CHAPTER III

MATERIALS AND METHODS

3.1 Subjects

Male and female subjects with type 2 diabetes who received the treatment at Public Health Center 66, Health Department, Bangkok Metropolitan Administration were recruited to participate in this study. All subjects aged at least 35 years old. They received the treatment with only sulfonylureas and/or biguanides medication and had FPG concentrations between 100-250 mg/dl. The concentrations of total cholesterol and triglyceride of all subjects were less than 240 mg/dl and 200 mg/dl respectively. They had blood pressure less than 160/100 mmHg and body mass index between 18.5-29.9 kg/m^2. In addition, they were able to read and write Thai language and willing to participate in this study.

The subjects were excluded if they had one of the following conditions: hepatic disease, renal disease, thyroid disease, cancer, serious infection, surgery, malnutrition, pregnancy, and breast feeding. The subjects with a history of allergy to bovine milk or lactose intolerance and with regular alcohol drinking or smoking were also excluded. Furthermore, this study excluded the subjects who were taking immunosuppressant, antiinflammatory medications, and any nutrition or herbal supplements that were likely to affect the study outcomes. During the study, the subjects were withdrawn if they were unable to tolerate the adverse effects of whey protein, unwilling or inability to comply with study protocol for any reasons (e.g. consumed the WPI less than 80% of dose requirement, loss to follow up).

The study protocol was reviewed and approved by the Ethical Committee of the Bangkok Metropolitan Administration, Bangkok (Appendix A). All subjects provided written consent (Appendix B) before participation in the study.

3.2 Experimental Design

This quasi-experimental study was conducted during the period from March to June 2009. The subjects participated in a 10-week study with two consecutive periods: a pre-experimental period (4 weeks) followed by a WPI supplementation period (6 weeks). The subjects were randomly divided into a treatment group or a control group by simple-random sampling method. The treatment group was supplemented with 30 g/day of whey protein isolate (WPI) for 6 weeks, and the control group received no supplement. All subjects were advised to consume a suitable diet for diabetic patients and informed about diabetic self-care. The blood biochemistry, insulin resistance, blood pressure and anthropometric parameters were measured at the beginning and the end of the WPI supplementation period. Dietary intake of each subject was assessed using the 3-day food records. Subject's compliance and adverse effects of WPI were also assessed in the treatment group.

3.3 Study Procedures

3.3.1 Pre-experimental Period

On the first appointment date, all subjects were informed about the purpose and procedures of the study. After obtaining informed consents, they were interviewed individually about their demographic characteristics, health history on diabetes, and dietary and physical activity behaviors. Blood pressure, weight, height

and anthropometric parameters were measured and recorded in the data record sheet (Appendix C). The nutritional status of each subject was screened using nutritional assessment form by the Nutrition Therapy Committee, Rajavithi Hospital (Appendix C). The subjects were informed about the importance of diet and diabetic control. Then, they received nutrition counseling and the diabetes self-care booklet (Appendix D). Moreover, they received the 3-day food records (Appendix E) and were instructed how to record a 3-day dietary intake. Each subject was scheduled for two subsequent study visits (4 and 10 weeks after the first appointment date). The subjects were asked to maintain amount of energy intake, to maintain level of physical activity, and to adhere to their oral hypoglycemic medications throughout the study.

3.3.2 Whey Protein Supplementation Period

At the beginning of the WPI supplementation period (week 0), fasting venous blood was obtained from each subject to determine baseline blood biochemistry. Blood pressure, body weight, height and anthropometric parameters were also measured. The 3-day food records were completed by the subjects prior to their visit. The subjects were randomly divided into the treatment and control groups. The subjects in the treatment group received 45 sachets of 30 g WPI/sachet for 6-week supplementation. They were advised to consume a sachet of WPI daily until the end of week 6.

At the end of the 6-week supplementation period (week 6), blood sample of the subjects were collected for determining biochemical parameters. Blood pressure and anthropometric measurements were also taken. Additionally, the 3-day food records were done by the subjects during the supplementation period. For the treatment group, the subject's compliance with the dosing regimen was assessed by

interviewing and counting the consumed and unconsumed sachets of WPI. Furthermore, they were interviewed about adverse effects of WPI consumption.

3.4 Research Instruments

3.4.1 Whey Protein Isolate

Whey protein isolate (Provon 290®; Glanbia Nutritionals, USA) containing 90.0% protein in powder form was packed in the seal aluminum foil packaging to protect moisture and light (30 g/sachet). One sachet of the WPI contained 26.7 g of protein and provided 111.9 kcal. The data of the WPI was presented in Appendix F. The subjects in the treatment group were advised to take 30 g of WPI after breakfast daily for 6 weeks by mixing a sachet of WPI with a glass (240 ml) of cold water or their favorite liquid foods such as juice, milk, coffee, yogurt, rice soup, or noodle soup. The WPI drink must be stirred thoroughly before consuming.

3.4.2 3-Day Food Record

The 3-day food record was used for assessment of food consumption and nutrient intake of the subjects. The subjects recorded their food intake for three days during the week (1 weekend day and 2 weekdays) prior to their visits. They were asked to record items and portions of food and beverages consumed including name, method of preparation. They estimated food portion size using standard household measuring cups and spoons. Then, investigator converted portion size into gram of foods. At each visit, the subjects were individually queried about their record's response by investigator for purposes of clarity and to eliminate any ambiguity in their responses. All raw dietary data were transformed to be energy, nutrients, and

distribution of protein, fat, and carbohydrate by the computerized program "Thai Nutrisurvey version 2" (Department of Health, Ministry of Public Health and Faculty of Tropical Medicine, Mahidol University, Thailand). Daily energy and nutrient intake were presented in kilocalories (kcal) and percentage of ratio of carbohydrate, protein, and fat.

3.4.3 Physical Activity Questionnaire

The physical activity questionnaire was modified from National Institute for Health and Clinical Excellence (NICE) guideline (NICE, 2006). The questionnaire was used to classify the subjects into four different levels of physical activity including inactive, moderately inactive, moderately active, and active. The subjects were classified the levels of physical activity by physical activity index (PAI). The PAI was classified by type of present work and level of physical activity (Appendix C). Physical activities included physical exercise, cycling, walking, housework, childcare, and gardening.

3.5 Blood Sample Collection

During the study period, venous blood sample of subjects were collected two times (at weeks 0 and 6) for biochemical tests. At each time, 15 ml venous blood samples of each subject were drawn from the antecubital vein using sterile needle syringe or vacuum tubes. The subjects were asked to fast overnight (about 10-12 hours) before blood samples were drawn in the morning. All blood samples were kept and transported in ice box until processing in the laboratory. All biochemical parameter determinations were taken within 24 hours after blood sample collection.

3.6 Determination of Biochemical Parameters

Biochemical parameters including FPG, HbA1c, TG, total-C, LDL-C, HDL-C, albumin, BUN, SCr, uric acid, AST, ALT, ALP, and electrolytes were assayed at laboratory unit, Public Health Laboratory Division, Bangkok Metropolitan Administration. All parameters were measured by colorimetric and turbidimetric methods using a ABX-Pentra 400 automated chemistry analyzer (HORIBA ABX, Montpellier, France). Serum insulin determination was taken at the laboratory unit of Bangkok Pathology-Laboratory Co., Ltd., Bangkok. Serum insulin was measured by solid-phase, two-site chemiluminescent immunometric assay using the IMMULITE-1000 Analyzer (Siemens Healthcare Diagnostics, Los Angeles, USA).

3.7 Insulin Resistance Assessment

The homeostasis model assessment (HOMA) method was used to assess insulin resistance and β-cell function of the subjects. The homeostasis model assessment of insulin resistance (HOMA-IR) and β-cell function (HOMA-B%) were calculated from fasting serum insulin (μIU/ml) and glucose (mmol/l) values with the following formulas (Matthews et al., 1985):

$$\text{HOMA-IR} = \frac{\text{fasting blood insulin} \times \text{fasting blood glucose}}{22.5}$$

$$\text{HOMA-B\%} = \frac{20 \times \text{fasting blood insulin}}{\text{fasting blood glucose} - 3.5}$$

3.8 Blood Pressure Measurement

Blood pressure was measured in the upper arm with the participant seated using a mercury sphygmomanometer (Hico Medical Co.Ltd., Tokyo, Japan) and a stethoscope (Spirit Medical, UK). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were reported in millimeter of mercury (mmHg).

3.9 Anthropometric Measurement

Anthropometric measurements in this study included the measurements of body weight, height, body mass index (BMI), waist circumference (WC), hip circumference (HC), Waist-to-hip ratio (WHR), triceps skinfold thickness (TSF), mid upper arm circumference (MAC), and mid arm muscle circumference (MAMC). The measuring technique employed complied with the National Health and Nutrition Examination Survey (NHANES) protocols (NHANES, 2004).

Body weight in kilometers (kg) was measured with the participant in light clothing and bare feet on a mechanical balance beam scale with height measuring rod, Health-O-Meter® Model 402KL (Health-O-Meter, Illinois, USA) calibrated to the nearest 0.1 kg. Height in centimeters (cm) was measured at one decimal. Then, BMI (kg/m^2) was calculated as body weight in kg divided by the square of the height in metres. The subjects were categorized into underweight, normal weight, overweight and obese by BMI according to the WHO Asia-Pacific guideline for Asian adults (International Obesity Task Force, 2000):

Underweight	BMI < 18.5 kg/m^2
Normal weight	BMI 18.5-22.9 kg/m^2
Overweight	BMI 23.0-24.9 kg/m^2
Obese	BMI ≥ 25.0 kg/m^2

WC was measured to the nearest centimeter with a measuring tape while the subjects were in a standing position at the end of gentle expiration. The following areas were used: laterally midway between the lowest rib and the top of the anterior iliac crest. Two measurements were taken. If the two measurements were identical, then that value was used. If the two values were different, then a third measurement was taken to determine which value was correct. Abdominal obesity was defined as ≥ 90 cm in men and ≥ 80 cm in women. The HC was measured with the tape measure passing round the widest part of the buttock and the symphysis pubis. Then, WHR was calculated as WC (in cm) divided by HC (in cm). The normal WHR was defined as WHR <0.9 in men and < 0.8 in women (Grundy et al., 2005).

MAC measures muscle mass and subcutaneous fat. MAC was measured to the nearest centimeter with a measuring tape at the midpoint of the upper dominant arm, between the acromion process and the tip of the olecranon.

Triceps skinfold thickness measurement provides an estimate of the size of the subcutaneous fat depot. In this study, TSF was measured to the nearest millimeters with a conventional skinfold caliper (Cambridge scientific industries®, Maryland). The triceps skinfold locus, which was halfway between the acromion and olecranon process on the back of the upper dominant arm, was measured with the elbow stretch out. The final results were reported as average values from triplicate reading.

MAMC provides an index of muscle mass and an indication of somatic protein stores. MAMC (in cm) was calculated by the following formula:

$$\text{MAMC (cm)} = \text{MAC (cm)} - [3.14 \times \text{TSF (cm)}]$$

3.10 Metabolic Syndrome Assessment

The metabolic syndrome is an important cluster of coronary heart disease risk factors with common insulin resistance. The presence of the metabolic syndrome is associated with an increased risk for cardiovascular morbidity and mortality. In this study, subjects were labeled as having metabolic syndrome when 3 or more factors in the modified criteria of the National Cholesterol Education Program–Adult Treatment Panel (NCEP–ATP III) were presented. The criteria are shown in **Table 8** (Grundy et al., 2005).

3.11 Compliance and Adverse Effects of Whey Protein Supplementation

Subjects in the treatment group were called once a week throughout the WPI supplementation period to ensure their compliance. At the end of study, the subjects returned WPI sachets. The eaten and uneaten sachets were counted and calculated for percent of compliance as follow:

$$\text{Percent of compliance} = \frac{(\text{number of eaten sachets} \times 100)}{45}$$

Adverse effects were assessed by direct questions during 6 weeks of WPI supplementation and at the end of study.

Table 8 Diagnosis of metabolic syndrome

Measures (any 3 of 5 constitute diagnosis of metabolic syndrome)	**Categorical Cut points**
Abdominal obesity	≥ 90 cm (35 inches) in men* ≥ 80 cm (31 inches) in women*
Elevated TG	≥ 150 mg/dl (1.7 mmol/l) or on drug treatment
Reduced HDL-C	< 40 mg/dl (1.03 mmol/l) in men < 50 mg/dl (1.30 mmol/l) in women or on drug treatment
Elevated blood pressure	≥ 130 mmHg systolic blood pressure or ≥ 85 mmHg or on drug treatment
Elevated FPG	≥ 100 mg/dl or on drug treatment

* The waist circumference cut point appears to be appropriate for Asians.
HDL-C = high density lipoprotein cholesterol, cm = centimeter, mg/dl = milligram/deciliter, mmol/l = millimole/liter, mmHg = millimeter of mercury
Source: Grundy et al., 2005

3.12 Statistical Analysis

The demographic data, eating pattern, work and exercise characteristics and adverse effect data of the subjects were analyzed by descriptive statistics and were presented as number and percentage. Chi-Square test was used to compare between groups differences. Outcome variables (continuous data) were analyzed by inferential statistics and were presented as mean and standard error of mean (mean ± SEM). Normal distribution of the data was checked by Shapiro-Wilk's statistics, and if p value ≥ 0.05, data was considered to be normally distributed. Mean and SEM at baseline and week 6 after treatment were compared between the treatment and control groups for all outcome variables. When the distribution of variables was normal, two-tailed paired and independent sample t-tests were used to compare within group and between groups differences respectively. Significance was accepted with $p < 0.05$.