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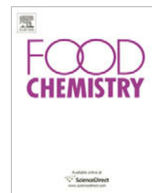

Fatty acid synthase and *in vitro* adipogenic response of human adipocytes inhibited by α and α′ subunits of soybean β-conglycinin hydrolysates

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ABSTRACT

The objective was to assess the effect of protein hydrolysates of β-conglycinin enriched soybean on fatty acid synthase (FAS) activity and adipogenic response of human adipocytes *in vitro*. The results showed that genotypic changes in soybean protein subunits produced peptide profiles that led to inhibition of FAS and lipid accumulation *in vitro*. FAS inhibitory potency (IC_{50}) of soy protein hydrolysates (SPH) ranged from 50 to 175 μM, while lipid inhibition from 15.6% to 45.9%. Protein hydrolysate C2H from a soybean containing the highest total β-conglycinin (46.9%) showed the most potent inhibitory effect on *in vitro* adipogenesis (46%) and FAS (IC_{50} = 50 μM). C2H was composed of dominant peptides from fragments *f*(85–112) and *f*(131–132) of β-conglycinin α subunit. Smaller peptides identified as fragments *f*(330–342) and *f*(329–342) of α′ subunit were also found. In conclusion, soybean genotypes enriched in β-conglycinin α and α′ subunits are suitable sources of active peptides that inhibit FAS activity and lipid accumulation.

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1. Introduction

Accumulation of body fat arises from a chronic imbalance between energy acquisition and expenditure that may lead to a pathologic growth of adipocytes, characterised by increased fat cell size and number (Shimomura, Hammer, Richardson, Ikemoto, & Bashmakov, 1998). The amount of adipose tissue can be regulated by the inhibition of adipogenesis and fat deposition (Rahman et al., 2008). Fat deposition in adipose tissue can be reduced by reducing lipid synthesis through inhibiting fatty acid synthase (FAS) (Rahman et al., 2008). FAS (EC 3.2.1.85) is a key enzyme for lipogenesis that catalyses the *de novo* synthesis of long chain fatty acids from acetyl-CoA and malonyl-CoA through a NADPH-dependent cyclic reaction (Maier, Leibundgut, & Ban, 2008). Pharmacological inhibition of FAS markedly reduced food intake and body weight in mice (Loftus et al., 2000) and prevented preadipocyte differentiation *in vitro* (Schmid, Rippmann, Tadayyon, & Hamilton, 2005). Therefore, FAS is currently considered as a promising molecular target for weight management (Buettner, 2007; Chakravarthy et al., 2007). Particularly, among foods, soy proteins have become attractive for weight management (Cope, Erdman, & Allison, 2007; Velasquez & Bhathena, 2007; Xiao, 2008). The major storage proteins in soybean are β-conglycinin (BC) and glycinin (GL); they represent about 60% of the total proteins (Martinez-Villaluenga, Berhow, Bringe, & Gonzalez de Mejia, 2008). These two proteins differ in their structure, amino acid composition and biological activity. BC appeared to be responsible for the satiety, antilipogenic and hypolipidemic effects of soy protein (Duranti et al., 2004; Kohno, Hirotsuka, Kito, & Matsuzawa, 2006). Reduction in body fat and food intake was significant when soy protein was hydrolysed into bioactive peptides (Aoyama et al., 2000; Nishi, Hara, & Tomita, 2003). Moreover, recent studies demonstrated that BC embeds active peptides that inhibit lipid accumulation in 3T3-L1 adipocytes (Martinez-Villaluenga et al., 2008), induce satiety by increasing cholecystokinin secretion in enteroendocrine cells (Hira, Maekawa, Asano, & Hara, 2009) and alter lipid metabolism to decrease secretion of apoliprotein B-100-containing protein from HepG2 cells (Mochuzuki et al., 2009).

Soybean lines with different protein composition have become available and have been studied to improve soybean quality (Mahmoud et al., 2006; Tezuka, Taira, Igarashi, Yagasaki, & Ono, 2000). The selection of soybean genotypes with specific protein composition and improved biological activities is a promising approach for the development of food products for health promotion and well being. The objective of the present research was to assess the effect of protein hydrolysates of β-conglycinin enriched soybean on fatty acid synthase (FAS) activity and adipogenic response by *in vitro* differentiated human adipocytes.

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2. Materials and methods

2.1. Materials

Four defatted soybean flour samples (A2–D2) were provided by the Monsanto Company (St. Louis, MO). They were derived from distinct soy genotypes that by selective breeding were designed to have different protein profiles, particularly in BC and GL profiles. Alcalase from *Bacillus licheniformis* (E.C. 3.4.21.62) and C75 (4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid) were purchased from Sigma–Aldrich (St. Louis, MO, USA). BC and GL were purified in our laboratory as described previously (Wang, Bringe, Berhow, & Gonzalez de Mejia, 2008). Isolation, purification and storage of FAS from chicken liver were performed as described previously (Tian, Hsu, & Wang, 1985). Human preadipocytes were obtained from Zen Bio (Research Triangle Park, NC, USA). The cells originated from human subcutaneous adipose tissue and were obtained from females (average 40-year old) with a body mass index (BMI) of 27.95 average who where not diabetic and not smokers. Preadipocyte medium PM-1 [DMEM/Ham's F-12 (1:1, v/v) medium containing HEPES buffer, faetal bovine serum, penicillin, streptomycin, amphotericin B], differentiation medium DM2 [DMEM/Ham's F-12 (1:1, v/v) medium containing HEPES buffer, faetal bovine serum, penicillin, streptomycin, amphotericin B, biotin, pantothenate, human insulin, dexamethasone, isobuthylmethylxanthine and PPARγ agonist] and adipocyte medium AM-1 [DMEM/Ham's F-12 (1:1, v/v) medium containing HEPES buffer, faetal bovine serum, penicillin, streptomycin, amphotericin B, biotin, pantothenate, human insulin, dexamethasone, and PPARγ agonist] were from Zen Bio (Research Triangle Park, NC, USA).

2.2. Preparation of soy protein hydrolysates (SPH)

Defatted soybean flour, purified BC and GL were suspended in deionised water (1:20 w/v) and brought to 50 °C at pH 8.0. Then, enzyme hydrolysis was carried out with alcalase (11 U/mg) using a 1:20 (w/v) enzyme/flour ratio. Hydrolysis was carried out for 3 h and the pH was constant maintained by adding 0.5 M NaOH. The 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 by ultra-filtration using 3000 Da MWCO membrane (Millipore) and freeze dried in a FreeZone freeze dry system (Kansas City, MO). The protein DC assay (Bio-rad) was followed for protein quantification using bovine serum albumin as standard ($y = 0.0002x - 0.0021$, $r^2 = 0.997$).

2.3. Sodium dodecyl sulphate–polyacrylamide gel electroforesis (SDS–PAGE)

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 (Bio-rad Laboratories, Hercules, CA, USA) was used as a molecular weight marker. The gels were stained in Colloidal Coomassie Blue G-250, destained in 10% acetic acid, and imaged using the GS 800 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, CA). Protein quantification was performed using Bio-Rad Quantity One Software. The software was used to determine the relative quantity of each band in the sample lane. Proteins were reported as the relative percent of the total protein bands in the lane. The theoretical molecular weight of each protein was calculated from the amino acid sequence with ProtParam program (http://ca.expasy.org/tools/protparam.html). Identification of protein bands of the four soybean genotypes was confirmed by comparing theoretical molecular weights with experimental data.

2.4. Peptide mass mapping by matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF)

Soybean hydrolysates as well as purified BC and GL hydrolysates were analysed by MALDI-TOF using an Applied Biosystems Voyager-DE STR (Foster City, CA, USA) for molecular mass peptide mapping. The following parameters were used in the analysis: linear mode of operation, positive polarity and 500–20,000 m/z scanning range.

2.5. Identification of peptides in hydrolysates by liquid chromatography–tandem mass spectrometry (LC–MS/MS)

Purified BC and GL hydrolysates (2 μg) were dissolved in a solution containing 5% acetonitrile and 0.1% formic acid. Samples were injected (10 μl) onto a dC_{18} Atlantis nanoAcquity column [75 × 150 mm, 3 mm particle size (Waters, Milford, MA)] using 0.1% aqueous formic acid as solvent A and acetonitrile with 0.1% formic acid as solvent B. A linear gradient from 1% to 60% B was run for 60 min and back to 1% B for 10 min with the flow rate maintained at 0.25 ml/min. Mass spectrometric analysis was carried out in a Q-tof API-US nanoAcquity UPLC (Waters) mass spectrometer equipped with an electron spray ion source. The Q-Tof instrument was operated in positive ion mode.

Peptide identification was carried out by searching against the NCBI or SWISS-PROT database [taxonomy = viridiplantae (green plants)] by use of MASCOT. Only peptides identified with a confidence of at least 95% were considered to be correct calls ($p < 0.05$).

2.6. Measurement of FAS activity

Fatty acid synthesis catalysed by FAS was measured by monitoring NADPH oxidation to $NADP^+$ by the decrease in absorbance at 340 nm at 37 °C in a 96-well clear-bottomed polystyrene plate (Corning, NY, USA) using a Biotek® Microplate Reader System equipped with temperature controller (Biotek® Instruments, Winooksi, VA). For each assay, references (blank of substrates and blank of inhibitors), control (no inhibitors) and samples (with inhibitors) were measured simultaneously. The reactions were performed at a final volume of 150 μl containing 3 μM acetyl-CoA, 10 μM malonyl-CoA, 35 μM NADPH in 100 mM potassium phosphate buffer containing 0.5 mM DTT and 1 mM EDTA. Initial rates were calculated from the slope of the progress curves during the first 6 min. The inhibition of FAS activity (%) was calculated from the ratio of the initial velocity in the presence and absence (control) of the inhibitor. The sigmoidal dose–response curves were plotted by non-linear regression using the GraphPad Prism software in order to calculate the concentration needed to inhibit 50% FAS activity (IC_{50}); the higher the potency the lower the IC_{50} value.

2.7. Preadipocyte culture and differentiation

The human preadipocyte culture was performed as reported previously (Halvorsen et al., 2001). For preadipocyte differentiation, cells were seeded in 96-well plates at a density of 3×10^4 cells/cm^2 in PM-1 medium (day 0). After 24 h (day 1) the medium was changed to a differentiation medium (DM-2). After a 3-day induction period (day 4), the cells were fed every 3 days with adipocyte medium (AM-1) for an additional 10 days. By day 13, cells contained large lipid droplets and were considered mature

adipocytes. Cells were incubated at 37 °C in a humidified incubator in a 5% CO_2 atmosphere.

2.8. In vitro inhibition of human preadipocytes differentiation as measured by lipid accumulation

Briefly, on day 1 preadipocytes were fed with DM-1 medium (negative control), DM-1 medium containing soy alcalase hydrolysates (30 μM) or DM-1 medium containing TNFα 40 nM, positive control. The cells were fed every 3 days with adipocyte medium (AM-1) for an additional 10 days. On day 13 cells were washed with phosphate buffered saline (PBS) and lysed with lysis buffer. A lypolytic reagent was added to each well (120 μl/well) and cells were incubated at 37 °C for 2 h. Aliquots of the lysates were diluted with PBS and glycerol content was determined using the triglyceride reagent A (GPO-Trinder, Sigma) following the manufacturer's instructions. Glycerol content was measured spectrometrically at 540 nm using a SpectraMax 250 microplate reader (MDS Analytical Technologies, Sunnyvale, CA). Inhibition (%) of preadipocyte differentiation was calculated using the following equation:

$$[\text{Glycerol } (\mu\text{M})_{\text{treatment}} - \text{Glycerol } (\mu\text{M})_{\text{negative control}}/\text{Glycerol } (\mu\text{M})_{\text{negative control}}] \times 100 = \% \text{ Inhibition of adipogenesis}$$

2.9. 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 PROC GLM procedure of SAS (SAS Institute, Cary, NC). 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

3.1. Characterisation of defatted flour from different soybean genotypes

Table 1 shows the total protein content and the percent contribution of each identified protein, to total protein, in defatted soy flours from different genotypes (A2–D2). The total protein concentration of defatted soy flours ranged from 45.3% to 50.6%, dry basis. Protein profiles showed nine major protein bands corresponding to lipoxygenase isoforms 2 and 3 (92.9 kDa), and 1 (89.1 kDa), BC subunits (α, α′ and β) ranging from 67 to 45 kDa, glycinin subunits (A3 chain and 1, 2, and 4 basic chains) ranging from 38.5 to 20.5 kDa and Kunitz trypsin inhibitor (18.6 kDa). The identities of these protein bands agreed with other publications (Wang et al., 2008). In soybean genotype A2 the major protein was GL while in B2, C2 and D2 the predominant protein was BC.

Lipoxygenases isoforms 2–3 and 1 were found in soy genotypes to have different levels of abundance, ranging from 4.2% to 6.9% of total protein, and 0.0% to 1.8% of total protein, respectively. Regarding BC, considerable variation was observed for the α′ (8.1–20.1% of total protein), α (10.4–20.0% of total protein) and β subunit (4.9–11.3% of total protein) among the soybean genotypes. Soybean genotype A2 had the lowest content of total BC (23.4% of extracted protein) compared to soybean genotypes B2, C2 and D2 containing an average BC of 43.4 ± 3.15% of the extracted protein. Soybean genotypes were bred to have a different ratio of α′, α and β subunits of BC (3:4:3, 4:4:2 and 5:3:2 for B2, C2 and D2, respectively) (Bringe & Jenkinson, 2009). Regarding GL, the protein percentage distribution of the A3 chain (0.0–5.5% of total protein), A1,2,4 chains (0.0–14.6% of total protein) and basic (0.0–17.5% of total protein) varied among soy genotypes. Soy genotype A2 contained 35.3% of extracted protein as total GL while soy genotypes B2, C2 and D2 presented 13.7%, 6.0% and 0.0%, of extracted protein, respectively. The Kunitz trypsin inhibitor was found in soy genotypes to have different levels of abundance, ranging from 5.1% (A2) to 7.5% (D2) of total protein.

3.2. Characterisation of soy alcalase hydrolysates

Table 2 shows the list of peptide masses of protein hydrolysates from different soybean genotypes (A2–D2), purified BC and GL by MALDI-TOF-MS. A comparison of the spectra revealed both similarities and differences between the peptide profiles of hydrolysates from different soybean genotypes. All soy hydrolysates displayed common ions to BCH (e.g. *m/z* 1529, 1794, 2703, 2832, 3225, 3381, 3824, 3897, 5028, 5543, 7514 and 7758). A2H exhibited intense ions at *m/z* 3824 and 3897 and common to BCH. Ion signals at *m/z* 1667, 1793, 1935, 2458, 3382, 3823 and 3896 were dominant in the B2H sample. In addition, A2H and B2H displayed several characteristic ion signals common to GLH (*m/z* 1044, 1306, 1671, and 1970), in agreement with GL content in the different soybean genotypes (Table 1). C2H was characterised by the presence of high intense signals at *m/z* 3382, 3824 and 3896 common to BCH and by the absence of ions common to GLH. Finally, D2H exhibited intense ions at *m/z* 2458, 3382, 3824 and 3897 also present in BCH and, similar to C2H, ions common to GLH were not detected.

To identify the peptide sequences released from BC and GL after hydrolysis, BCH and GLH were further analysed by liquid

Table 1
Total protein and contribution of identified proteins to total proteins of defatted soybean flours derived from different genotypes (A2–D2).[A]

Proteins identified	A2	B2	C2	D2	LSD ($p < 0.05$)
Total protein (% dry basis)	49.0	46.4	50.6	45.3	
	% of total protein				
Lipoxygenases 2 and 3	5.1^{b}	5.2^{b}	4.2^{c}	6.9^{a}	0.66
Lipoxygenase 1	1.6^{b}	0.0^{c}	1.6^{b}	1.8^{a}	0.14
α′ subunit of β-conglycinin	8.1^{c}	13.5^{b}	17.7^{a}	20.1^{a}	3.74
α subunit of β-conglycinin	10.4^{b}	17.7^{a}	20.0^{a}	13.1^{b}	4.18
β subunit of β-conglycinin	4.9^{c}	11.3^{a}	9.2^{ab}	7.6^{b}	2.25
Total β-conglycinin	23.4^{c}	42.5^{ab}	46.9^{a}	40.8^{b}	5.99
Glycinin A3 chain	3.1^{b}	5.5^{a}	0.0^{c}	0.0^{c}	1.23
Glycinin A1,2,4 chains	14.6^{a}	1.8^{c}	3.0^{b}	0.0^{d}	0.63
Glycinin basic chains	17.5^{a}	6.5^{b}	3.0^{c}	0.0^{d}	2.37
Total glycinin	35.3^{a}	13.7^{b}	6.0^{c}	0.0^{d}	3.90
Kunitz trypsin inhibitor	5.1^{b}	5.9^{b}	5.6^{b}	7.5^{a}	1.43

[A] Means with different superscript letters in the same row are significantly different ($p < 0.05$).

Table 2
Peptides masses detected in alcalase hydrolysates from different soybean genotypes (A2H–D2H), purified β-conglycinin (BCH) and glycinin (GLH) by MALDI-TOF-MS.[A]

Peptide mass (Da)	A2H	B2H	C2H	D2H	BCH	GLH
1044.6	×	×				×
1305.7	×	×				×
1529.2	×	×	×	×	×	
1666.9	×	×××	×	×		
1670.6	×	×				×
1690.1	×	×	×	×	×	
1750.7						×××
1793.8	×	××	×	×	×	
1934.9	×	×××	×			
1949.1	×		×	×		
1970.2	×	×				×××
2458.7	×	××	×	××		
2703.1	×	×	×	×	×	
2748.4						×
2775.4	×	×	×			×
2832.2	×	×	×	×		
2990.7						×
3086.7			×	×	×	
3181.5						×
3225.6	×	×	×	×	×	
3275.8	×	×	×	×		
3381.6	×	×××	×××	××	×××	
3417.0						××
3537.4			×	×	×	
3823.5	××	××	××	×××	×	
3896.7	×××	×××	×××	×××	××	
4155.9	×	×	×	×		
4874.3	×	×	×			
5028.8	×	×	×	×	×	
5543.1	×	×	×	×	×	
7514.2	×	×	×	×		
7758.2	×	×	×	×		

[A] Peptide masses found in soybean hydrolysates, purified β-conglycinin (BCH) and glycinin (GLH) hydrolysates with intensities <40% are indicated with '×'; peptides masses with intensities between 40% and 70% are indicated with '××' and peptides masses with intensities >70% are indicated with '×××'.

chromatography–tandem mass spectrometry (LC–MS/MS) (Tables 3 and 4). Most of the identified peptides from BCH were derivatives of α and α′ subunits although a few peptides from the β subunit were also identified (Table 3). Predominant signal masses at m/z 3381 and 3897 in purified BC and A2H, B2H, C2H and D2H were identified as fragments *f*(85–112) and *f*(131–132) of α subunit of BC (REPQQPGEKEEDEDEQPRPIPFPRPQPR and KRGEKGSEEEDED EDEEQDERQFPFPRPPHQK, respectively). Noteworthy, these dominant peptides were constituted of a high percentage of acidic amino acid residues [Asp (D) and Glu (E)]. In addition, signal masses at m/z 1528 and 1960 in purified BC and A2H, B2H, C2H and D2H were identified as fragments *f*(330–342) and *f*(329–342) of the α′ subunit of BC (VVNPDNDENLRMI and YVVNPDNDENLRMI, respectively). Regarding GLH, most of the identified peptides were derivatives of GL G1 (A1a and Bx subunits) although some peptides were released from GL G4 (A5, A4 and B3 subunits) and G5 (A3 and B4 subunits) after alcalase hydrolysis (Table 4). Predominant peaks at m/z 1670, 1750 and 1970 in GLH (Table 2) were identified as fragments *f*(244–259), *f*(263–277) and *f*(260–277) of GL G1 (QGENEGEDKGAIVTVK, SVIKPPTDEQQQRPQ and GGLSVIKPPT-DEQQQRPQ, respectively). Thus, signals at m/z 1670 and 1970 observed in protein hydrolysates A2H and B2H (Table 2) were assigned to GL peptides QGENEGEDKGAIVTVK and GGLSVIKPP-TDEQQQRPQ.

3.3. Inhibitory effect of soy protein hydrolysates on FAS activity

Soy hydrolysates were able to inhibit FAS with a significantly ($p < 0.05$) higher inhibitory potency (lower IC_{50} values) from enriched BC (40.8–46.9%) soybean genotypes (B2H, C2H and D2H) than from 23% BC soybean genotype (A2H). Table 5 shows that the concentration of soy hydrolysates which inhibit 50% the FAS activity (IC_{50}) ranged from 50 to 175 μM, with C2H exhibiting the highest potency. Interestingly, soy hydrolysates from enriched BC soybean genotypes showed a similar (B2H, D2H; $p > 0.05$) or a significantly higher (C2H; $p < 0.05$) potency than the positive control (C75). These results suggest that BC embeds more active peptides, exhibiting FAS inhibitory activity, than GL. As shown in Table 5, BCH was more potent at inhibiting FAS activity than GLH. In addition, BCH showed a 6.5-fold lower IC_{50} (35 μM) compared to GLH (226 μM) (Table 5). Consistent with the experimental data, a higher total BC soybean genotype correlated ($r^2 = -0.95$) with a higher potency on FAS inhibitory activity. In addition, higher total GL soybean genotypes correlated ($r^2 = +0.93$) with a lower potency on FAS inhibitory activity (Table 5). On the other hand, protein hydrolysates from soybean genotypes with a similar content in BC and different GL content showed large variations on their FAS inhibitory activity. These results suggest that the subunit composition of BC and GL in soybean have an effect on FAS inhibitory activity of protein hydrolysates. Correlations between subunits in

Table 3
Peptides identified in the purified β-conglycinin alcalase hydrolysate (BCH) by LC–MS/MS.[A]

Experimental mass (Da)	Theoretical mass (Da)	Putative sequence	Protein source	Accession no.
754.18	754.44	KNPQLR	*f*(428–434) α subunit	P13916
754.18	754.44	KNPQLR	*f* (263–268) β subunit	P25974
1282.71	1282.63	VVNPDNNENLR	*f*(295–305) α subunit	P13916
1283.67	1283.61	VVNPDNDENLR	*f*(311–321) α′ subunit	P11827
1323.79	1323.72	EITPEKNPQLR	*f*(424–434) α subunit	P13916
1323.79	1323.72	EITPEKNPQLR	*f* (258–268) β subunit	P25974
1446.76	1446.68	YVVNPDNDENLR	*f*(329–340) α′ subunit	P11827
1527.83	1527.73	VVNPDNDENLRMI	*f*(330–342) α′ subunit	P11827
1533.91	1533.83	RQFPFPRPPHQK	*f*(151–162) α subunit	P13916
1551.90	1551.83	EITPEKNPQLRDL	*f*(424–436) α subunit	P13916
1551.90	1551.83	EITPEKNPQLRDL	*f* (258–270) β subunit	P25974
1558.87	1558.78	YVVNPDNNENLRL	*f*(294–306) α subunit	P13916
1577.81	1577.71	YVVNPDNDENLRM	*f*(329–341) α′ subunit	P11827
1690.90	1690.78	YVVNPDNDENLRMI	*f*(329–342) α′ subunit	P11827
1846.88	1846.79	RKQEEDEDEEQQRE	*f*(165–178) α subunit	P13916
2742.42	2742.34	PGEKEEDEDEQPRPIPFPRPQPR	*f*(90–112) α subunit	P13916
3380.61	3380.65	REPQQPGEKEEDEDEQPRPIPFPRPQPR	*f*(85–112) α subunit	P13916
3896.97	3896.74	KRGEKGSEEEDEDEDEEQDERQFPFPRPPHQK	*f*(131–132) α subunit	P13916

[A] Peptide sequences listed were found with a score >63 indicating identity with a confidence of at least 95% ($p < 0.05$).

Table 4
Peptide sequences identified in the purified glycinin alcalase hydrolysate (GLH) by LC–MS/MS.[A]

Experimental mass (Da)	Theoretical mass (Da)	Putative sequence	Protein source	Accession no.
834.50	834.42	VFDGELR	*f*(468–474) Glycinin G4 (A5, A4 and B3 subunits)	P02858
834.50	834.42	VFDGELR	*f*(434–440) Glycinin G5 (A3 and B4 subunits)	P04347
1322.74	1322.62	PPTDEQQQRPQ	*f*(267–277) Glycinin G1 (A1a and Bx subunits)	P04776
1197.67	1197.57	VNPESQQGSPR	*f*(547–557) Glycinin G4 (A5, A4 and B3 subunits)	P02858
1309.68	1309.56	AGNPDIEHPETM	*f*(190–201) Glycinin G5 (A3 and B4 subunits)	P04347
1330.81	1330.69	NFREGDLIAVPT	*f*(135–146) Glycinin G1 (A1a and Bx subunits)	P04776
1563.93	1563.93	IKPPTDEQQQRPQ	*f*(265–277) Glycinin G1 (A1a and Bx subunits)	P04776
1672.96	1672.83	QGENEGEDKGAIVTVK	*f*(244–259) Glycinin G1 (A1a and Bx subunits)	P04776
1750.04	1749.90	SVIKPPTDEQQQRPQ	*f*(263–277) Glycinin G1 (A1a and Bx subunits)	P04776
1977.19	1977.03	GGLSVIKPPTDEQQQRPQ	*f*(260–277) Glycinin G1 (A1a and Bx subunits)	P04776

[A] Peptide sequences listed were found with a score >63 indicating identity with a confidence of at least 95% ($p < 0.05$).

Table 5
Protein hydrolysates from enriched β-conglycinin soybean genotypes exhibited enhanced FAS inhibitory potency.[A]

Inhibitor	IC_{50} (μM)	β-Conglycinin (% total proteins)	Glycinin (% total proteins)
A2H	175.10 ± 16.07^{a}	23.4 ± 0.3^{c}	35.3 ± 0.6^{a}
B2H	83.29 ± 11.83^{b}	42.5 ± 0.5^{ab}	13.7 ± 2.6^{b}
C2H	50.40 ± 6.18^{cd}	46.9 ± 1.4^{a}	6.0 ± 0.8^{c}
D2H	63.19 ± 8.02^{bc}	40.8 ± 0.7^{b}	0.0 ± 0.0^{d}
BCH	34.86 ± 3.84^{d}	67.0	33.0
GLH	226.16 ± 20.96^{a}	17.7	83.3
C75 (+control)	80.34 ± 19.53^{b}	–	–
		Linear correlation analysis[B]	
Equation		$y = -5.3x + 296.6$	$y = 3.5x + 44.7$
r^2 coefficient		−0.95	+0.93

[A] Mean ± standard deviation of three independent replicates. Data with different superscript letters are significantly different ($p < 0.05$).
[B] Linear correlations were carried out between total BC and GL concentrations (% total protein) in soybean flours derived from different genotypes and IC_{50} for FAS inhibition (μM) of protein hydrolysates; the higher the potency, the lower the IC_{50} value.

soybean genotypes and IC_{50} values of protein hydrolysates were carried out. It was observed that a higher potency (lower IC_{50} values) of soy hydrolysates correlated with a higher content in α′ ($r^2 = -0.85$) and α ($r^2 = -0.58$) subunits of BC as well as a lower content in A1,2,4 ($r^2 = +0.88$) and basic ($r^2 = +0.93$) chains of GL in soybean genotypes. Finally, there was no correlation between FAS inhibitory activity of soy hydrolysates and the soybean A3 chain of glycinin ($r^2 = +0.22$).

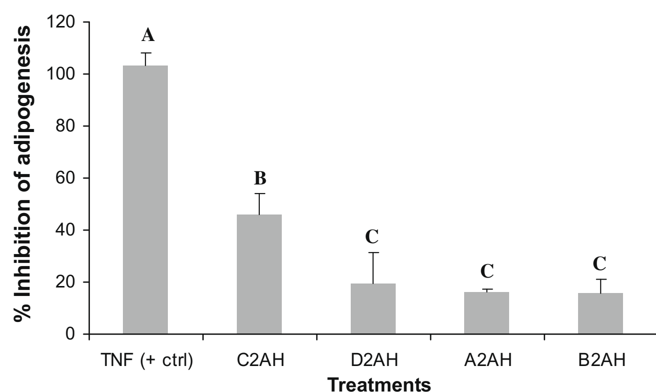

Fig. 1. Protein hydrolysates from soybean genotypes with enriched β-conglycinin α and α′ subunits show a more potent effect on inhibition of preadipocytes differentiation (adipogenesis). Preadipocytes were differentiated in the presence of DM-1 media containing 30 μM soy hydrolysates or 40 nM of TNFα on day 1. After 3 days the media was replaced by adipocyte medium (AM-1). On day 13, lipids were extracted and glycerol was determined. Bars represent mean ± standard deviation based on data from three independent experiments. Different letters indicate significant difference ($p < 0.05$).

3.4. Inhibitory effect of soy protein hydrolysates on adipogenesis in vitro

Fig. 1 presents the effect of alcalase hydrolysates from different soybean genotypes (A2H–D2H) and TNF α on inhibition of human preadipocytes differentiation (adipogenesis) as measured by lipid accumulation. Primary human preadipocytes were treated with soy protein hydrolysates or TNF-α during the differentiation period (days 1–13). Cell treatments were performed at non-cytotoxic concentrations (30 μM for soy protein hydrolysates and 40 nM for TNF α) based on our preliminary studies. The inflammatory cytokine TNF-α inhibits adipocyte differentiation and maintenance of matured adipocyte phenotype and therefore it was used as a positive control. Non-treated cells were used as a negative control of adipogenesis inhibition. The efficiency of TNFα was confirmed as it showed 103 ± 1.5% inhibition of adipogenesis vs. negative control levels related to lipid accumulation. Soy protein hydrolysates A2H, B2H and D2H inhibited preadipocyte differentiation by 16.0%, 15.6%, and 19.7% vs. the negative control, respectively. Interestingly, soy protein hydrolysate C2H gave rise to more than 2.5-fold higher inhibition of preadipocyte differentiation (50% of negative control levels; $p < 0.05$), as measured by lipid accumulation compared to A2H, B2H and D2H.

4. Discussion

Scientific evidence concerning the role of soy protein and body weight reduction is increasing (Cope et al., 2007; Velasquez & Bhathena, 2007; Xiao, 2008). However, despite intense studies in this area the active protein component responsible for the effects of soy protein on weight and adiposity reduction is still unclear. BC has received more attention in this context because it was

shown to have a role in the induction of satiety, reduction of adiposity and plasma LDL-cholesterol and triglycerides (Duranti et al., 2004; Hira et al., 2009; Kohno et al., 2006; Martinez-Villaluenga, Dia, Berhow, Bringe, & Gonzalez de Mejia, 2009; Martinez-Villaluenga et al., 2008). The native BC is a randomly assorted heterotrimer of α′, α, and β subunits which are the products of a multigene family (Harada, Barker, & Goldberg, 1989). The relative percentages of α′, α, and β subunits in the trimer are ∼35%, 45%, and 20%, respectively (Maruyama et al., 1999). However, soybean genotypes used in the present study revealed a variation on BC or GL fractions, and their corresponding subunits as a result of a selective breeding program to improve soybean quality for human consumption. For this purpose the effect of protein hydrolysates from different soybean genotypes were tested on the inhibition of FAS activity and adipogenesis *in vitro*. Thus, it was observed that the protein hydrolysate C2H with the highest total BC content (47% of total proteins) and low glycinin content (6% of total proteins) showed the highest inhibition of adipogenesis and FAS activity *in vitro*. This observation suggests that unique protein sequences in BC may embed more potent bioactive peptides. In addition, we observed a significantly higher FAS inhibitory activity in the BCH compared to GLH. These results are in accordance with other studies suggesting that BC appears to be primarily responsible for the effects on LDL-cholesterol and adiposity reduction of soy protein (Hira et al., 2009; Kohno et al., 2006; Martinez-Villaluenga et al., 2008, 2009; Mochuzuki et al., 2009) whereas GL appeared essentially inactive (Lovati, Manzoni, Gianazza, & Sirtori, 1998; Martinez-Villaluenga et al., 2008). Differences in the biological activity of BC and GL may be explained by the difference in amino acid sequence and composition. Within GL or BC, different subunits share sequence homology. However, between GL and BC, the sequences are largely different. The percentages of acidic (Asp and Glu), basic (Arg, His, and Lys) and aromatic (Phe and Tyr) amino acids in BC are higher than those in GL (Mahmoud et al., 2006). On the other hand, GL contains much higher percentages of sulphur-containing amino acids Cys and Met (Mahmoud et al., 2006). In agreement with these statements our study showed that identified peptides in the BCH were composed of a higher proportion of acidic, basic and aromatic amino acids compared to GL identified peptides in the GLH.

Other proteins, such as whey, may also contribute to body weight maintenance as recently demonstrated in mice by Huang et al. (2008). These researchers established that whey high-protein diet may contribute to a better stabilisation of body mass than soy high-protein diet in a chronic high-fat diet-induced obesity model. As demonstrated in our study, soy protein composition had a significant impact on reducing adipogenesis. Higher β-conglycinin concentration in soybean improved fatty acid synthase inhibition and adipogenesis reduction by protein hydrolysates. Therefore, the contribution to body weight maintenance of soymilk enriched in β-conglycinin in comparison to dairy proteins should be further investigated.

The results of the present study are intriguing because they also show for the first time that soybean genotypes with a high content in α' and α subunits of BC (17% and 20% of total proteins, respectively) improved the potency of protein hydrolysates to inhibit FAS activity and adipogenic response of human adipocytes *in vitro*. Similarly, previous studies showed that certain subunits in BC play a major role in lipid metabolism (Duranti et al., 2004; Lovati et al., 1998). Lovati et al. (1998) reported that incubation of HepG2 cells with purified α + α′ subunits from BC sharply increased uptake and degradation of LDL added to the culture medium, whereas the β chains were ineffective. Later the same researchers confirmed *in vivo* that the α′ subunit of BC is responsible for the biological activity observed *in vitro* (Duranti et al., 2004).

Vaughn, Rizzo, Doane, Beverly, and Gonzalez de Mejia (2008) studied the effect of soy peptides on weight management in a validated rat model and found a decreased body weight independently from food intake. These findings suggested that soy peptides may play a role on body weight loss by regulating energy expenditure. Hydrolysates from soy genotypes enriched in BC significantly inhibited lipid storage in mature adipocytes through downregulation of lipoprotein lipase and fatty acid synthase gene expression preventing adipocyte hypertrophy (Martinez-Villaluenga et al., 2009). In the present research we showed, for the first time, that BC peptides are inhibitors of FAS activity. Inhibition of FAS is a promising strategy for obesity prevention (Buettner, 2007; Chakravarthy et al., 2007). The central inhibition of FAS suppresses food intake and leads to dramatic weight loss in mice (Loftus et al., 2000), suggesting that hypothalamic accumulation of cellular malonyl-CoA levels is involved in energy homeostasis (Buettner, 2007; Hu, Cha, Chohnan, & Lane, 2003). In addition, it has been shown that FAS inhibition in adipose tissue prevents preadipocyte differentiation (Schmid et al., 2005) in agreement with our results. C2H protein hydrolysate had the highest FAS inhibitory activity and also inhibited 50% preadipocyte differentiation at a concentration of 50 μM. This result confirmed the efficiency of BC peptides as inhibitors of FAS. The adipogenesis process is controlled by transcription factors such as C/EBPα and PPARγ that are activated within hours, which in turn upregulates gene expression of key lipogenic enzymes (FAS, acetyl-CoA-carboxilase, ACC, among others). Several lines of evidence suggest that during the early phase of differentiation FAS products are used to maintain and sustain signalling for preadipocyte differentiation (Amri, Ailhaud, & Grimaldi, 1994), and that C/EBPα and PPARγ expression depends on FAS activity (Schmid et al., 2005). Therefore, the mechanism whereby inhibition of FAS prevents preadipocyte differentiation might be due to downregulation of gene expression of the transcription factors C/EBPα and PPARγ (Schmid et al., 2005).

To exert the physiological effect, BC bioactive peptides need to remain in an active form after gastrointestinal digestion and be absorbed by the gastrointestinal tract. We have shown that after *in vitro* gastrointestinal digestion of soy alcalase hydrolysates, 86% of the original inhibition of lipid storage on mature adipocytes remained (Martinez-Villaluenga et al., 2008). Studies in animals have shown that larger peptides (10–51 amino acids), generated by food protein digestion, can also be absorbed intact through the intestine and produce biological effects (Roberts, Burney, Black, & Zaloga, 1999). However, the bioavailability of peptides and their mechanism of absorption is still a topic under investigation. This aspect, as well as the identification of specific FAS inhibitory peptides is underway in our laboratory. It is also envisioned that the use of bioactivity assays such as the ones presented in this study will be valuable for comparing the effects of processing and formulations on various commercial food products and meals designed for weight management benefits.

5. Conclusions

The results presented in this study demonstrated that soybean genotypic changes in protein subunit composition give rise to characteristic peptide profiles that affect the biological activity of the hydrolysates. In particular, soybean genotypes enriched in BC α and α′ subunits are suitable sources of active peptides that inhibit FAS activity and adipogenesis *in vitro*. This will allow the development of genotypes tailored for specific food applications such as the development of functional ingredients for weight management.

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