (100 μM) inhibited lipid accumulation from 7.6% to 14.7% in 3T3-L1 adipocytes after 72 h compared to negative control (Table 2), about one-third of the inhibition of SAH. Similarly to SAH, soybean genotypes richer in total BC correlated ($R^2 = 0.70$) with higher inhibition of lipid accumulation (Table 2). This correlation for SGIH samples was improved when the comparison was made with the amount of the α subunit of BC in the genotypes ($R^2 = 0.91$).

Several *in vivo* studies focused in the role of BC on lipid metabolism. A randomized double-blind placebo-controlled study with 138 volunteers aged 26 – 69 years showed that the intake of 5 g BC *per* day decreased visceral fat [28]. Moreover, mice and rats that were fed BC down regulated FAS, increased the activities of β-oxidation enzymes such as carnitine palmitoyl transferase and acyl-coA oxidase in the liver, and increased fecal excretion of TG [29, 30].

Table 2. Inhibitory effect of SAH (100 μM) and SGIH (100 μM) on lipid accumulation relative to negative control (untreated cells) in 3T3-L1 adipocytes after 72 h

Soybean genotypes	BC (% total protein)	Lipid accumulation (% inhibition)	
		SAH	SGIH
A1	24.5 ± 0.6^{c}	29.2 ± 3.0^{c}	7.6 ± 2.1^{c}
A2	23.4 ± 0.3^{c}	29.6 ± 1.0^{c}	7.9 ± 2.3^{c}
B1	40.9 ± 0.0^{b}	32.9 ± 1.9^{abc}	11.7 ± 2.1^{abc}
B2	42.5 ± 0.5^{ab}	32.9 ± 2.7^{abc}	11.2 ± 0.4^{abc}
C1	46.5 ± 0.0^{a}	37.0 ± 1.7^{a}	13.3 ± 2.9^{ab}
C2	46.9 ± 1.4^{a}	34.7 ± 2.4^{a}	14.7 ± 0.4^{a}
D1	43.0 ± 0.1^{ab}	34.2 ± 1.5^{ab}	9.6 ± 2.1^{abc}
D2	40.8 ± 0.7^{b}	32.8 ± 2.9^{abc}	9.5 ± 1.6^{bc}
Linear correlation equation		$y = 0.26x + 23.05$	$y = 0.22x + 2.01$
R^2 coefficient		0.85	0.70

Means with different superscript letters in the same column are significantly different ($p < 0.0001$). Linear correlations were carried out between total BC concentration (% total protein) in soybean flours derived from different genotypes and inhibition values on lipid accumulation in adipocytes of SPH.

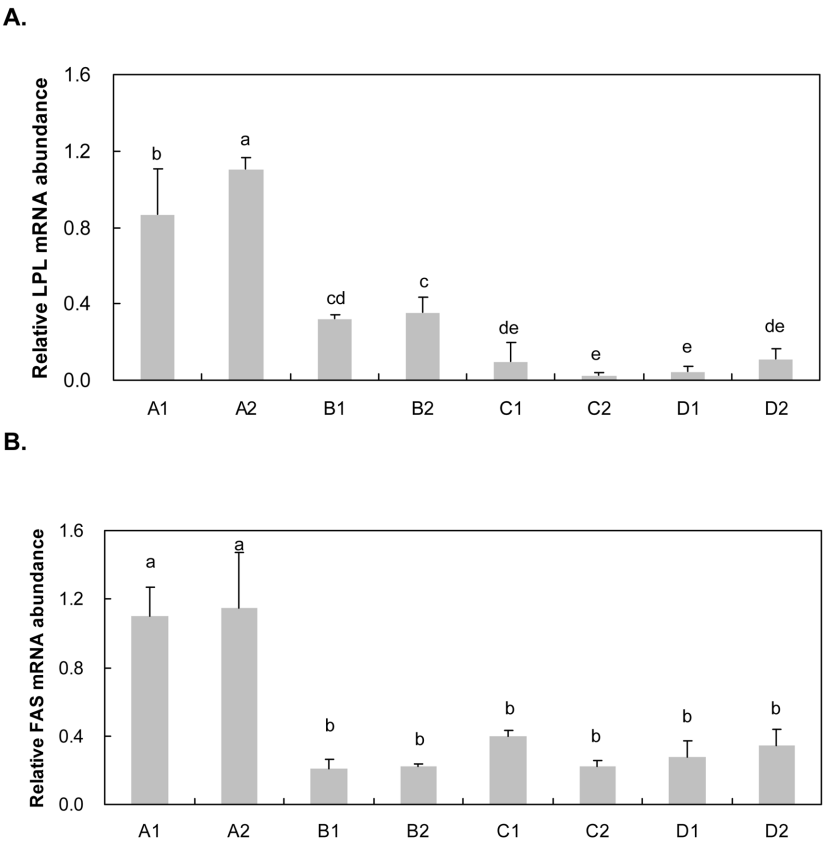

Figure 1. Effect of SAH (100 μM) on LPL (A) and FAS (B) mRNA abundance relative to negative control (untreated cells) in 3T3-L1 adipocytes after 72 h. Means with different letters are significantly different ($p < 0.0001$, $n = 3$ for LPL and FAS mRNA abundance). Bars indicate SD.

3.3 Effect of SPH on LPL and FAS gene expression

The present study showed that SAH and SGIH exerted an inhibitory effect on lipid accumulation in 3T3-L1 adipocytes. This suggested that soy hydrolysates may affect lipid metabolism in 3T3-L1 adipocytes. Therefore, the effect of SPH on gene expression of enzymes involved in lipid uptake by adipocytes (LPL and FAS) and *de novo* fatty acid synthesis were studied.

Figure 1 shows the effect of SAH on LPL (A) and FAS (B) gene expression in 3T3-L1 adipocytes. SAH derived from 43% BC group showed a marked decrease of LPL and FAS mRNA abundance relative to the negative control (values < 1); however, SAH from 24% BC did not affect LPL and FAS gene expression compared to negative control and LPL and FAS mRNA abundance was significantly higher compared to SAH from 43% BC group ($p <$

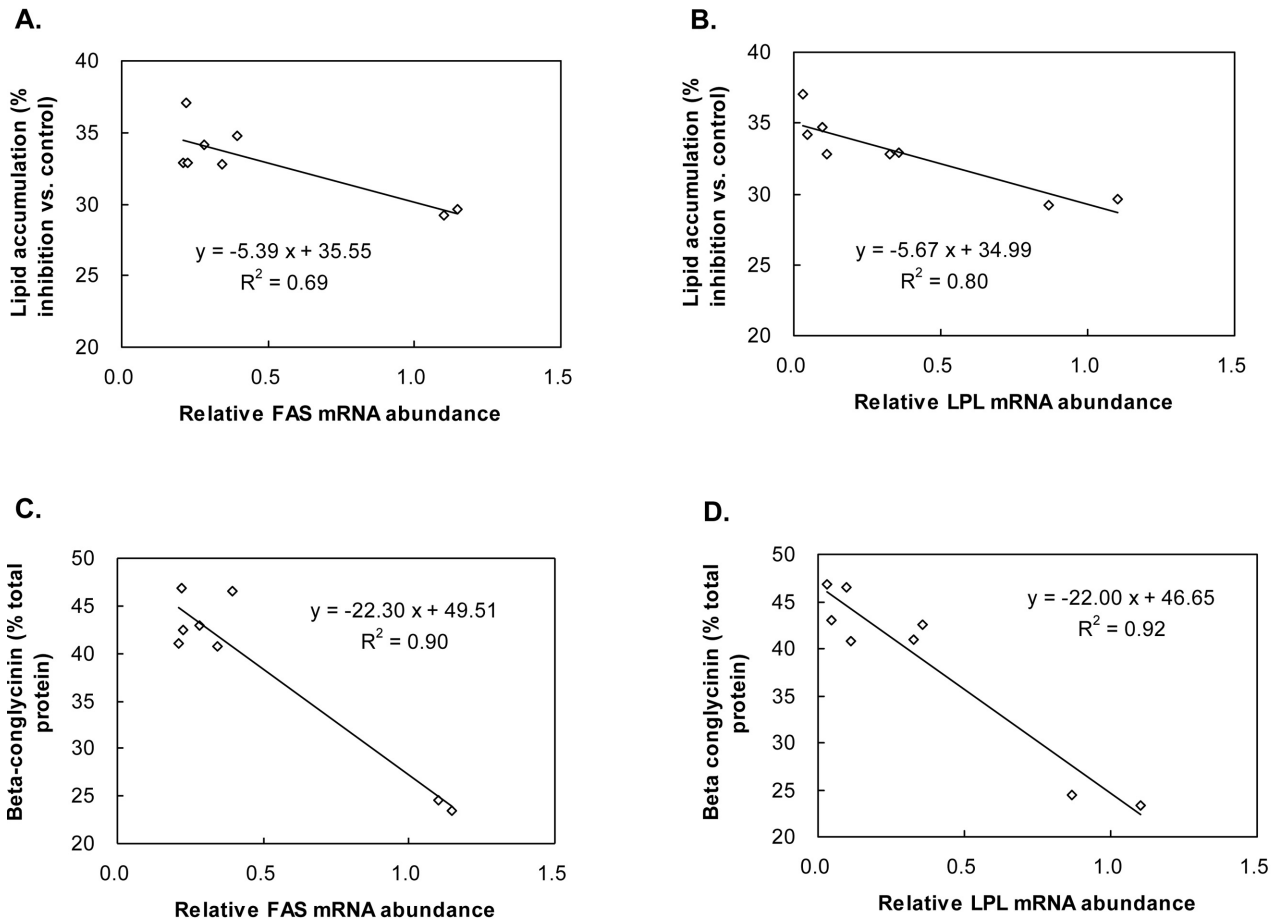

Figure 2. Single linear correlations of (A) lipid accumulation inhibition with FAS mRNA expression (B) lipid accumulation inhibition with LPL mRNA expression (C) BC (% total protein) with FAS mRNA expression and (D) BC (% total protein) with LPL mRNA expression of SAH.

0.0001). Inhibitory effect of alcalase SAH on lipid accumulation was negatively correlated with FAS ($R^2 = -0.69$) and LPL ($R^2 = -0.80$) mRNA relative abundance (Figs. 2A and B). Moreover, BC concentration in soybean lines used to produce SAH was negatively correlated to FAS ($R^2 = -0.90$) and LPL ($R^2 = -0.92$) mRNA relative abundance (Figs. 2C and D). This indicated that the higher BC concentration in soybean lines, the higher the downregulation of FAS and LPL gene expression in 3T3-L1 adipocytes will be exerted by SAH which is consistent with previous results of inhibitory effect of SAH on lipid accumulation showed in Table 2.

Figure 3 shows the effect of SGIH on LPL (A) and FAS (B) gene expression in 3T3-L1 adipocytes. SGIH decreased LPL mRNA abundance compared to the negative control (values < 1) and no significant differences were observed between SPH from 43 and 24% BC groups. In contrast, SGIH did not change FAS mRNA abundance compared to negative control (values ≥ 1) and 24% BC group was not statistically different from 43% BC group ($p > 0.05$). Inhibitory effect of SGIH on lipid accumulation was weakly correlated to LPL ($R^2 = -0.3$) mRNA relative abundance (data not shown). Furthermore, BC concentrations in soybean lines were also weakly correlated to LPL ($R^2 = -0.5$) mRNA relative abundance (data not shown). This suggested that SGIH may inhibit lipid accumulation by inhibiting LPL gene expression.

GI digestion of bioactive peptides may modify their activity as consequence of a continued proteolytic hydrolysis by GI enzymes [2]. Therefore, a further step in the present study was to study the bioactivity of SAH from 43% BC group after simulation of GI digestion. Figure 4 shows the effect of SAH followed by simulated GI digestion and SGIH on FAS (A) and LPL (B) gene expression in 3T3-L1 adipocytes. Results showed that SAH retained their biological activity related to down regulation of FAS ($p < 0.0001$) and LPL ($p = 0.0108$) gene expression in 3T3-L1 adipocytes after simulated GI digestion. Moreover, a significantly higher inhibitory effect of SAH compared to SGIH on FAS ($p < 0.0001$) and LPL ($p = 0.0108$) gene expression in 3T3-L1 adipocytes was observed.

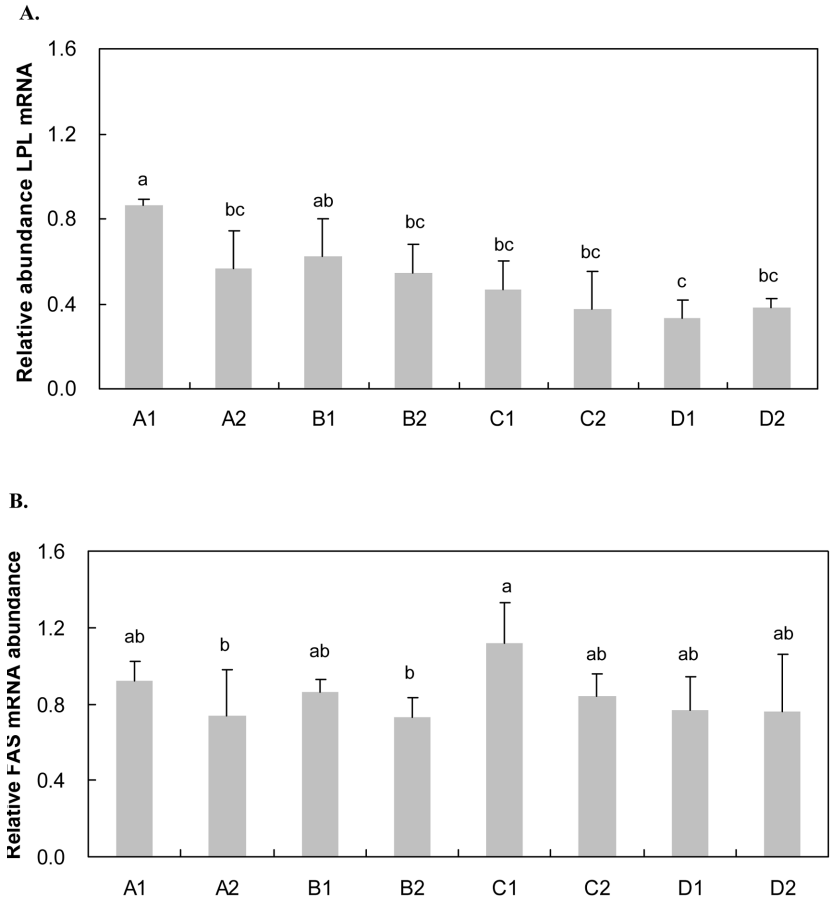

Figure 3. Effect of SGIH (100 μM) on LPL (A) and FAS (B) mRNA abundance relative to negative control (untreated cells) in 3T3-L1 adipocytes after 72 h. Means with different letters are significantly different ($p < 0.05$, $n = 3$ for LPL mRNA abundance and $p > 0.05$, $n = 3$ for FAS mRNA abundance). Bars indicate SD.

Several studies in cell lines and animals have reported the potential effect of soy peptides to inhibit lipid absorption and regulate lipid metabolism [31–33]. In the present study, we demonstrated that SAH (100 μM) from soybean lines enriched in BC exerted an inhibitory effect on lipid accumulation by down regulating LPL and FAS gene expression in 3T3-L1 adipocytes. LPL is produced mainly in adipocytes and transported to the luminal surface of the capillary where it exerts its activity [23]. Chylomicrons and very low-density lipoproteins activate LPL through C-II apoprotein. Free fatty acids produced by the action of LPL on these lipoproteins are, then, taken up by adipocytes through a concentration-dependent mechanism involving a transmembrane transport protein. *In vitro* and *in vivo* studies have demonstrated that downregulation of LPL expression or/and suppression of LPL activity, are mechanisms that may cause fat mass-loss, preventing free fatty acids from entering adipocytes [7]. This activity has been reported for bioactive compounds such as conjugated linoleic acid *in vitro* and *in vivo*, however, to our knowledge this is the first evidence that soy hydrolysates inhibit LPL gene expression or activity.

Inside the adipocytes, free fatty acids are re-esterified to triglyceride and, in part, β-oxidized [7]. FAS catalyzes *de novo* synthesis of fatty acids from acetyl-CoA and malonyl-CoA in the presence of NADPH through the reaction, which elongates the acetyl group by C2 units that are derived from malonyl-CoA in a stepwise and sequential manner [34]. Moreover, the amino acid sequence of human FAS has 79% identity with that of rodents [34]. Increased expression and activity of FAS in adipose tissue may contribute to the development of obesity [35]. As a central enzyme in lipogenesis, the gene encoding FAS has been identified as a candidate gene for determining body fat in humans. Increased FAS expression associated with hyperinsulinemia, dyslipidemia, and altered adipokine profile may explain the resulting accumulation of body fat [35]. Profound weight loss and reduced food intake were induced in rodents by inhibiting FAS, suggesting that FAS may regulate feeding behavior and energy homeostasis [36–39]. Soy protein consumption reduced adiposity *in vivo* by reducing the expression of hepatic sterol regulatory element binding proteins (SREBPs), and its target genes such as FAS [40].

3.4 Effect of alcalase SPH on inflammatory responses

Soybean lines lacking glycinin subunits (D1–D2) were selected as raw material to study the effect of SPH as potential anti-inflammatory agents. An examination of the cyto-

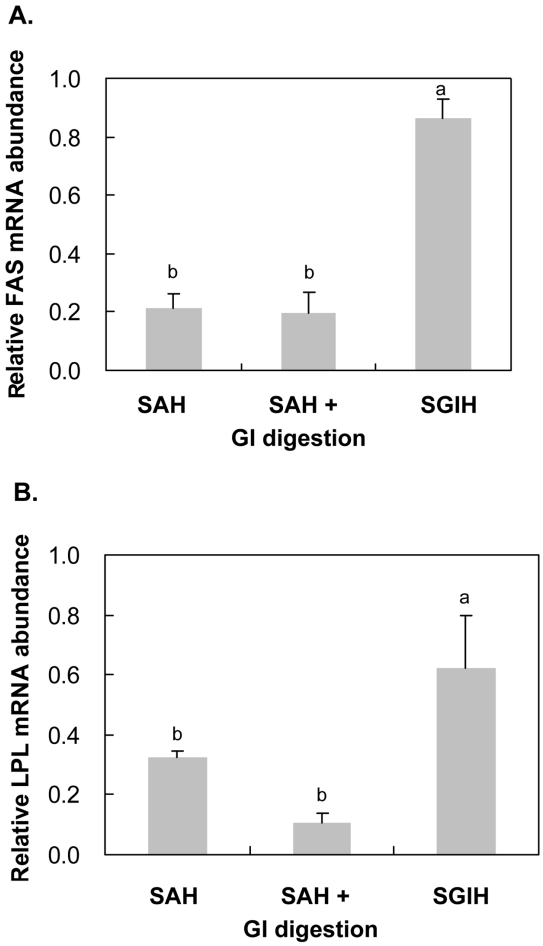

Figure 4. Effect of SAH (100 μM), SAH followed by simulated GI digestion and SGIH on FAS (A) and LPL (B) gene expression in 3T3-L1 adipocytes after 72 h. Means with different letters are significantly different ($p < 0.0001$, $n = 3$ for FAS gene expression and $p = 0.0106$, $n = 3$ for LPL gene expression). Bars indicate SD.

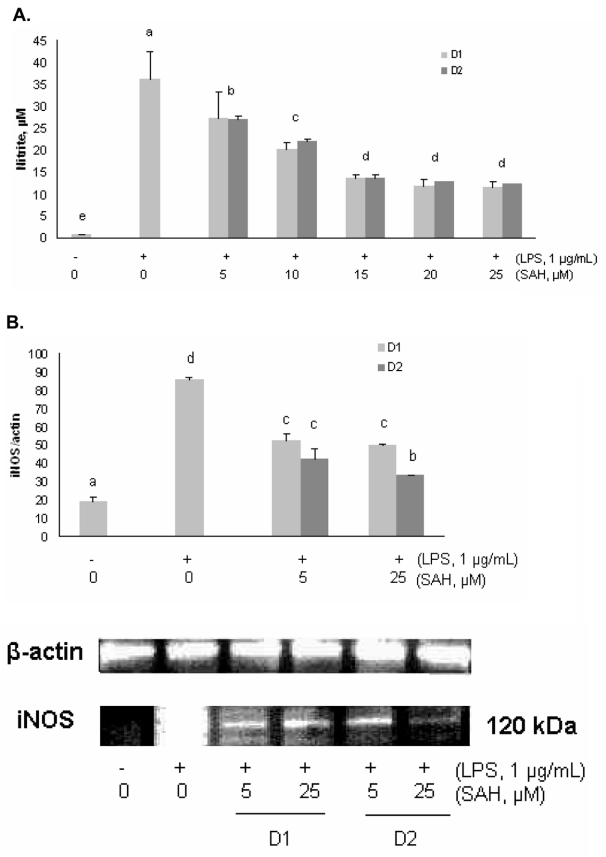

Figure 5. Effect of different concentrations of SAH (D1 and D2) in nitrite production (A) and iNOS protein expression by Western blot (B) in LPS-stimulated RAW 264.7 macrophages. Means with different letters are significantly different from the positive control ($p < 0.0001$, $n = 3$ for nitrite production; and $p < 0.0001$, $n = 2$ for iNOS expression). Bars indicate SD.

toxicity of SAH in RAW 264.7 macrophages by the MTT assay indicated that even the highest concentration tested (25 μM) did not affect the viability of the cells (data not shown). It was also found that SAH of D1 and D2 can inhibit production of the pro-inflammatory responses at concentrations as low as 5 μM. The effect of SAH on NO production was determined by measuring the level of nitrite accumulation (the stable metabolite of NO) in culture media. LPS (1 μg/mL) induced significant nitrite production as compared with the negative control ($p < 0.0001$). This was inhibited by SAH treatment in a dose dependent manner ($p < 0.0001$) (Fig. 5A). Since, the production of NO is controlled by iNOS, we studied the expression of iNOS protein in SAH-treated RAW 264.7 cells using Western blot. Figure 5B shows the expression of iNOS as affected by different concentrations of SAH. As shown in Western blot, the expression of the iNOS protein was barely detected in the non-stimulated cells. However, the level increased markedly 24 h after the LPS treatment. SAH exerted a significant inhibition of iNOS protein expression in the LPS-stimulated RAW 264.7 macrophages ($p < 0.0001$). Higher concentrations of SAH D2 (25 μM) resulted in a more marked inhibition of iNOS ($p < 0.0001$).

To further understand the role of SAH in inflammation, we also measured the production of PGE_2 and expression of COX-2 on LPS-stimulated RAW 264.7 cells. COX-2 is induced by a wide variety of stimuli such as LPS, certain serum factors, cytokines, and growth inhibitors and is expressed predominantly at the sites of inflammation [34]. Therefore, there is increasing interest in the use of COX-2 inhibitors for treating inflammatory diseases among which obesity is included.

Figures 6A and B show the effect of different concentrations of SAH on PGE_2 production and COX-2 expression. Western blot shows that the expression of COX-2 protein was significantly lower in the nonstimulated cells compared to LPS-stimulated RAW 264.7 macrophages 24 h after LPS treatment. SAH effectively suppressed PGE_2 production

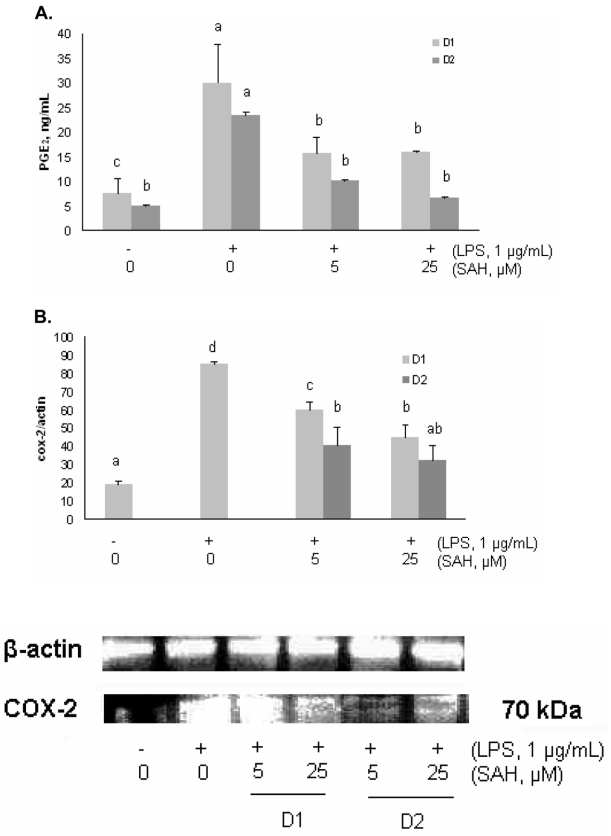

Figure 6. Effect of different concentrations of SAH (D1 and D2) in PGE_2 production (A) and COX-2 protein expression by Western blot (B) in LPS-stimulated RAW 264.7 macrophages. Means with different letters are significantly different from the positive control ($p < 0.0001$, $n = 3$ for PGE_2 production, and $p = 0.0001$, $n = 2$ for COX-2 protein expression. Bars indicate SD.

($p < 0.0001$) and COX-2 protein expression ($p < 0.0001$) at 5 and 25 μM (Figs. 6A and B) in comparison with the positive control.

Treatment of macrophages with genotypes A2, B1, and C1 alcalase hydrolysates showed also a significant reduction on the production of NO and PGE_2 and expression of iNOS and COX-2 when compared to the positive control. NO production was inhibited from 18.1 ± 0.9 to $35.7 \pm 2.5\%$ while PGE_2 production was inhibited from 56.1 ± 6.2 to $71.3 \pm 0.9\%$. This reduction was also accompanied by reduction of the inducible forms of iNOS (31.2 ± 0.6 to $53.5 \pm 2.6\%$) and cyclooxygenase (35.7 ± 18.7 to $49.8 \pm 16.4\%$). Furthermore, at 5 μM no significant differences on the anti-inflammatory activities were observed when compared to hydrolysates from D1 and D2. These results indicated that low concentration of hydrolysates were not capable of differentiating among the genotypes tested with respect to their potential anti-inflammatory activity. The use of higher concentrations of the hydrolysates is recommended to further characterize the anti-inflammatory potential of these soybean samples.

Inflammation is a part of the normal host response to infection and injury. However, excessive or aberrant inflammation contributes to a range of acute and chronic human diseases and is characterized by the production of inflammatory cytokines, arachidonic acid-derived eicosanoids, reactive oxygen species, and adhesion molecules [38]. Several evidences from *in vitro*, animal and human studies have shown a potential anti-inflammatory effect of soybean attributed to several of its bioactive compounds including isoflavones [41 – 43], saponins [44], and lunasin [45]. This investigation observed that soy hydrolysates have potential to inhibit inflammation. Food-derived peptides may be absorbed directly from the intestine by peptide-specific transport systems [46] and act as regulatory compounds with hormone-like activities [47]. Therefore, it is thought that in the intestine, the peptide transporter constitutes a major mechanism for absorption of the products of protein digestion [48]. The anti-inflammatory effect of several human endogenous peptides has been investigated. Kim *et al*. [49] have shown that the human prothrombin fragment-2-derived peptide, NSA9, can inhibit inflammation in LPS-activated microglia. They observed that at a concentration as low as 20 μM of NSA9, pro-inflammatory responses like NO, PGE_2, and COX-2 and iNOS mRNA expressions were significantly reduced. They suggested that NSA9 can be a powerful therapeutic candidate for neurodegenerative disease caused by microglial activation. Also, a vasoactive intestinal peptide, at a concentration of 10^{-8} M, reduced COX-2 expression and PGE_2 production by macrophages, dendritic cells, and microglia activated with different inflammatory stimuli [50].

The level of PGE2 was measured, and not PGD2, as this prostanoid correlates better to aberrant inflammation and consequently to certain types of chronic diseases such as colon cancer. PGE2 can induce the growth, migration and invasiveness of colorectal carcinoma cells [51, 52]; also COX-2/PGE2 may exert pro-oncogenic effects [53]. In our study, we demonstrated that hydrolysates from soy protein can reduce the production of PGE2 in LPS-induced macrophage which is more related to diseases associated with chronic inflammation.

4 Concluding remarks

In conclusion, protein hydrolysates derived from soybeans containing in average 43% of the total protein as BC, markedly inhibited lipid accumulation in adipocytes and inflammation indicators, *in vitro*. The bioactivities of soy hydrolysates depend on the peptides profile (amino acid sequence and concentration) as influenced by the protein profile of soybeans and enzymes involved in the hydrolysis. SAH from soybean enriched in BC decreased markedly lipid

accumulation in 3T3-L1 adipocytes, down regulating gene expression of enzymes involved in lipid uptake by adipocytes (LPL) and FAS. Furthermore, GI digestion of these hydrolysates did not cause a loss of bioactivity. SGIH inhibited lipid accumulation in a lesser extent by inhibiting LPL gene expression in 3T3-L1 adipocytes.

In addition, SAH of soybean genotypes significantly inhibited expression of LPS-induced iNOS/NO and COX-2/PGE_2 pathways in RAW 264.7 macrophages.

Soybeans enriched in BCs can provide hydrolysates that limit fat accumulation in fat cells and inflammatory pathways in macrophages *in vitro* and therefore warrant further studies as a healthful food.

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Conflict of interest statement: Neal A. Bringe is an employee at Monsanto Co., St. Louis, MO, USA. E. Gonzalez de Mejia has received grant support from Monsanto Co., St. Louis, MO, USA to conduct this study.

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