

# Capsanthin Inhibits both Adipogenesis in 3T3-L1 Preadipocytes and Weight Gain in High-Fat Diet-Induced Obese Mice

Sung Jun Jo<sup>1</sup>, Jeung Won Kim<sup>1</sup>, Hye Ok Choi<sup>1</sup>, Jung Hwan Kim<sup>1</sup>, Hyung Joong Kim<sup>1</sup>, Sun Hee Woo<sup>2</sup> and Byung Hoon Han<sup>1,\*</sup>

<sup>1</sup>Life Science Research Institute, E.S. Biotech. Co. Ltd., Cheonan 31257,

<sup>2</sup>Department of Agronomia, Chungbuk National University, Cheongju 28644, Republic of Korea

## Abstract

Adipogenesis in murine preadipocyte 3T3L-1 has been used as a model system to study anti-obese bioactive molecules. During adipogenesis in 3T3-L1 preadipocytes, we found that capsanthin inhibited adipogenesis (IC<sub>50</sub>; 2.5 μM) and also showed lipolytic activity in differentiated adipocytes from the preadipocytes (ED<sub>50</sub>; 872 nM). We identified that the pharmacological activity of capsanthin on adipogenesis in 3T3-L1 was mainly due to its adrenoceptor-β<sub>2</sub>-agonistic activity. In high-fat diet animal model study, capsanthin significantly enhanced spontaneous locomotive activities together with progressive weight-loss. The capsanthin-induced activation of kinetic behavior in mice was associated with the excessive production of ATP initiated by both the enhanced lipolytic activity together with accelerated oxidation of fatty acids due to the adrenoceptor β<sub>2</sub>-agonistic activity of capsanthin. Capsanthin also dose-dependently increased adiponectin and p-AMPK activity in high fat diet animals, suggesting that capsanthin has both anti-obesity and insulin sensitizing activities.

**Key Words:** Capsanthin, Anti-adipogenic, Lypolytic activity, Spontaneous locomotive activity, Adiponectin

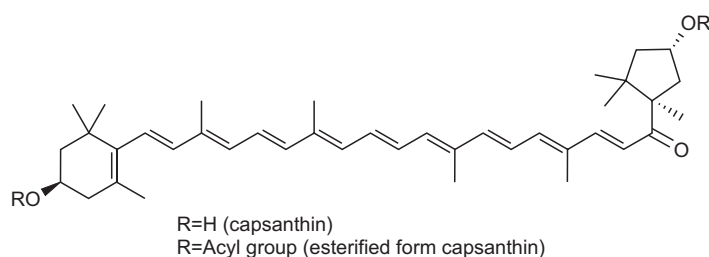
## INTRODUCTION

The use of red pepper (*Capsicum annum* L.) as food supplements is gradually increased world-widely. Red pepper contains two noticeable components, i.e., the first one is capsanthin (intense red colored xanthophyll) and the other one is highly pungent capsaicin (Fig. 1). Most of pharmacological studies on anti-adipogenic potential of red pepper have been

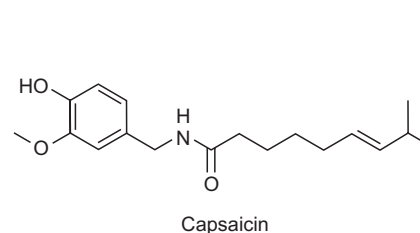
directed with capsaicin (Hsu and Yen, 2007; Joo *et al.*, 2010; Kang *et al.*, 2010; Lee *et al.*, 2011). However, the effect of capsanthin on this metabolic disease has not been fully studied, although capsanthin was reported to the anti-oxidant and anti-cancer potential (Matsufuji *et al.*, 1998; Maoka *et al.*, 2001).

This study was directed to elucidate whether capsanthin affects adipocyte-related biological functions like capsaicin. The effect of capsanthin on adipocyte functions was determined in

A



B



**Fig. 1.** Structures of capsanthin (A) and capsaicin (B).

**Open Access** <https://doi.org/10.4062/biomolther.2017.048>

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0/>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Received Mar 6, 2017 Revised Mar 18, 2017 Accepted Mar 18, 2017  
Published Online May 1, 2017

**\*Corresponding Author**

E-mail: bhhan3312@yahoo.co.kr

Tel: +82-41-556-9166, Fax: +82-41-556-9165

the adipogenesis model of murine preadipocyte cell line 3T3-L1. In addition, the pharmacological activity of capsanthin was validated with high-fat diet-induced obesity mouse models. In this study, we first demonstrated that capsanthin may have both anti-obese and anti-diabetic potentials.

## MATERIALS AND METHODS

### Extraction of red pepper powder

Red pepper powder (10 kg) was extracted 3 times with ethyl acetate (EtOAc, 50 L) by refluxing under nitrogen replacement for 3 hours for each extraction processes and vacuum evaporated to give 1.53 kg of EtOAc extract [A].

### Elimination of capsaicin from [A]

[A] (1.5 kg portion) was dissolved in 7 L hexane and washed with 0.1N-NaOH until the capsaicin spot was completely disappeared on the TLC (CHCl<sub>3</sub>:EtOH=10:1) of the extract. The hexane fraction (capsaicin free) was concentrated to a small volume, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated to obtain 1.27 kg anhydrous extract [A-1]. [A-1] is intensely red oily liquid composed of di-fatty-acyl-esters of capsanthin with a bulky amount of triglycerides together with some other xanthophyll components.

### Elimination of triglycerides from [A-1]

[A-1] (1.2 kg) in 5 L absolute ethanol was treated for one hour at room temperature with catalytic amount of NaOCH<sub>3</sub> under nitrogen replacement to convert triglyceride into fatty-acyl-ethyl-ester by room temperature trans-esterification (Schuchardt *et al.*, 1998). Complete reaction could be checked via the disappearance of interphase of two liquid phases to ensure the conversion of triglycerides into fatty-acyl-ethyl-esters. At the end of the reaction, the reaction mixture was neutralized by d-HCl. The reaction mixture was concentrated in vacuo and chromatographed on silica-gel column (hexane:EtOAc) by gradient process elution to isolate 34 g mixture of fatty-acyl-ester of xanthophylls [A-2]. [A-2] fraction contains the mixture of fatty-acyl-esters of capsanthin, capsorubin, zeaxanthin, cantaxanthin, lutein and  $\beta$ -cryptoxanthin. Those fatty-acyl-esters of xanthophylls are resisting to room temperature trans-esterification, since their ester bonds are based on the secondary alcoholic groups of xanthophylls.

### Separation of capsanthin di-fatty-acyl-ester mixture from [A-2]

[A-2] (10 g) was chromatographed over silica column to obtain 1.54 g of capsanthin di-fatty-acyl-ester (deep red) by using hexane: EtOAc (20:1) as an eluent. Capsanthin di-fatty-acyl-ester was identified by PMR spectra analysis (data is not shown) (Rüttimann *et al.*, 1983).

### Preparation of capsanthin and other xanthophyll components by heated trans-esterification

[A-2] (20 g) was dissolved in absolute ethanol (100 mL) with catalytic amount of NaOCH<sub>3</sub> and refluxed under nitrogen replacement for 30 min. (heated trans-esterification). After cooling to room temperature, the reaction mixture was neutralized by d-HCl. The crude product was concentrated in vacuo and separated by silica column chromatography (CHCl<sub>3</sub>: acetone:methanol=5:1:0.1) to obtain capsanthin 1.3 g, ca-

**Table 1.** Anti-adipogenic and lipolytic activity of carotenoids (unit:  $\mu$ mol)

Material Name	IC <sub>50</sub> *	ED <sub>50</sub> **
Capsanthin	2.50 $\pm$ 0.45	0.872 $\pm$ 0.06
Esterified form Capsanthin	12.5 $\pm$ 3.44	9.80 $\pm$ 2.14
Capsorubin	>100	>100
Zeaxanthin	>200	>200
Cantaxanthin	>100	>100
Lutein	97.2 $\pm$ 0.44	77.5 $\pm$ 1.01
$\beta$ -Cryptoxanthin	>100	>100
$\beta$ -Carotene	69.2 $\pm$ 2.87	72.1 $\pm$ 3.17

\*Inhibition of preadipocyte differentiation, \*\*lipolytic activity.

psorubin 200 mg and zeaxanthin 12 mg as chromatographically pure states. Those chemical identities were confirmed by PMR-spectral analysis (Rüttimann *et al.*, 1983; Sompong and Trakanrunroj, 2010). Other three xanthophylls (cantaxanthin, lutein and  $\beta$ -cryptoxanthin) and  $\beta$ -carotene were purchased from Sigma Aldrich Co (St. Louis, MO, USA).

### Analysis of fatty acids composition of capsanthin di-fatty-acyl-ester

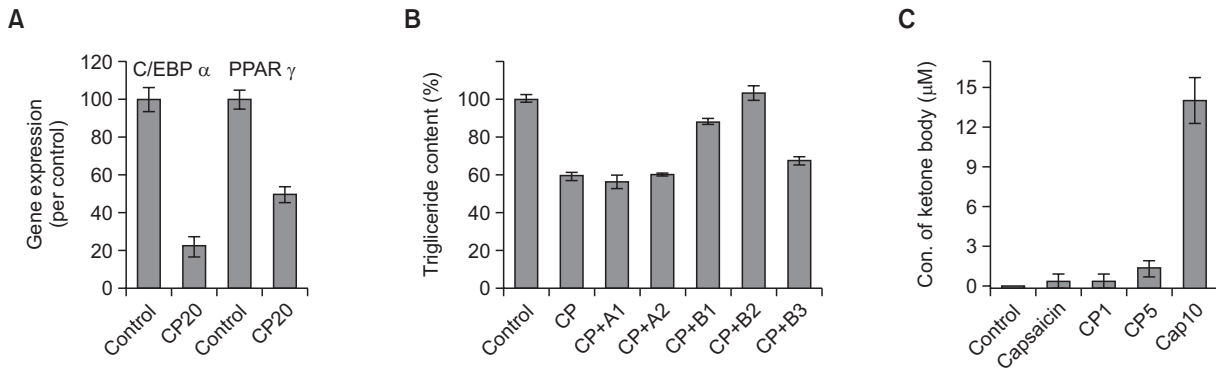
Capsanthin di-fatty-acyl-ester 10 mg dissolved in absolute ethanol was treated by heated trans-esterification with catalytic amount of NaOCH<sub>3</sub> under N<sub>2</sub>-replacement. After appropriate dilution of the reaction mixture with hexane, the hexane layer was analyzed by gas liquid chromatography (GLC) to find the composition of fatty-acyl-ethyl esters; lauric acid 8.7%, myristic acid 50.6%, palmitic acid 40.7%. GLC conditions; HP-5890-II Series, detector; FID, column; DB-23 capillary (Agilent technologies [Santa Clara, CA, USA], 60 m, 0.25 mm ID, 0.25  $\mu$ m), Oven Temp. initial 130°C, 2.7°C/min gradient to ~230°C, 3 min, inlet Temp.; 270°C, detector Temp.; 300°C, carrier gas; N<sub>2</sub>, 30psi, split ratio; 1/50, flow rate; 30 ml/min.

### Capsanthin inhibits differentiation of 3T3-L1 Cell

The cell line 3T3-L1 pre-adipocytes was purchased from the Korean Cell Line Bank. Cells were cultured (Zebisch *et al.*, 2012) in Dulbecco's Modified Eagle's Medium (DMEM containing 10% fetal bovine serum, Gibco), 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin and maintained at 37°C under 5% CO<sub>2</sub> atmosphere. At 2 days post-confluence (denoted as Day 0), previous medium was replaced with adipocyte induction medium (DMEM containing 10% fetal bovine serum (FBS, Gibco), supplemented with 2  $\mu$ g/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1  $\mu$ M dexamethasone and treated with 10  $\mu$ M capsanthin in DMSO (final concentration; 0.5%). The induction medium was changed every 2 days for 2 times and counted differentiated adipocyte after Oil Red O (ORO) staining. IC<sub>50</sub>-value was determined as 2.5  $\mu$ M for free capsanthin and 12.5  $\mu$ M for ester-form capsanthin by serial dilution assay technique (Table 1).

### Reverse transcription-PCR

The fully differentiated adipocytes under the presence of capsanthin was centrifuged at 3000 g for 10 min to harvest the cultured cells. Total RNA was isolated from cultured cells using GeneAll Ribospin (Cat. No. 304-150; GeneAll Biotechnology, Co., Ltd., Seoul, Korea). The extracted RNA was quantitated



**Fig. 2.** (A) Inhibition of adipogenic gene expression by capsanthin. (B) Lipolytic activity of capsanthin 20  $\mu$ M under the presence of each 5  $\mu$ M of various adrenoceptor antagonists. Control: DMSO, CP: capsanthin, A1:  $\alpha$ 1-antagonist, A2:  $\alpha$ 2-antagonist, B1:  $\beta$ 1-antagonist, B2:  $\beta$ 2-antagonist, B3:  $\beta$ 3-antagonist. (C) Effect of capsanthin on ketone body production in mice fed with high fat diet. Control: high fat diet (HFD), CP1: HFD+capsanthin 1  $\mu$ mol, CP5: HFD+capsanthin 5  $\mu$ mol, Cap10: HFD+capsanthin 10  $\mu$ mol.

by absorbance using a nanodrop spectrophotometer (Maestro nanodrop) and processed for RT-PCR (Kong and Park, 2008). All PCR primers were obtained from BioNEER (Daejeon, Korea) which included (Farmer, 2006; Lin and Lane, 1994; Fig. 2A)

PPAR $\gamma$  (F:GAGATGCCATTCTGGCCACCAACTTCGG, R:TATCATAAATAAGCTTCAATCGGATGGT TC)  
 C/EBP $\alpha$  (F:TCATCCACTTCACCAGTGACAA, R:AAACCATCCTCTGGGTCTCC).

#### Western blot analysis

Cellular proteins were extracted from control and capsanthin treated 3T3-L1 cells. Cells were collected by centrifugation and washed once with phosphate buffered saline (PBS). The washed cell pellets were resuspended in extraction-RIPA-lysis buffer and incubated for 60 min at 4 $^{\circ}$ C, with gentle shaking. Cell debris was removed by centrifugation (15000 g), followed by quick freezing of the supernatants for 10 min at 4 $^{\circ}$ C. The cellular protein from treated and untreated cell extracts were electroblotted onto a nitrocellulose membrane following separation on 10% SDS-polyacrylamide gel electrophoresis (Mahmood and Yang, 2012). The immunoblot was incubated overnight with blocking by 5% skim milk at 4 $^{\circ}$ C, followed by incubation with diluted polyclonal antibodies against p-AMPK (Lizcano *et al.*, 2004) and uncoupling protein-1 (UCP-1) (Joo *et al.*, 2010) for 60 min at room temperature with gentle shaking. Blot were incubated with a dilution of horseradish peroxidase conjugated mouse anti-rabbit IgG (5127, Cell signaling, Boston, MA, USA) secondary antibody. The blots were exposed to autoradiography films, which were analyzed with the Chemidoc (EZ-capture ST, ATTO Co., Tokyo, Japan).

#### In vitro anti-adipogenic activity assay on 3T3-L1 cell culture system

All of the isolated four carotenoids from the extract of red pepper together with other four purchased carotenoids were subjected to in vitro evaluation of anti-adipogenic activity by using 3T3-L1 cell culture system to obtain IC<sub>50</sub>-values for the anti-adipogenic activities and ED<sub>50</sub>-values for the lipolytic potentials. The cells were cultured in the same way as described above and after 4 times replacement of induction media (8 days incubation), the culture system was treated with 10  $\mu$ M

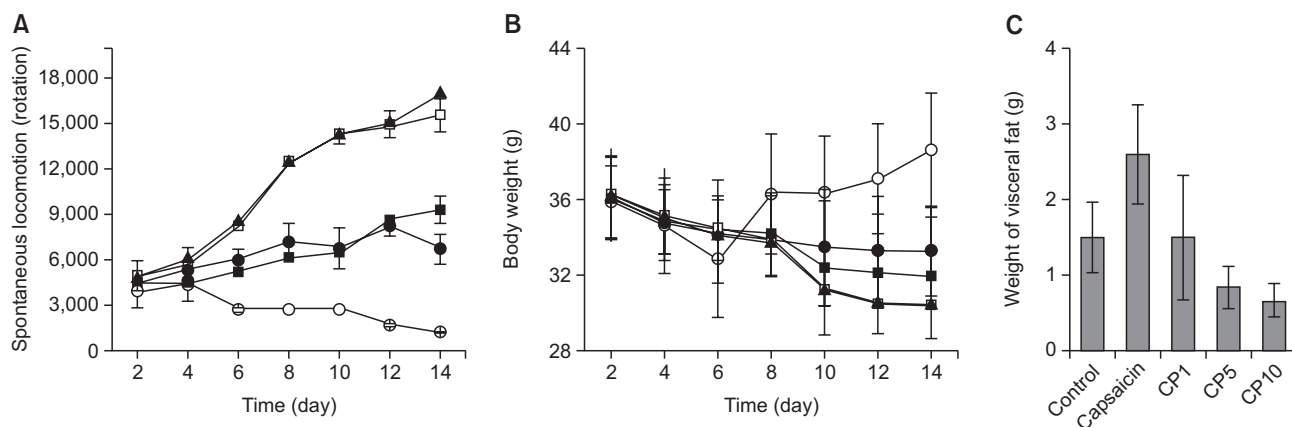
capsanthin in 0.5% DMSO and incubated for two days. Following that lipolytic activity of capsanthin were assessed by staining triglycerides in the attached cells on plate with ORO. The attached cells (capsanthin treated) were fixed with 10% paraformaldehyde for 1 h. After being washed well with PBS (pH 6.8), cells were incubated with ORO for 2 h at 37 $^{\circ}$ C under 5% CO<sub>2</sub>. Then, the plate was rinsed thoroughly with PBS at least 5 times to remove unbound ORO. ORO was washed with PBS buffer 3 times. The triglyceride bound ORO was extracted with isopropanol and assayed the ORO-content. The extracted ORO was transferred to 96 well plates and absorbances were measured using ELISA reader (at 510 nm). Potent lipolytic activity was observed, hence ED<sub>50</sub>-value was determined as 872 nM for free capsanthin and 9.80  $\mu$ M for ester-form capsanthin by serial dilution assay techniques (Table 1).

#### Adrenoceptor- $\beta$ 2-agonistic activity of capsanthin on 3T3-L1 cell culture system

The lipolytic activities of 20  $\mu$ M capsanthin under the presence of each 5  $\mu$ M of various adrenoceptor-antagonist i.e.;  $\alpha$ 1-antagonist (doxazosin-mesyate),  $\alpha$ 2-antagonist (yohimbine. HCl),  $\beta$ 1-antagonist (metoprolol-tartrate),  $\beta$ 2-antagonist (C118,551.HCl) and  $\beta$ 3-antagonist (SR59230A) (Sigma Aldrich Co.), were assayed by incubation overnight at 37 $^{\circ}$ C, 5% CO<sub>2</sub> atmosphere. Triglycerides in adipocyte were stained by incubation with ORO. Unbound ORO was washed with PBS buffer 3 times. The triglyceride bound ORO was extracted with isopropanol and assayed the ORO content by HPLC (Fig. 2B). HPLC (waters 486) condition: wavelength 450 nm, eluent acetonitrile : water (9:1), column Mightysil RP-18 GP (150 $\times$ 4.6 mm, 5  $\mu$ M; Kanto Co., Inc., Tokyo, Japan; Fig. 2B).

#### Animal feeding studies

Female C57BL/6C mice (four weeks) were obtained from Orientbio Co (Seongnam, Korea). The mice were fed a laboratory chow and water *ad libitum* (45% kcal from fat) for 6 weeks and housed in a 12 h light/dark cycle. Capsanthin feeding animal experiments were conducted to find further pharmacological activities of capsanthin. Mice were fed with high fat diet during six weeks in advance and including whole experimental periods to increase body weights due to the accumulation of fat in the mice body.



**Fig. 3.** (A) Effect of capsanthin on spontaneous-locomotive activity. (B) Effect of capsanthin on body weight. (●) HFD+cooking oil 50  $\mu$ L, (○) HFD+capsaicin 1  $\mu$ mol, (■) HFD+capsanthin 1  $\mu$ mol, (□) HFD+capsanthin 5  $\mu$ mol, (▲) HFD+capsanthin 10  $\mu$ mol, respectively. (C) Effect of capsanthin on visceral fat weight. CP1: High fat diet (HFD)+capsanthin 1  $\mu$ mol, CP5: HFD+capsanthin 5  $\mu$ mol, Cap10: HFD+capsanthin 10  $\mu$ mol.

### Spontaneous locomotive activity of mice

Single mouse was accommodated in a cage which was equipped with a wheel on which one way running exercise is possible together with automatic digital counting system. Each mouse in the cage is freely accessible to water and high fat diet. Animal cages equipped with same facilities were order-made to test the spontaneous locomotive activities of each mouse. Thirty five mice were allocated to five specific groups consisting of seven mice per group; Group-1 (control group); high fat diet (HFD)+cooking oil 50  $\mu$ L, Group-2 (positive control group); HFD+capsaicin 1  $\mu$ mol, Group-3; HFD+capsanthin 1  $\mu$ mol, Group-4; HFD+capsanthin 5  $\mu$ mol and Group-5; HFD+10  $\mu$ mol capsanthin per day, respectively. All capsanthin samples were orally administered as the cooking oil solution. The test was conducted during 14 days. Every each other day afternoon at 1:00 PM the cumulative data of spontaneous locomotive activities of each mouse (running score) during past 24 h together with the body weight of each mouse were recorded and the digital counters were reset. The average values for the seven mice/group for running score (Fig. 3A) and average body weights were recorded on (Fig. 3B).

### Serum separation and abdominal fat

After the completion of running experiments the mice were anesthetized with diethyl ether by the bell-jar technique and sacrificed by decapitation to collect trunk blood. To prepare serum, the blood was clotted at 4°C overnight and the clotted blood was centrifuged at 3000 g for 20 min. Serums were stored at -80°C until analysis. At the end of feeding experiment the weight of abdominal fat pads were measured after laparotomy operation (Fig. 3C).

### Analysis of adipokines

LDL-cholesterol, HDL-cholesterol, total cholesterol, ketone body, adiponectin, alanine-aminotransferase (ALT) TNF- $\alpha$  and p-AMPK content in the mouse serum participated to the spontaneous locomotive activity tests were assayed according to the instruction manuals of respective assay kits; LDL-cholesterol assay kit (#5607-02, Bio Scientific, MD, USA), HDL-cholesterol assay kit (#5607-01, Bio Scientific, MD, USA), total cholesterol assay kit (STA-390, Cell Biolabs, San Diego, CA,

USA), ketone body assay kit (EKBD-100, Bioassay system, Hayward, CA, USA), adiponectin assay kit (R&D system, Minneapolis, MN, USA) and TNF- $\alpha$  ELISA Kit (KMC3012, Invitrogen, Carlsbad, CA, USA).

### Statistical analysis

All data are recorded with mean value  $\pm$  standard deviation calculated by Origin Program (Origin ver. 8.0, Origin Lab, Northampton, MA, USA).

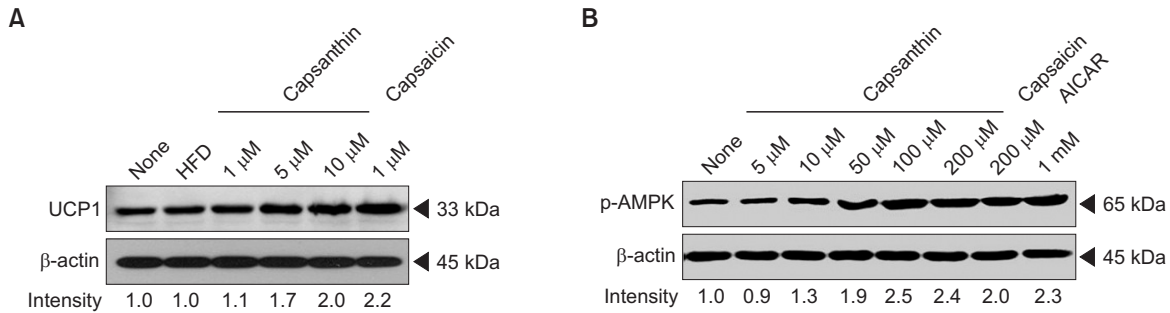
## RESULTS

### Isolation of carotenoids from red pepper extract

EtOAc extract of red pepper is composed of large amount of triglycerides, fairly good amount of capsaicin and a little amount of unstable carotenoids. After evaporation of EtOAc, intense red oily liquid was obtained. We could eliminate capsaicin which has already been reported as a potent anti-adipogenic substance (Diepvens *et al.*, 2007), very easily by simple repeated alkali washing. Thus obtained capsaicin-free extract was subjected to trans-esterification in absolute ethanol to convert bulky amount of triglycerides into fatty-acyl-ethyl-ester (Schuchardt *et al.*, 1998). In this reaction condition fatty-acyl-esters of carotenoids remained intact, however, large amount of triglyceride was converted to fatty-acyl-ethyl-ester, which could be easily eliminated from xanthophyll-esters by simple silica column chromatographic purification. The pure isolated carotenoids including capsanthin, capsorubin and zeaxanthin were identified by PMR spectra assignment referring to references (Rüttimann *et al.*, 1983; Sompong and Trakanrunroj, 2010).

### Gene expression under the presence of capsanthin

C/EBP $\alpha$  and PPAR $\gamma$  are well known as the most reliable gene-expression factors for the adipocyte differentiation. It is well known fact that down regulation of both expression factor C/EBP $\alpha$  and PPAR $\gamma$  (Kong and Park, 2008) are concerned with the inhibition of differentiation from preadipocyte to adipocyte (Fig. 2A, Table 1). As shown in Table 1, capsanthin (free form) shows the most potent anti-adipogenic and lipolytic ac-



**Fig. 4.** (A) Effect of capsanthin and capsaicin on UCP-1 induction in 3T3-L1 cell line. (B) Effect of capsanthin and capsaicin on p-AMPK expression in 3T3-L1 cell line.

tivity, however, esterified form capsanthin showed very weak activity. The other xanthophyll components showed very weak activities for both the inhibition of adipocyte differentiation and for the lipolytic potencies. During the incubation of 3T3-L1 cells with free capsanthin, we found a thick layer of free fatty acids floating on the surface of cell culture system due to the powerful lipolytic activity of capsanthin.

#### Lipolytic mechanism of capsanthin

In order to see the mode of capsanthin-action on the lipolytic activity, 3T3-L1-cell cultures were treated with capsanthin under the presence of various adrenoceptor-antagonists as doxazocin ( $\alpha_1$ ), yohimbrin-HCl ( $\alpha_2$ ), metoprolol.tartrate ( $\beta_1$ ), ICI118,551-HCl ( $\beta_2$ ) and SR59230A ( $\beta_3$ ) (Otton *et al.*, 1984; Babamoto and Hirokawa, 1992). As shown in Fig. 2B, the lipolytic activity of capsanthin is most highly inhibited in the presence of  $\beta_2$ -antagonist and lesser inhibition with  $\beta_1$ -antagonist, hence, capsanthin must be potent adrenoceptor  $\beta_2$ -specific agonist with somewhat lesser  $\beta_1$ -agonistic activity. Practically  $\beta_3$ -agonistic activity could not be detected (Fig. 2B). From the above results the activation of hormone sensitive lipase must be based on the adrenoceptor- $\beta_2$ -agonistic activity. It is well known fact that adrenoceptor- $\beta_2$ -agonistic activity is linked to the activation of hormone-sensitive lipase in adipocytes and also to the activation of  $\beta$ -oxidation of fatty acids in muscle cells (Otton *et al.*, 1984; Lee *et al.*, 2015).

#### Comparison of capsanthin and capsaicin on their spontaneous locomotive activities

Capsanthin fed mice showed highly enhanced spontaneous locomotive activities with dose dependency (Fig. 3A), and continuously progressing weight loss during 14 days of experimental period (Fig. 3B), however, capsaicin fed animals (positive control group) showed unexpectedly no spontaneous locomotive activity rather obvious sleeping behavior all through the experimental period (Fig. 3A) and body weight change showed first 4days slight loss and thereafter until the end of experimental period the positive control group showed highest increase of body weight (Fig. 3B). Hence, the highly activated spontaneous locomotive activity of capsanthin group must be due to the increased production of ATP as the result of increased oxidation of fatty acids in muscle cell (Otton *et al.*, 1984; Lee *et al.*, 2015). The enhanced  $\beta$ -oxidation of fatty acids could be exemplified by the sudden increase of ketone body in the serum of mice fed more than 10  $\mu$ mol of capsanthin (Fig. 2C). Actually ketone body production in the

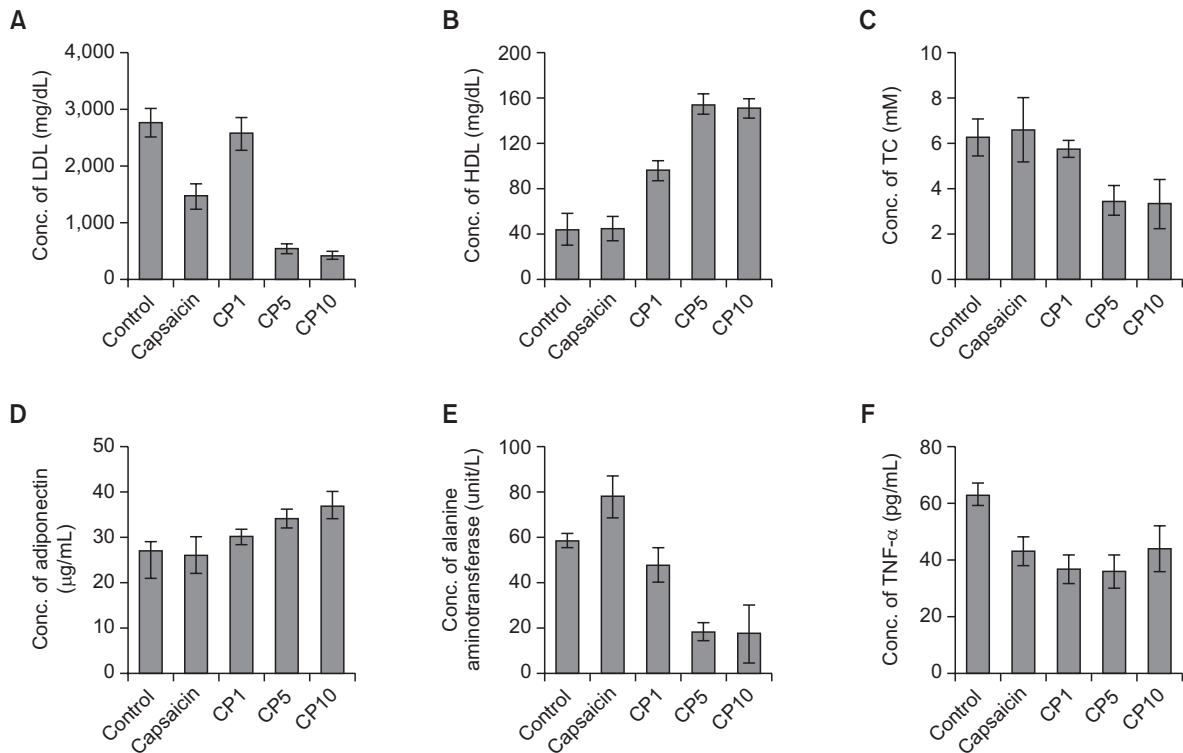
serum of mice fed with capsanthin under 5  $\mu$ mol were found to be base line level, however, mice fed with 10  $\mu$ mol showed abruptly high production of ketone body due to the overflow of acetyl-CoA to the maximum capacity of TCA-cycle. This may suggest that the branch point for the ketone body production will be near 5 to 10  $\mu$ mol per kg of mouse. This fact may be very useful in the future determination of optimum dosage of capsanthin for animal and human clinical experiments. These contrasting differences between capsaicin-fed and capsanthin fed animals may be explained based on the presence of potent UCP-1 induction due to  $\beta_3$ -agonistic activity in capsaicin fed mice (Yoshida *et al.*, 1998; Yoshioka *et al.*, 2001) and very weak or practically no UCP-1 induction due to the very weak  $\beta_3$ -agonistic activity in capsanthin fed mice (Fig. 4A). Due to potent  $\beta_3$ -agonistic activity of capsaicin, UCP-1 must be induced during first 4 days of experiments (Fig. 3B), hence ATP-deficit condition must be created during the later stage of experimental periods resulting in decreased spontaneous locomotive activity together with progressive body weight increase of capsaicin fed animal group (Fig. 3B).

#### Laparotomic view of mice after spontaneous locomotive running

At the end of capsanthin feeding experiments, all mice were sacrificed to obtain mice serum and to see the laparotomic view for visceral fat contents and found that capsaicin fed mice (positive control group) showed large size of white adipose tissue pad (WAT), however, capsanthin fed mice showed only highly shrunk brown adipose tissue (BAT) instead of WAT as shown in Fig. 3C. These results suggest that the anti-adipogenic activity of capsanthin may be directly concerned with anti-obesity activity of red pepper, however, capsaicin's thermogenic property is not concerned with anti-obesity activity in our present experiments.

#### Adipokine distribution in the serum of mice after capsanthin feeding experiments

Enhanced lipolytic activity of capsanthin will induce temporary overflow of fatty-acids in the blood stream which might influence negatively due to cyto-toxicities (Shimabukuro *et al.*, 1998; Bergman and Ader, 2000) to the normal physiology of mice depending on the distribution pattern of adipokines as adiponectin, p-AMPK, TNF- $\alpha$ , LDL, HDL, ALT, total cholesterol and triglyceride. Adiponectin contents in the serum of capsanthin fed mice showed dose dependently increased contents as shown in Fig. 5D, however, capsaicin fed positive



**Fig. 5.** Analysis for adipokines. Control: High fat diet (HFD), Capsaicin: HFD+Capsaicin 1 µmol, CP1: HFD+capsanthin 1 µmol, CP5: HFD+capsanthin 5 µmol, Cap10: HFD+capsanthin 10 µmol, respectively. (A) LDL content in serum, (B) HDL content in serum, (C) Total cholesterol content in serum, (D) Adiponectin content in serum, (E) ALT activity, (F) TNF-α activity.

control group showed unchanged. These apparently smaller increase of adiponectin concentration must be considered to be very meaningful, when we consider that the initial pool size of adiponectin has been well known very big in the animal and human bodies (Gil-Campos *et al.*, 2004) compared to other adipokines. The dose dependently increased adiponectin due to repeated feeding of capsanthin will induce the activation of p-AMPK as appeared in Fig. 4B, which will show balancing function of glucose utilization to fatty acid oxidation in the energy metabolism (Fruebis *et al.*, 2001; Wu *et al.*, 2003; Whitehead *et al.*, 2006). Adiponectin has further biological activities as anti-atherogenic (Yamauchi *et al.*, 2003), anti-inflammatory (Ye *et al.*, 2007), and antagonizing activity to the effect of TNF-α (Whitehead *et al.*, 2006). These activated fatty acid metabolism must be reflected, as shown in Fig. 5B. These results suggest highly beneficial effects of capsanthin to the normal physiology of mice by showing, dose-dependently increased serum level of HDL (Yamamoto *et al.*, 2002) and p-AMPK (Yamauchi *et al.*, 2002) and adiponectin (Whitehead *et al.*, 2006). The above mentioned beneficial adipokine distribution must be played by high molecular oligomerization of adiponectin. When the oligomerization of adiponectin will be incomplete due to some unidentified reason, the beneficial effect of adiponectin will be turned to be toxic to host animals and the incomplete oligomerization of adiponectin will be suggested by the elevation of ALT-level (Hickman *et al.*, 2007; Liu *et al.*, 2007) (Fig. 5E). As shown in Fig. 5E, capsaicin treated group showed highly enhanced ALT activity suggesting the liver toxicity of capsaicin, however, capsanthin treated group showed dose dependent regression of ALT activity despite to

the continuous feeding of capsanthin during 14 days. Furthermore TNF-α level, which is known to have antagonizing activity to adiponectin, remains unchanged as shown in Fig. 5F (Liu *et al.*, 2007). LDL-level (Fig. 5A) shows dose dependent decreasing tendency, HDL level (Fig. 5B) dose dependently increasing and total cholesterol (Fig. 5C) dose dependent decreasing tendency are shown. Those data suggest well balanced function of adiponectin to fatty acid metabolism due to adrenoceptor-β<sub>2</sub>-agonistic activity of capsanthin. Collectively, based on the above data, the the problem of temporarily overflow of fatty acid will not be occurred due to highly enhanced fatty acid oxidizing capacity. Finally TNF-α level (Hotamisligil, 1999) (Fig. 5F) which has been known to antagonize adiponectin activity showed no change. These data on adipokine distribution are suggesting highly beneficial contribution of capsanthin to the obesity reduction and also possibly to the improvement or amelioration (Whitehead *et al.*, 2006) of type-2 diabetic syndrome. These facts strongly suggest that capsanthin may be useful in the obesity reducing program and also to the amelioration of type 2 diabetic disorders (Antuna-Puente *et al.*, 2008).

## DISCUSSION

Capsanthin is contained in red pepper or paprika in the form of long chain fatty-acyl-esters (diacyl-ester; 80.8%, monoacyl-ester; 17.2%, free-form; 2.0%) (Schweiggert *et al.*, 2007). Esterified form capsanthin (98%) will be almost inactive to the in vitro anti-adipogenic activity screening test. This fact may be

the cause of worldwide negligence in the past screening of anti-adipogenic activity of capsanthin.

By the way to the capsanthin studies, we found an unexpected example in which the well known thermogenic (Diepvens *et al.*, 2007) capsaicin is not always concerned with anti-obese activity. Capsanthin has no concern with thermogenic property, since it has no adrenoceptor- $\beta_3$ -agonistic activity, hence it has no UCP-1 inductive activity.

Capsanthin the red xanthophyll pigment shows potent anti-adipogenic, lipolytic and fatty-acid burning activities due to its potent adrenoceptor- $\beta_2$ -agonistic activity. In the animal feeding experiment, mice showed highly enhanced spontaneous locomotive activity due to the excessive production of ATP from activated burning fatty acid. Together with sustained weight loss. Capsaicin which was adopted as the positive control in our experiment is highly anti-adipogenic and thermogenic substance, hence, showed no spontaneous locomotive activity and showed rather sleeping behaviors due to the deficit of ATP and showed no weight-loss, but rather weight increase. Capsanthin is potent anti-adipogenic but not thermogenic substance, hence it may be a good candidate for the development of new bioactive agent effective as a new anti-obese or insulin sensitivity enhancing substance.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGMENTS

The authors are deeply appreciating to our government, The Ministry of Science and Technology, Republic of Korea, for the financial support (Grant No. A004400069).

## REFERENCES

- Antuna-Puente, B., Feve, B., Fellahi, S. and Bastard, J. P. (2008) Adipokines: the missing link between insulin resistance and obesity. *Diabetes Metab.* **34**, 2-11.
- Babamoto, K. S. and Hirokawa, W. T. (1992) Doxazosin: a new alpha 1-adrenergic antagonist. *Clin. Pharm.* **11**, 415-427.
- Bergman, R. N. and Ader, M. (2000) Free fatty acids and pathogenesis of type 2 diabetes mellitus. *Trends Endocrinol. Metab.* **11**, 351-356.
- Diepvens, K., Westerterp, K. R. and Westerterp-Plantenga, M. S. (2007) Obesity and thermogenesis related to the consumption of caffeine, ephedrine, capsaicin, and green tea. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **292**, R77-R85.
- Farmer, S. R. (2006) Transcriptional control of adipocyte formation. *Cell Metab.* **4**, 263-273.
- Fruebis, J., Tsao, T. S., Javorschi, S., Ebbets-Reed, D., Erickson, M. R., Yen, F. T., Bihain, B. E. and Lodish, H. F. (2001) Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 2005-2010.
- Gil-Campos, M., Canete, R. R. and Gil A. (2004) Adiponectin, the missing link in insulin resistance and obesity. *Clin. Nutr.* **23**, 963-974.
- Hickman, I. J., Whitehead, J. P., Prins, J. B. and Macdonald, G. A. (2007) Raised alanine transaminase and decreased adiponectin are features of the metabolic syndrome in patients with type 2 diabetes. *Diabetes Obes. Metab.* **9**, 438-440.
- Hotamisligil, G. S. (1999) The role of TNF-alpha and TNF receptors in obesity and insulin resistance. *J. Intern. Med.* **245**, 621-625.
- Hsu, C. L. and Yen, G. C. (2007) Effects of capsaicin on induction of apoptosis and inhibition of adipogenesis in 3T3-L1 cells. *J. Agric. Food Chem.* **55**, 1730-1736.
- Joo, J. I., Kim, D. H., Choi, J. W. and Yun, J. W. (2010) Proteomic analysis for antiobesity potential of capsaicin on white adipose tissue in rats fed with a high fat diet. *J. Proteome. Res.* **9**, 2977-2987.
- Kang, J. H., Goto, T., Han, I. S., Kawada, T., Kim, Y. M. and Yu, R. (2010) Dietary capsaicin reduces obesity-induced insulin resistance and hepatic steatosis in obese mice fed a high-fat diet. *Obesity (Silver Spring)* **18**, 780-787.
- Kong, C. S. and Park, K. Y. (2008) Anti-obesity effect of garlic-added kochujang in 3T3-L1 adipocytes. *J. Food Sci. Nutr.* **13**, 66-70.
- Lee, M. S., Kim, C. T., Kim, I. H. and Kim, Y. (2011) Effects of capsaicin on lipid catabolism in 3T3-L1 adipocytes. *Phytother. Res.* **25**, 935-939.
- Lee, P., Birzniece, V., Umpleby, A. M., Poljak, A. and Ho, K. K. (2015) Formoterol, a highly beta2-selective agonist, induces gender-dimorphic whole body leucine metabolism in humans. *Metabolism* **64**, 506-512.
- Lin, F. T. and Lane, M. D. (1994) CCAAT/enhancer binding protein alpha is sufficient to initiate the 3T3-L1 adipocyte differentiation program. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8757-8761.
- Liu, Y., Retnakaran, R., Hanley, A., Tungtrongchitr, R., Shaw, C. and Sweeney, G. (2007) Total and high molecular weight but not trimeric or hexameric forms of adiponectin correlate with markers of the metabolic syndrome and liver injury in Thai subjects. *J. Clin. Endocrinol. Metab.* **92**, 4313-4318.
- Lizcano, J. M., Goransson, O., Toth, R., Deak, M., Morrice, N. A., Boudeau, J., Hawley, S. A., Udd, L., Makela, T. P., Hardie, D. G. and Alessi, D. R. (2004) LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. *EMBO J.* **23**, 833-843.
- Mahmood, T. and Yang, P. C. (2012) Western blot: technique, theory, and trouble shooting. *N. Am. J. Med. Sci.* **4**, 429-434.
- Maoka, T., Mochida, K., Kozuka, M., Ito, Y., Fujiwara, Y., Hashimoto, K., Enjo, F., Ogata, M., Nobukuni, Y., Tokuda, H. and Nishino, H. (2001) Cancer chemopreventive activity of carotenoids in the fruits of red paprika *Capsicum annuum* L. *Cancer Lett.* **172**, 103-109.
- Matsufuji, H., Nakamura, H., Chino, M. and Takeda, M. (1998) Anti-oxidant activity of capsanthin and the fatty acid esters in paprika (*Capsicum annuum*). *J. Agric. Food Chem.* **46**, 3468-3472.
- Otton, S. V., Inaba, T. and Kalow, W. (1984) Competitive inhibition of sparteine oxidation in human liver by beta-adrenoceptor antagonists and other cardiovascular drugs. *Life Sci.* **34**, 73-80.
- Rüttimann, A., Englert, G., Mayer, H., Moss, G. P. and Weedon, B. C. (1983) Synthesis of optically active natural carotenoids and structurally related compounds. X. Synthesis of (3R,3'S,5'R)-Capsanthin, (3S,5R,3'S,5'R)-Capsorubin, (3'S,5'R)-Cryptocapsin, and some related compounds. A new approach to optically active, five-membered-ring carotenoid building units by hydroboraton. *Helv. Chim. Acta* **66**, 1939-1960.
- Schuchardt, U., Sercheli, R. and Vargas, R. M. (1998) Transesterification of vegetable oils: a review. *J. Braz. Chem. Soc.* **9**, 199-210.
- Schweiggert, U., Kurz, C., Schieber, A. and Carle, R. (2007) Effects of processing and storage on the stability of free and esterified carotenoids of red peppers (*Capsicum annuum* L.) and hot chilli peppers (*Capsicum frutescens* L.). *Eur. Food Res. Technol.* **225**, 261-270.
- Shimabukuro, M., Zhou, Y. T., Levi, M. and Unger R. H. (1998) Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 2498-2502.
- Sompong, D. and Trakanrunroj, P. (2010) The flower of *Radermachera ignea* (kurz) steenis, a new source of zeaxanthin. *Suranaree J. Sci. Technol.* **17**, 303-308.
- Whitehead, J. P., Richards, A. A., Hickman, I. J., Macdonald, G. A. and Prins, J. B. (2006) Adiponectin-a key adipokine in the metabolic syndrome. *Diabetes Obes. Metab.* **8**, 264-280.
- Wu, X., Motoshima, H., Mahadev, K., Stalker, T. J., Scalia, R. and Goldstein, B. J. (2003) Involvement of AMP-activated protein kinase in glucose uptake stimulated by the globular domain of adiponectin in primary rat adipocytes. *Diabetes* **52**, 1355-1363.
- Yamamoto, Y., Hirose, H., Saito, I., Tomita, M., Taniyama, M., Matsubara, K., Okazaki, Y., Ishii, T., Nishikai, K. and Saruta, T. (2002)

- Correlation of the adipocyte-derived protein adiponectin with insulin resistance index and serum high-density lipoprotein-cholesterol, independent of body mass index, in the Japanese population. *Clin. Sci.* **103**, 137-142.
- Yamauchi, T., Kamon, J., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., Eto, K., Akanuma, Y., Froguel, P., Foufelle, F., Ferre, P., Carling, D., Kimura, S., Nagai, R., Kahn, B. B. and Kadowaki, T. (2002) Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat. Med.* **8**, 1288-1295.
- Yamauchi, T., Kamon, J., Waki, H., Imai, Y., Shimozawa, N., Hioki, K., Uchida, S., Ito, Y., Takakuwa, K., Matsui, J., Takata, M., Eto, K., Terauchi, Y., Komeda, K., Tsunoda, M., Murakami, K., Ohnishi, Y., Naitoh, T., Yamamura, K., Ueyama, Y., Froguel, P., Kimura, S., Nagai, R. and Kadowaki, T. (2003) Globular adiponectin protected ob/ob mice from diabetes and ApoE-deficient mice from atherosclerosis. *J. Biol. Chem.* **278**, 2461-2468.
- Ye, J., Gao, Z., Yin, J. and He, Q. (2007) Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. *Am. J. Physiol. Endocrinol. Metab.* **293**, E1118-E1128.
- Yoshida, T., Umekawa, T., Kumamoto, K., Sakane, N., Kogure, A., Kondo, M., Wakabayashi, Y., Kawada, T., Nagase, I. and Saito, M. (1998)  $\beta_3$ -Adrenergic agonist induces a functionally active uncoupling protein in fat and slow-twitch muscle fibers. *Am. J. Physiol.* **274**, E469-E475.
- Yoshioka, M., Doucet, E., Drapeau, V., Dionne, I. and Tremblay, A. (2001) Combined effects of red pepper and caffeine consumption on 24 h energy balance in subjects given free access to foods. *Br. J. Nutr.* **85**, 203-211.
- Zebisch, K., Voigt, V., Wabitsch, M. and Brandsch, M. (2012) Protocol for effective differentiation of 3T3-L1 cells to adipocytes. *Anal. Biochem.* **425**, 88-90.