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Enhancing the anti-adipogenic activity of soy protein by limited hydrolysis with Flavourzyme and ultrafiltration

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ABSTRACT

Limited hydrolysis of soy protein isolate (SPI) with Flavourzyme for 2 h to obtain the hydrolysate (FH2h) revealed much higher suppression of glycerol-3-phosphate dehydrogenase (GPDH) activity and relative lipid accumulation (RLA) than intact SPI in 3T3-L1 preadipocytes during differentiation. Lower GPDH activity or RLA indicates higher anti-adipogenic activity. The GPDH significantly decreased from 673 to 477 U/mg protein ($p < 0.05$). Sequentially fractionating FH2h with 30–1 kDa (kilo-daltons) molecular weight cut-off (MWCO) membranes to obtain the 1 kDa permeate resulted in further reduction of 59% GPDH activity. When comparing the high-performance size-exclusion chromatography (HPSEC) profiles, the most active peptide fraction for the anti-adipogenic activity was primarily composed of small peptides with molecular weight less than 1300 Da. According to the Western immunoblot analysis, 1 kDa permeate inhibits adipogenesis by affecting the expression of peroxisome proliferators-activated receptor γ (PPARγ) and the CCAAT/enhancer binding protein α (C/EBPα) during 3T3-L1 cells differentiation.

1. Introduction

Obesity arises from the imbalance between energy intake and energy expenditure that may lead to a pathologic growth of adipocytes (Aoyama, Fukui, Takamatsu, Hashimoto, & Yamamoto, 2000). It is known that the amount of adipose tissue can be regulated by the inhibition of adipogenesis from precursor cells. 3T3-L1 preadipocytes are one of the most well-characterised and reliable models for studying adipogenesis. Treatment with adipogenic agents including 3-isobutyl-1-methylxanthine, dexamethasone, and insulin induces postconfluent mitotic clonal expansion and begin to express adipocyte-specific genes (Rubin, Hirsch, Fung, & Rosen, 1978). A member of the C/EBP family transcription factor, C/EBPβ, and the PPARγ play critical roles in adipogenesis (Rosen et al., 1999), and C/EBPβ activates the expression of PPARγ and C/EBPα mRNA that coordinately activate the transcription of adipocyte-specific genes (Rosen, Walkey, Puigserver, & Spiegelman, 2000).

GPDH occupies a key position in the metabolism linking glycolysis to phospholipids and triglycerides biosynthesis (Harding, Pyeritz, Copeland, & White, 1975). It has been reported that in murine adipocytes the GPDH and mRNA levels can be regulated by insulin and that the enzyme activity also serves as a late marker of differentiation (Bhandari, Saini, & Miller, 1991). The activity of GPDH is increased several 100-fold during preadipocyte differentiation (Flores-Delgado, Marsch-Moreno, & Kuri-Harcuch, 1987). Since suppression of GPDH activity resulted in the inhibition of differentiation as well as reduction of lipid accumulation in 3T3-L1 cells (Hirai, Yamanaka, Kawachi, Matsui, & Yano, 2005; Kim et al., 2001), determining GPDH activity could be employed as a convenient way to evaluate the effect of protein hydrolysate on anti-adipogenic activity of 3T3-L1 cells.

Enzymatic hydrolysis coupled with membrane fractionation of soy protein might result in releasing and enhancing their biological activity and could be used as potential nutraceutical for the prevention of hypertension (Chiang, Tsou, Tsai, & Tsai, 2006), reduction of serum cholesterol and body fat (Nagaoka et al., 1997) and for the increase of calcium bioavailability (Bao, Song, Zhang, Chen, & Guo, 2007). During hydrolysis, a wide variety of smaller peptides and free amino acids are generated, depending on enzyme specificity and hydrolysis conditions. Preliminary studies from our lab (unpublished data) indicated that the hydrolysate obtained from the enzymatic hydrolysis of soy protein with Flavourzyme may decrease the lipid accumulation in 3T3-L1 preadipocyte differentiation and 3T3-L1 adipocytes.

In the present study the effect of limited hydrolysis of SPI with Flavourzyme on GPDH activity and lipid accumulation in 3T3-L1 preadipocytes was evaluated. The effect of membrane fractionation of Flavourzyme-SPI hydrolysate on its ability to inhibit adipogenesis was also investigated. During 3T3-L1 preadipocyte differentiation,

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some of the transcription factors such as C/EBPα, C/EBPβ and PPARγ were shown to reveal the possible pathway for protein hydrolysate to inhibit lipid accumulation.

2. Materials and methods

2.1. Chemicals

SPI was purchased from Gemfont Company (Taipei, Taiwan). Flavourzyme® 1000 MG (1000 unit/g) was obtained from Novo Nordisk A/S (Copenhagen, Denmark). HPSEC standards including cytochrome c, aprotinin, gastrin I, substance P, and $(glycine)_3$ with a molecular weight of 125,000, 6500, 2126, 1329.8 and 189 Da, respectively were purchased from Merck (Damdstadt, Germany) or Sigma Chemical Co. (St. Louis, MO, USA). Polyethersulfone spiral-wound membranes with a MWCO of 1–30 kDa and with a membrane area of 0.2 m^2 were purchased from Osmonics Inc. (California, USA). Dulbecco's Modified Eagle Medium (DMEM) and all tissue culture materials were from Gibco (Grand Island, New York, USA). The fetal bovine serum (FBS) was obtained from Biological Industries (Kibbutz Beit Haemek, Israel).

2.2. Preparations of Flavourzyme-SPI hydrolysates (FH)

SPI at 2.5% (w/v) and 1% (w/w of SPI) Flavourzyme were employed to produce and compare each hydrolysate with the GPDH activity in the 3T3-L1 cells. The optimum conditions of temperature and pH for the Flavourzyme were 50 °C and 7.0, respectively, according to the internal document of the supplier. Samples, withdrawn at 0.5, 1, 2, 4, 6 h, were immediately heated in a boiling water bath for 10 min. Parts of each heated sample were used to determine the degree of hydrolysis (DH) using the ortho-phthaldialdehyde (OPA) method (Church, Swaisgood, Porter, & Catignani, 1983). The remainder of each heated sample was subjected to centrifugation at 8000*g* in a microcentrifuge for 15 min. The supernatants were freeze-dried and assayed for GPDH activity and lipid accumulation in the 3T3-L1 cells.

2.3. Fractionation of selected FH with ultrafiltration membranes

Selected FH was subjected to ultrafiltration using a spiral-wound membrane with 30 kDa, 10 kDa and 1 kDa MWCO, sequentially. Fresh water were added into each concentrate twice to replace 1/10 of the permeate volume to remove permeate components from the concentrate. Each concentrate or permeate was collected and freeze-dried to determine its protein concentration, profile of molecular weight distribution, and to feed it cell culture.

2.4. Cell culture

The 3T3-L1 preadipocytes (American Type Culture Collection, Rockville, MD, USA) were seeded in a 24-well plate at a density of 1×10^4 cells/well. Cells were grown in DMEM supplemented with 10% FBS at 37 °C in a 5% CO_2 humidified atmosphere. To induce differentiation, 2-day postconfluent 3T3-L1 preadipocytes (day 0) were stimulated for 48 h with 0.5 mM 3-isobutyl-1-methylxanthine, 0.1 μM dexamethasone, and 10 μg/ml insulin in DMEM supplemented with 10% FBS. Then the preadipocytes were maintained and were fed every 2 days with DMEM supplemented with 10% FBS and 10 μg/ml insulin. Two treatments were employed to examine the effect of Flavourzyme-SPI hydrolysate or its fractions on lipid accumulation and GPDH during preadipocyte differentiation. Treatment 1, which was denoted as (++), employed 2-day postconfluent 3T3-L1 preadipocytes receiving 50–2000 ppm FH at day 0 and every 2 days afterwards until the end of the experiment at day 8 (from day 0 to day 8). Treatment 2, which was denoted as (+−), also employed 2-day postconfluent 3T3-L1 preadipocytes but they only received FH at day 0 during the early phase of differentiation. The effect of FH fractions on GPDH activity was determined only using cell culture with treatment 2.

2.5. Assay for RLA

On day 8 after differentiation, 3T3-L1 cells were stained with Oil Red O to determine intracellular lipid or triglyceride (TG) content (Green & Kehinde, 1975). The cells were fixed with 3.7% formaldehyde for 1 h at 4 °C. After fixation, cells were washed twice with deionized water and stained using 0.3% Oil Red O in isopropanol for 15 min at room temperature. A preliminary study indicated that the absorbance of the dye-TG complex was proportional to the concentration of TG present in each sample (data not shown) at 540 nm. RLA (%) was expressed as:

$$\text{RLA } (\%) = \frac{A_c - A_s}{A_c} \times 100 \quad (1)$$

where A_c denotes the absorbance of control cell culture and A_s denotes the absorbance of treatment 1 or treatment 2 cell culture.

2.6. Assay for glycerol-3-phosphate dehydrogenase activity

On day 8 after differentiation, 3T3-L1 cells were washed twice with ice-cold PBS, and harvested into 25 mM Tris buffer (pH 7.5) containing 1 mM EDTA and 1 mM DTT. Cells were disrupted by sonication and then centrifuged at 12,000*g* for 20 min at 4 °C. The supernatants were analysed for GPDH activity according to the procedure of Wise and Green (1979). Protein concentration was measured using a BCA™ protein assay kit (Pierce, Rockford, IL, USA). One unit of enzyme activity was defined as the amount of protein required for the use of 1 nmol NADH min^{-1} mg $protein^{-1}$.

2.7. Molecular weight distribution

The molecular weight distribution of the hydrolysate was analysed using HPSEC. The HPSEC was equipped with a Superdex HR 10/30 column (Amersham Biosciences Ltd., Pittsburgh, PA., USA) connected to a UV detector (Shimadzu Co., Kyoto, Japan) set at 214 nm. The mobile phase was 0.02 M phosphate buffer (pH 7.2) containing 0.25 M NaCl, and the flow rate was set at 0.5 ml/min.

2.8. Stability of SPI derived peptides for Suppressing GPDH Activity

The stability against *in vitro* gastric proteases was assessed as described by Wu and Ding (2002) with some modifications. One percent (w/v) of hydrolysate solution was treated in 0.1 M KCl–HCl (pH 2.0) buffer with pepsin for 4 h in a water bath at 37 °C, then stopped by boiling in a water bath for 15 min and neutralised to pH 7.0 with the addition of 2 N NaOH solution. One millilitre of neutralised suspension was centrifuged (10,000*g* for 40 min) and the supernatant was used to treat 3T3-L1 preadipocytes to measure its GPDH activity. The remaining neutralised suspension was digested further by 2% (w/w) pancreatin at 37 °C for 4 h. The enzyme was inactivated by boiling for 15 min followed by centrifugation. The supernatant was used to treat cells to determine GPDH activity.

2.9. Western immunoblot analysis

3T3-L1 cells were harvested and lysed in 25 mM Tris/1 mM EDTA, pH 7.5 by sonication. The protein concentration of cell lysate was determined and 20 μg of protein was subjected to 12.5% SDS–