CHARPTER III

MATERIALS AND METHODS

1. Preparation of Low lactose Milk-based Medical Food

Materials : -Low fat pasteurized milk (Foremost(R))

-Lactase enzyme, Lactaid (R)

(Lactaid Co., Pleasantville, NJ.)

-Maltodextrin (Goodman Fielder Limited)

-Soybean oil, Salad oil (Best Foods(R))

-MCT oil (Mead Johnson)

-Lecithin (DSP, USA)

Methods :

1.1 Preparation of Low Lactose Milk

Lactase was added to low fat pasteurized milk, 946 ml, and left refrigerated (4° C) for 24 hr. Lactose content in milk was analyzed with high pressure liquid chromatographic method.[30-32] Suitable amount of lactase added to milk was studied.

1.2 Preparation of Spray-dried Low Lactose Milk-based Medical Food

Low lactose milk, 946 ml, from 1.1 was evaporated to approximately 450 ml in rotary vacuum evaporator (Rotavapor-R, Buchi, Switzerland) at 70° C.

Ten grams of soybean oil and 10 g of MCT oil were mixed and lecithin were added to make an emulsion. Then 90 g of maltodextrin and concentrated low lactose milk were added to the emulsion and

were homogenized for 10 sec with homogenizer (Polytron, Kinematica GmbH, Switzerland). Suitable amount of lecithin was determined by apparent colloidal stability of the emulsion.

Emulsion of low lactose milk-based medical food was spray-dried in mini spray dryer Buchi 190. Inlet air temperature, outlet air temperature and spraying rate of sample were adjusted to obtain dried fine powder. Physical properties and chemical compositions of spray-dried product were evaluated. Finished products were kept in tight light resistant container.

2. Determination of Physical Properties

2.1 Bulk Density [33]

Twenty grams of powder were placed in a 100 ml measuring cylinder. The cylinder was dropped 150 mm on to a soft pad ten times. Level of powder in g/ml was measured as bulk density.

2.2 Solubility Index [33]

Thirteen grams of powder were dissolved in 100 ml distilled water (24° C). Three drops of defoaming agent (Silicon Antifoam, Fluka) were added, the contents were homogenized for 90 sec in blender (National, MX-SII N). After standing for 15 min, the contents were mixed and poured into specially graduated conical centrifuge tubes to the 50 ml mark. The tubes were centrifuged in GS 100, Flements. Centrifuge for 5 min at 1000 r.p.m.. The supernatant liquid in each tube was removed to within 5 ml of the surface of the centrifuged sediment. Water (24° C) was added to each tube with agitation to disperse the sediment and filled to the 50 ml mark. Centrifuge for 5 min at 1000 r.p.m.. The volume of sediment in a tube in ml to the nearest scale division gave the Solubility Index.

2.3 Apparent Colloidal Stability [34]

Thirteen grams of powder were dissolved in 100 ml of distilled water. It was left undisturbed for at least 24 hr in refrigerator. Judge was made when a visible line of demarcation was seen.

3. Chemical Analysis of Spray-dried Product

3.1 Moisture Determination (Air Oven Method) [33,35,37]

The empty dish and lid were dried in the oven at 100° C until its weight became constant. Two grams of sample was accurately weighed and dried at 100° C in hot air oven for 3 hr then it was cool in desiccator and weighed. The sample was returned to the oven and dried until its weight became constant. The moisture content was calculated.

$$\text{moisture (\%)} = \frac{\text{loss of weight (g)}}{\text{initial sample weight (g)}} \times 100$$

3.2 Protein Determination (Kjeldahl Method) [36,37]

Materials : -Concentrated sulphuric acid, A.R. (E.Merck)
-Catalyst Tablet, 3.5 g K_2SO_4, 0.4 g $CuSO_4$ (Kjeltabs C. 3, 5, Tecator, Sweden)
-Sodium hydroxide 40%, G.R. (E. Merck)
-Boric acid 4%. G.R. (E. Merck)
-Sulphuric acid 0.1 N.
-Modified methyl red indicator, 0.2% methylred and 0.1% methylene blue in ethanol

Methods :

1. The homogenized sample was accurately weight 0.1-0.2 g and transfered to Kjeldahl tube.

2. One catalyst tablet and 4 ml of concentrated sulphuric acid were added.

3. Sample was digested at 370-420° C (System 12 1009 Digestor, Tecator, Sweden) until the liquid became completely clear. Remove the tube and allow to cool.

4. Fifteen milliliters of nitrogen-free water and 15 ml ofsodium hydroxide solution were added.

5. Sample was distilled at least 150 ml of distillate (Kjeltec System 1002 Distilling Unit, Tecator, Sweden) into an erlenmeyer flask with 20 ml of boric acid and 5 drops of indicator.

6. Distillate was titrated with 0.1 N sulphuric acid. Carry out a blank determination.

Calculation : Total nitrogen (%) $= \frac{(v_2 - v_1)N \times 0.014 \times 100}{W}$

Crude protein (%) $= \frac{(v_2 - v_1)N \times 1.4 \times \text{factor}}{W}$

whereas : N = normality of sulphuric acid

W = weight of sample (g)

v_1 = volume (ml) of sulphuric acid required for the blank test

v_2 = volume (ml) of sulphuric acid required for the test portion

6.25 is the general factor

6.38 is the factor for milk

3.3 Fat Determination (Rose - Gottlieb Method)[33,37,38]

Materials : -Aqueous amonia 35% w/v (Carlo Erba)

-Ethanol 95% v/v, A.R. (E. Merck)

-Diethyl ether, A.R. (E. Merck)

-Petroleum ether, A.R. (BDH)

-Mixed ether, equal volume of diethyl ether and petroleum ether

Methods :

1. Sample was accurately weighed 0.9-1 g into Rohrig tube and 10 ml of water was added. Shake until homogeneous.

2. Then 1.25-1.5 ml of ammonia was added. Sample was warmed to 60-70° C and maintained at this temperature for 15 min, cool and 10 ml of ethanol was mixed.

3. Diethyl ether 25 ml and petroleum ether 25 ml were added. The sample was shaked for 1 min and let stand unil the ethereal layer was completely seperated from the aqueous layer. The ethereal layer was decanted into a flask.

4. The extraction and decantation were repeated twice by adding mixed ether 30 and 25 ml, respectively.

5. All of the ethereal layers were added in the same flask. The ether was evaporated on water bath.

6. The residue fat was dried at 100 ± 2°C in the oven for 1 hr, cool in desiccator and accurately weighed.

$$\text{Fat (\%)} = \frac{\text{weight of extracted fat (g)}}{\text{weight of sample (g)}} \times 100$$

3.4 Ash Determination[33,37,39]

Sample was accurately weighed 2 g into a constant weight crucible and placed on hot plate until smoking ceased. Then the crucible was placed inside the muffle furnace (Gallenkamp, Size 2, Germany) at 550° C until sample was white appearance. The sample was cool in desiccator and accurately weighed.

$$\text{Calculation : ash (\%)} = \frac{\text{weight of ash (g)}}{\text{weight of sample (g)}} \times 100$$

3.5 Carbohydrate Determination (By Calculation)

Total contents in food was 100 percent.

Carbohydrate (%) = 100 - [moisture (%) + protein (%) + fat (%) + ash (%)]

3.6 Lactose Determination (High Pressure Liquid Chromatographic Method)[30-32]

Meterials : -Ethanol, 80% and absolute, A.R.(E. Merck)

-Standard sugar solution,freshly prepared. glucose, lactose, sucrose, maltose (Fluka Co.)

-Mobile phase HPLC grade acetonitrile/H_2O, 75/25 V/V, was filter through 0.45 µm membrane and degased under vacuum for 2 min.

Apparatus : -HPLC. Rheodyne injector, LC-3A pump, 2A RI detector, and Shi Chromatopac C-R1A recorder integrater. Shimadzu.

-Column. LC-NH_2 Supelco (4.6 mm i.d.x 25 cm) Shimadzu.

-Membrane filter, 0.2 µm and 0.45 µm

-Microsyringe, 20 µl.

Methods :

1. For analysis of powder, 4 g were dissolved in 4 ml of water. In liquid form, pipette 6 ml of sample into centrifuge tube.

2. Thirty milliliters of absolute ethanol was added. Sample was mixed on mixer at high speed for 1 min. The samples were centrifuged for 10 min at 2000 r.p.m. (GS 100, Glements Centrifuge).

3. The supernatant was decanted, and the pellet was washed with 2 ml of water. Then 8 ml of ethanol were added and the centrifuging was repeated.

4. Two supernatants were combined in a 50 ml volumetric flask with was brought to volume with 80% ethanol. A portion of sample was filtered through a 0.2 µm membrane filter.

5. Ten to twenty microliters of sample were analyzed by HPLC, flow rate of mobile phase was 2.0 ml/min.

6. Sample peaks were identified by compared with chromatogram of standard sugar. Concentrations of lactose in the samples were calculated by extrapolating the peak height of chromatogram against the concentration in the standard curve.

3.7 Amino Acid Composition (Performic Oxidation and HCl Hydrolysis)[40]

Meterials : -Hydrogen peroxide. 30 % v/v
-Formic acid. 89 % w/w
-Hydrochloric acid. 6 N
-Phenol
-Lithium loading buffer pH 2.2
-Peroxide mixture, hydrogen peroxide 0.5 ml was added with formic acid 45 ml and phenol 25 mg. The sample was incubated at 30° C for 60 min, then cool in ice bath for 15 min.

Methods

1. Sample was weighed approximately 10 mg nitrogen and oxidized with peroxide mixture 7 ml at 0° C for 16 hr. Excess formic acid was destroyed by evaporation.

2. Sample from 1 was refluxed with 50 ml 6 N hydrochloric acid which added phenol at 145° C for 24 hr and re-evaporated to dryness.

3. Six normal hydrochloric acid was added to make up 100 ml, then 25 ml of sample were taken and re-evaporated to dryness.

4. Residue was diluted to 50 ml with Lithium loading buffer pH 2.2, and 5 ml were taken. AGPA (internal standard) 400 µl, was added and diluted 10 ml with Lithium loading buffer.

5. Sample was loaded into amino acid analyzer (Hitachi 835-50)

Condition : Analytical column : 2.6 x 250 mm (Resin # 2619)
Flow rate : 0.275 ml/min
Sample : 5 n mol/ 50 µl
Ammonia filter column : 4 x 200 mm (Resin # 2650)

6. The concentration of amino acid was evaluated by comparing with standard chromatogram.

4. Biological Assay (Rat Bioassay) [42,43]

4.1 Determination of Protein Efficiency Ratio (PER)

Nutritive value of food in this method was determined by measuring the growth rate of young animals fed a test food.

4.1.1 Protein evaluation basal diet consisted of 10% protein, 8% soybean oil, 5% salt mixture (Appendix B), 1% vitamin mixture (Appendix B), 1% cellulose, 5% water and 70% carbohydrate (sucrose : corn starch 1:1). Protein content was determined by Kjeldahl method.

4.1.2 Ten Wistar male rats (National Laboratory Animal Center, Mahidol University), 21-24 days of age, between 50-70 g of weight, were used for each diet. Rats were acclimated for 2-3 days before weaning, provided diet and water ad libitum.

4.1.3 Throughout assay period (four weeks), each rat was kept in individual metabolic cage and provided with appropriate diet and water ad libitum. The rats were maintained in a temperature controlled environment with a 12 light-dark cycle.

Three groups of rats were assayed. Protein sources were:

Group 1 (Casein reference group): Casein, Sigma Co.

Group 2 (Low lactose group) : Spray-dried low lactose milk-based medical food.

Group 3 (Lactose group) : Spray-dried milk-based medical food (untreated milk was substitued for low lactose milk)

4.1.4 Body weight and food intake of each rat were recorded at regular interval, not exceed 7 days and on the 28th day after beginning of assay period.

The PER for each rat was calculated as follow :

$$PER = \frac{\text{gain in weight of test animal (g)}}{\text{protein consumed (g)}}$$

If a corrrection to casein = 2.5 was to be made, proceed as follow:

$$\text{Correct PER} = \frac{\text{PER} \times 2.5}{\text{determined PER for reference standard casein}}$$

4.2 Determination of Net Protein Ratio (NPR)

Criteria for evaluation was the same as PER, but a zero protein control group was added.

Group 4 (Zero protein control group) : Diet composition was 8% soybean oil, 5% salt mixture, 1% vitamin mixture, 1% cellulose, 5% water, and 8% carbohydrate.

Rats were fed ad libitum for ten-day assay period. The NPR for each rats was calculated as follow :

$$NPR = \frac{\text{gain of test animal (g) + loss of control animal (g)}}{\text{protein consumed by test animal(g)}}$$

$$RNPR = \frac{\text{NPR test}}{\text{NPR reference}} \times 100$$

whereas : RNPR = Relative net protein ratio

4.3 Determination of Net Protein Utilization (NPU) by Nitrogen Balance Technique

Assays based upon nitrogen balance method which nitrogen intake and excretion were determined for rats fed diets containing the test protein or a protein - free diet.

Protein was fed at a level of 10 percent of the diet.

Group 1 : Low lactose group
Group 2 : Lactose group
Group 3 : Zero protein control group

Rats were fed ad libitum for ten-day assay period. Body weight and food intake of each rat were recorded. Urine and feces were collected daily throughout assay period. Nitrogen contents in food, urine and feces were analyzed with Kjeldahl method.

Calculation : $$NPU = \frac{\text{N retained}}{\text{N intake}} = \frac{I - (F - F_o) - (U-U_o)\ /100}{I}$$

$$= (BV \times TD)/100$$

$$TD = \frac{\text{N absorbed}}{\text{N intake}} = \frac{I - (F - F_o)}{I} \times 100$$

$$BV = \frac{\text{N retained}}{\text{N absorbed}} = \frac{I - (F - F_o) - (U-U_o)}{I - (F-F_o)} \times 100$$

whereas : TD = True digestibility

BV = Biological value

I = intake nitrogen

F = fecal nitrogen

Fo = fecal nitrogen at zero nitrogen intake

U = urinary nitrogen

U_o = urinary nitrogen at zero nitrogen intake

4.4 Statistical Analysis

Results were analyzed statistically by analysis of variance (ANOVA) and paired t-test.[44]