CHAPTER IV
MATERIALS AND METHODS

4.1. Materials

4.1.1. Chemicals

All chemicals and reagents including pyridine, 5–5'–dithiobis(2–nitrobenzoic acid) (DTNB), *N*–phenacyl–4,5–dimethylthiazolium bromide (DMPTB), Triton X–100, potassium chloride (KCl), ethylenediaminetetraacetic acid (EDTA), eserine, β-mercaptoethanol, *o*-phthaldialdehye, acarbose, *p*-nitrophenyl-α-D-maltopentaoside (PNPG-5), *p*-nitrophenyl-α-D-glucopyranoside (pNPG) and hippuryl–histidyl–leucine (HHL) were received from Sigma–Aldrich (St. Louis, MO, USA).

Standards including lutein, α-carotene and zeaxanthin were received from Carotenature (Lupsingen, Switzerland). Capsanthin, β-carotene, β-cryptoxanthin, lycopene, myricetin, luteolin, isorhamnetin, quercetin, hesperitin, kamepferol, apigenin chlorogenic acid, 4-hydroxybenzoic acid, caffeic acid, galic acid, syringic acid, vanillic acid, *p*-coumaric acid, sinapic acid, *t*-cinnamic acid, naringenin and ferulic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Solvents including hexane, ethyl acetate, ethanol, acetonitrile, dichloromethane and methanol were received from RCI Labscan (Bangkok, Thailand). Tertiarybutylhydroquinone (tBHQ) and ascorbic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Trifluoroacetic acid (TFA) and hydrocroloc acid (HCl) were received from Merck (New Jersey, USA).

Enzymes and proteins used in this research including *Candida rugosa* lipase (Type 8, ≥700 unit/mg), porcine pancreatic α-amylase (Type 7, ≥10 unit/mg), *Saccharomyces cerevisiae* α-glucosidase (Type 1, ≥10 unit/mg), rabbit lung angiotensin-converting enzyme (≥2 unit/mg) and bovine serum albumin (BSA, ≥98% agarose gel electrophoresis) were received from Sigma–Aldrich (St. Louis, MO, USA).

4.1.2. Equipments

The samples were blended using a Philips 600W blender from Philips Electronics Co., Ltd. (Jakarta, Indonesia). The samples were dried using a Heto Powerdry PL9000 from Heto Lab Equipment (Allerod, Denmark). The evaporation was performed using a N-1200B series rotary evaporator with OSB-2100 bath from EYELA (Tokyo, Japan).

The large volume centrifugation was performed using a ROTINA 38R Centrifuge from Hettich Lab Technology (Tuttlingen, Germany). A small volume (microcentrifuge scale) was centrifuged using a tabletop Spectrafuge 16M microcentrifuge from Labnet International, Inc. (New Jersey, USA).

A high performance liquid chromatography (HPLC) system consisted of an Agilent 1100 series HPLC with a photodiode array detector and an Eclipase XDB-C18 guard column (4.6 mm x 12.5 mm, 5 µm) from Agilent Technologies (Santa Clara, CA, USA). The column for analyses of flavonoids and phenolic acids was a Zorbax Eclipase XDB-C18 column (4.6 mm x 150 mm, 5 µm) from Agilent Technologies (Santa Clara, CA, USA), while the column for carotenoids analysis was an isocratic reverse phase column, Vydac 201TP54-C18 (4.6 mm x 250 mm, 5 µm) from Grace Davison Discovery Science (Columbia, MD, USA). The PTFE syringe filters (0.2 µm) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The quality and quantity of chromatogram were analyzed by ChemStation (Agilent Technologies, Santa Clara, CA, USA). Water used in all HPLC experiments was Milli-Q water (18.2 MΩ-cm conductivity).

The identification of volatile compounds was performed using a gas chromatography-mass spectrometry (GC-MS) consisted of an Agilent 7890A gas chromatograph equipped with a 5975C mass spectrometry inert XL MSD with triple-axis detector (Agilent Technologies, Santa Clara, CA, USA) supplied by the Salaya Central Instrument Faculty (SCIF), Mahidol University. Volatile compounds were absorbed on a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (Sigma-Aldrich, *St. Louis, MO,* USA) and separated on a HP-5MS fused silica capillary column (30 m × 0.25 mm, 0.25µm) from Agilent Technologies (Santa Clara, CA, USA). The compounds were identified using NIST library in mass spectrometry (Scientific Instrument Services, Inc., Ringoes, NJ, USA).

The enzymatic assay was performed on a Synergy^{TM} HT 96-well UV-visible spectrophotometer using a Gen5 data analysis software (BioTek Instruments, Inc., Winooski, VT).

4.1.3. Plant materials

Sweet peppers (green, yellow, orange and red fruit colors) used in this study were purchased from Baan Rai Paovaris, Pak Chong, Nakhon Ratchasima, Thailand during October - November (2013). All samples have been carefully checked for quality regarding species, maturity stages and physical appearances. Sweet pepper fruits were examined for integrity and purity (dust and insect contamination).

4.2. Experimental Procedure

4.2.1 Preparation of sweet pepper extracts

Sample preparation and extraction were summarized in Appendix A. Sweet peppers were washed with deionized water. The samples were cut into small pieces (about 1 cm x 1 cm) and analyzed color by C.I.E. LAB (L*, a*, b*) system using ColorFlex EZ spectrophotometer from HunterLab (Reston, Virginia, USA). Then, the samples were freeze-dried and stored in aluminum foil bag at -20 ^{o}C until analysis.

The moisture content of fresh and freeze-dried samples was determined using Association of Analytical Communities Method 930.15 (AOAC, 2005). Air oven was regulated to the temperature of 135±2 ^{o}C. The samples (approx. 2 g) were evenly distributed and placed in a low, covered aluminium dishes (≥50 mm diameter and 40 mm deep). The samples were incubated in the oven for 2 hours before being cool in a desiccator. The percentage of moisture content and the percentage of dry mass were calculated using the following equation;

$$\%\ \text{Moisture content (w/w)} = \left(\frac{\text{weight loss on drying}}{\text{weight test portion}}\right) \text{x } 100,$$

$$\% \text{ Dry matter} = 100 - \% \text{ moisture content}$$

Freeze-dried sweet peppers (11 g dry weight) were extracted under three solvents (400 mL), including hexane, ethyl acetate and 70% (v/v) aqueous ethanol, using a Soxhlet extractor for 6 hours. The extractant was evaporated, and the remaining was resuspended with a minimal volume of 50% (v/v) DMSO and stored at -40 °C.

The variation of extraction was investigated using sweet peppers (green, yellow, orange and red fruit colors), which was extracted in triplicate under particular solvents. The quality of the extraction condition was examined using macadamia nut (with known % recovery yield) as a control.

4.2.2 Determination of phytochemical compounds

Sample extraction was performed using the method of Judprasong *et al.*, 2013 [70]. Freeze-dried samples (0.2 g) were boiled with ethanolic potassium hydroxide with 10% (w/v) ascorbic acid (10 mL) for 30 min. Then, the samples were extracted with hexane 70 mL (shake for 2 min at room temperature). The extractant was evaporated, and the remaining was resuspended in mobile phase solvent (3 mL of acetronitrile:methanol:dichloromethane (80:11:9 v/v/v) containing 0.01 %(v/v) triethylamine and 0.01 (w/v) ammonium acetate) and filtered through a 0.2 µm PTFE syringe filter. The determination of carotenoid content was performed using the method of Judprasong *et al.*, 2013 [70]. The HPLC analysis was performed using the isocratic reverse phase column, 0.5 µm Vydac 201TP54-C18 (4.6 x 250 mm) with a flow rate of 0.7 mL/min. Individual carotenoids were identified by comparing retention time with standards (capsanthin, lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene).

Flavonoids and phenolic acids was performed using the method of Judprasong *et al.*, 2013 [70]. Freeze-dried samples (2.5 g) were hydrolyzed with 62.5% (v/v) methanol (40 mL) containing 0.5 g/L tBHQ and 6 N HCl at 80 °C for 2 hours. To the hydrolyzed sample, 1% (v/v) ascorbic acid (100 µL) was added. The sample was then filtered through a 0.2 µm PTFE syringe filter. The HPLC analysis was performed using 5 µm Zorbax Eclipase XDB-C18 column (4.6 mm x 150 mm).

The mobile phases were Milli-Q water containing 0.05% (w/w) trifluoroacetic acid (TFA) (solvent A), methanol containing 0.05% (w/w) TFA (solvent B), acetronitrile 0.05% (w/w) TFA (solvent C) was used at flow rate of 0.6 mL/min. Flavonoids and phenolic acids were identified by comparing retention time with standards (gallic acid, 4-hydroxybenzoic acid, chlorogenic acid, syringic acid, vanillic acid, caffeic acid, *p*-courmaric acid, ferulic acid, sinapic acid, *t*-cinnamic acid, quercetin, kamepferol, isorhamnetin, myricetin, apigenin, luteolin, naringenin and hesperitin).

Bioactive compound identification was performed using a liquid chromatography-mass spectrometry (LC-MS) including MicrOTOF spectrometer (ESI-TOF MS) from Bruker Daltonics (Billerica, MA, USA) supplied by Biotec, National Science and Technology Development agency (NSTDA). The column for LC-MS analysis of flavonoids and phenolic acids was a Kinetex™C18 column 100 Å, 3-column Batch Val. Kit, (4.6 mm x 150 mm, 2.6μm) from Phenomenex, Inc. (Torrance, CA, USA). Hystar software is a mass spectrometry software supported from Bruker Daltonics (Billerica, MA, USA) used for analyzed quantity and quality of chromatogram in LC-MS.

Volatile compound analysis utilizing headspace solid phase microextraction (HS-SPME) and GC-MS was adapted from Azar *et al*., 2010 [71]. The small pieces (about 1 cm x 1 cm) of pericarps of fresh sweet pepper (1.5 g) in a vial were incubated in air bath at 70 ˚C for 30 minutes. Volatile compounds were then absorbed on a DVB/CAR/PDMS fiber for 20 minutes and separated by gas chromatography. The oven temperature was held at 60 ˚C for 3 minutes then programmed at rate of 6 °C/min until reaching 250 °C. The flow rate of helium gas, a carrier gas, was 1 mL/min. The temperature in split/splitless injector mode was set at 250 °C. Electron-impact mode (EI) was set at 70 eV. The compounds were identified using NIST library in mass spectrometry.

4.2.3 Determination of lipase inhibitory activity

High throughput analysis of lipase activity was adapted from the method of Choi *et al*., 2003 [72]. The lipase inhibitory activity was determined by the colorimetric couple assays, utilizing the reaction of sulfhydryl product from lipase reaction and Ellman’s reagent (DTNB) as an indicator (Appendix B). The assay was consisted of lipase (0.5 µg) in 50 mM Tris (pH 8.0) containing 0.1% (w/v) BSA, DMPTB (0.05 mM) in 50 mM Tris (pH 7.2) containing 10 mM KCl and 1 mM EDTA, DTNB (0.8 mM) in 50 mM KPB (pH 7.0) and sweet pepper extract. The reaction was monitored at a wavelength of 412 nm using a microplate reader. The results were calculated as a percentage of inhibitory activity using the following equation;

$$\%\text{ inhibition} = \left(1 - \frac{B-b}{A-a}\right) \text{ x } 100,$$

where A is an initial velocity of the control reaction with lipase (control), a is an initial velocity of the control reaction without lipase (control blank), B is an initial velocity of the enzyme reaction with extract (sample) and b is an initial velocity of the reaction with extract but without lipase (sample blank) (Table 4.1).

Table 4.1 The control (*A*), control blank (*a*), sample (*B*) and sample blank (*b*) reactions used in the enzyme assay

Reactions	Enzyme	Substrate	Indicator	Plant extract
A	√	√	√	-
a	-	√	√	-
B	√	√	√	√
b	-	√	√	√

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4.2.4 Determination of α-amylase inhibitory activity

High–throughput α-amylase inhibition assay was performed according to the method of Funke and Melzig, 2006 [73]. The α-amylase inhibitory activity was determined by the colorimetric assay, utilizing the *p*-nitrophenol from α-amylase reaction (Appendix C). The assay was consisted of α-amylase (4 mg), PNPG-5 (6.25 mM) and sweet pepper extract in assay buffer (50 mM KPB (pH 7.0) and 200 mM KCl). The reaction was monitored at a wavelength of 405 nm using the microplate reader. The results were calculated as a percentage of inhibitory activity as above.

4.2.5 Determination of α-glucosidase inhibitory activity

High–throughput α-glucosidase inhibitory assay was investigated according to the protocol described by You *et al.*, 2011 [74]. The α-glucosidase inhibitory activity was determined by the colorimetric assay, utilizing the *p*-nitrophenol from α-glucosidase reaction (Appendix D). The assay was consisted of α-glucosidase (0.1 U/mL), pNPG (0.5 mM) and sweet pepper extract in assay buffer (50 mM KPB, pH 7.0). The reaction was monitored at a wavelength of 405 nm using the microplate reader. The results were calculated as a percentage of inhibitory activity as above.

4.2.6 Determination of ACE inhibitory activity

High–throughput ACE inhibitory assay was determined according to the method of Schwager *et al*., 2006 [75] with some modifications. The ACE inhibitory activity was determined by utilizing the reaction of L-His-L-Leu (HL) from ACE reaction with *o*-phthaldialdehye (Appendix E). The assay was consisted of ACE (0.5 U/mL), HHL (3 mM), *o*-phthaldialdehye (20 mg/mL) and sweet pepper extract in the 96–well black microplate. The reaction was incubated at 37 °C for 30 minute. The reaction was monitored with an excitation wavelength of 360 nm and an emission wavelength of 485 nm using the microplate reader. The results were calculated as a percentage of inhibitory activity as above.

4.3. Statistical Analysis

All of the experiments were carried out in triplicate. The data are expressed as mean ± standard deviation (SD). All statistical analyses were carried out using a GraphPad Prism version 5.1 (GraphPad Software, Inc., La Jolla, CA). One way analysis of variance (ANOVA) and Tukey's multiple comparison test performed to determine the significant differences between values. Significance of difference was defined at $p<0.05$. Pearson's correlation coefficient was calculated using Microsoft Excel 2007.