CHAPTER III

MATERIALS AND METHODS

Materials and methods

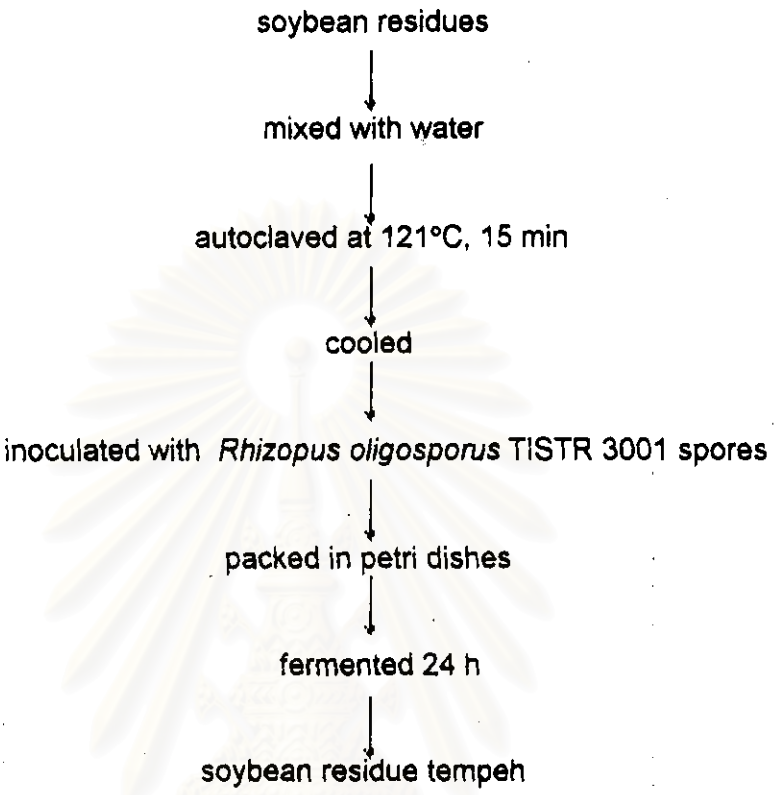
1. Preparation of tempeh

Tempeh was prepared by a modification of the methods of Lavanaya Kraidej (ลาวัณย์ ไกรเดช, 2519) and Zamora and Veum (1988) using aseptic technique.

1.1. Soybean residue tempeh

Soybean residues from Lactasoy Limited Company were mixed with distilled water to increase moisture content in the ratio of soy bean residues to distilled water 1:1. The soybean residues were then autoclaved at 121°C for 15 min, cooled to 37°C and inoculated with *Rhizopus oligosporus* TISTR 3001 spores at approximately 1 x 10^7 spores per 100 g of soybean residues (dry weight) and mixed thoroughly. The spore suspension of a 3-day-old culture of *Rhizopus oligosporus* TISTR 3001 grown on potato dextrose agar slants was used in this procedure. The inoculated soybean residues were packed in petri dishes and fermented at room temperature (30°C) for 24 h. After fermentation, there was a firm cake like structure due to profuse growth of the fungus on soybean residues. It has a clean, mushroomy or nutty odor (Nout and Rombouts, 1990). The fresh fermented soybean residues were dried in hot-air oven at 80°C for 2-3 days, ground and refrigerated for analysis of nutritive value.

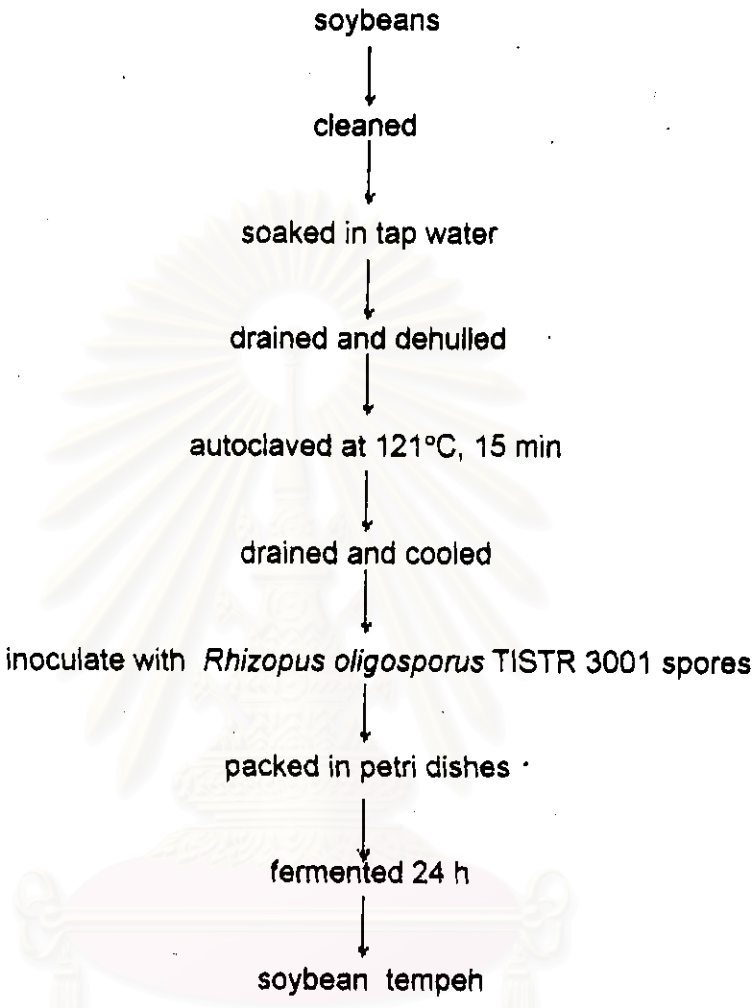
Figure 1 : Production of soybean residue tempeh



1.2. Soybean tempeh

Soybeans (*Glycine max* L.) were used in the fermentation procedure. Whole soybeans were cleaned to remove dirt, stones, weed seeds, damaged and possibly decomposed beans, and any other foreign matter. After cleaning, whole soybeans were soaked overnight in tap water (1 kg of soybeans per 3 l of water) to loosen the hulls. The soaked soybeans were drained and manually dehulled and the hulls were separated by floatation, accompanied by gentle stirring of the beans. The soybeans were then autoclaved at 121°C for 15 min, drained and cooled to 37°C and inoculated with *Rhizopus oligosporus* TISTR 3001 spores at approximately 1 x 10^7 spores per 100 g of soybeans (dry weight) and mixed thoroughly. The spore suspension of a 3-day-old culture of *Rhizopus oligosporus* TISTR 3001 grown on potato dextrose agar slant was used in this procedure. The inoculated soybeans were packed in petri dishes and fermented at room temperature (30°C) for 24 h. After fermentation, there was a firm cake like structure due to profuse growth of the fungus on soybeans. It has a clean, mushroomy or nutty odor (Nout and Rombouts, 1990). The fresh fermented soybeans were then dried in a hot-air oven at 80°C for 2-3 days, ground and refrigerated for analysis of nutritive value.

Figure 2 : Production of soybean tempeh



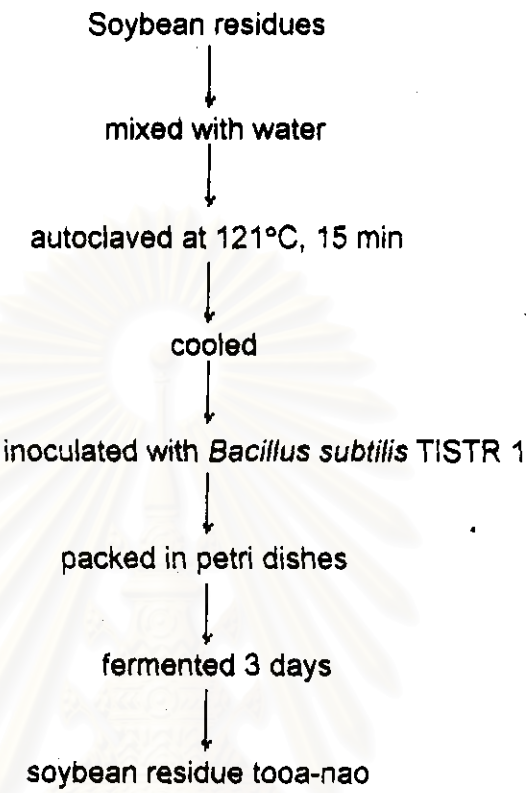
2. Preparation of tooa-nao

Tooa-nao was prepared by a modification of Bulan Phithakpol, Suparat Reungmaneepaitoon, and Warunee Varanyanon' s method using aseptic technique (Bulan Phithakpol, Suparat Reungmaneepaitoon, and Warunee Varanyanon, 1995) .

2.1. Soybean residue tooa-nao

Soybean residues were obtained from Lactasoy Limited Company. Mixed soybean residues with distilled water to increase moisture content in the ratio of soybean residues to distilled water 1:1. The soybean residues were then autoclaved at 121°C for 15 min, cooled to 37°C and inoculated with pure culture inocula of *Bacillus subtilis* TISTR 1 at approximately $1x10^6$ cells per g of soybean residues (dry weight) and mixed thoroughly. The cell suspension of 2-day-old culture of *Bacillus subtilis* TISTR 1 grown on nutrient agar slant (Sigma Chemical Co.,MO,USA) was used for fermentation. The inoculated soybean residues were packed in petri dishes and fermented at room temperature (30°C) for 3 days. The fermented soybean residues had brownish yellow color with strong smell. After fermentation, the fresh fermented mass were dried in a hot-air oven at 80°C for 2-3 days, ground and refrigerated for analysis of nutritive value.

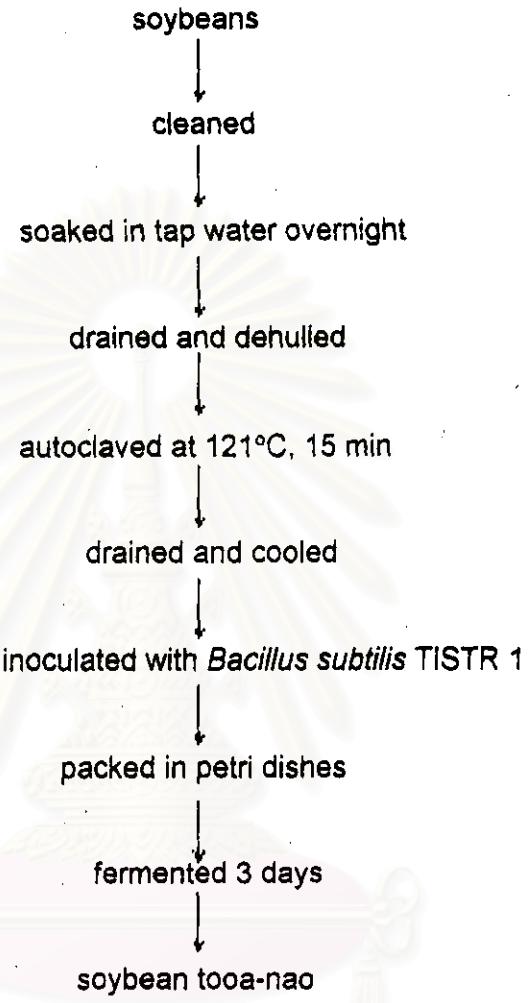
Figure 3 : Production of soybean residue tooa-nao



2.2. Soybean tooa-nao

Soybeans (*Glycine max* L.) were cleaned, washed, and soaked overnight in tap water (1 kg of soybeans per 3 l of water) to loosen the hulls. The soaked soybeans were drained and manually dehulled. The hulls were separated by floatation, accompanied by gentle stirring of the beans. The soybeans were then autoclaved at 121°C for 15 min, drained and cooled to 37°C and inoculated with *Bacillus subtilis* TISTR 1 at approximately $1x10^6$ cells per g of soybeans (dry weight) and mixed thoroughly. The cell suspension of 2-day-old culture of *Bacillus subtilis* TISTR 1 grown on nutrient agar slant was used for fermentation. The inoculated soybean were packed in petri dishes and fermented at room temperature (30°C) for 3 days. The fermented soybean residues have brownish yellow color with strong smell. After fermentation, the fresh fermented mass were dried in a hot-air oven at 80°C for 2-3 days, ground and refrigerated for analysis of nutritive value.

Figure 4 : Production of soybean tooa-nao



3. Heated unfermented soybean residues

The heated unfermented soybean residues were prepared in the same manner of soybean residues tempeh and soybean residues tooa-nao except the inoculation and fermentation steps.

4. Heated unfermented soybeans

The heated unfermented soybeans were prepared in the same manner of soybeans tempeh and soybeans tooa-nao except the inoculation and fermentation steps.

5. Determination of nutritive value

Each processed soybean residues and soybeans were analyzed as follows:

5.1. Proximate analysis (Osborne and Voogt,1972 ; AOAC,1990 ; James,1995)

5.1.1. Moisture was determined by hot-air oven method.

5.1.2. Crude protein was determined by Macro Kjeldahl method.

5.1.3. Crude fat was analyzed by Soxhlet method.

5.1.4. Ash was measured by dry ashing method.

5.1.5. Crude fiber was determined by Extraction unit for determining raw fiber content (VELP Scientifica, 1994).

5.1.6. Carbohydrate was calculated by difference method.

% Carbohydrate = 100 - (% moisture + % crude protein + % crude fat + % ash + % crude fiber)

All steps of analysis are shown in appendix I.

5.2. Amino acids composition

Amino acids composition were measured by amino acid analyzer, Beckman system 6300 series (Matheson,1974 ; Meason, Bech, and Rudemo,1980). Analysis procedure is shown in appendix I

The value of essential amino acids were conversed into amino acid score. Amino acid score was calculated by ratio of essential amino acid (mg) in 1 g of test protein to the same amino acid (mg) in 1 g of reference standard protein.

$$\text{Amino acid score} = \frac{\text{mg amino acid per g protein in test protein}}{\text{mg amino acid per g protein in reference standard protein*}} \times 100$$

* Standard protein of FAO/WHO 1973 (Joint FAO/WHO Ad Hoc Expert Committee,1973) is shown in appendix I.

5.3. Protein digestibility

The method of analysis was modified from Romero and Ryan (1978) and Nutjira Imanothai (1986).

Chemicals

Bovine serum albumin (fraction V powder), trypsin (type II), chymotrypsin (type II), and porcine pepsin were obtained from Sigma Chemical Co. (MO, USA). 1.0 M TNBS (2,4,6 - trinitrobenzenesulfonic acid) was supplied by Fluka AG (Buchs, Switzerland). Other chemicals were of best quality as possible.

All solutions used *in vitro* digestibility were made up with deionized distilled water.

solution A : 100 ml of 0.1 M Na_2SO_3 (made fresh weekly)

solution B : 1 litre of 0.1 M NaH_2PO_4

solution C : 1 litre of 0.1 M $Na_2B_4O_7$ in 0.1 M NaOH

solution D : 1.5 ml of solution A plus 98.5 ml of solution B (made fresh daily)

1.0 M TNBS : the solution is kept stoppered and is frozen when not in use.

Analytical procedure

Protein content of all samples were determined with Macro Kjeldahl method. *In vitro* protein digestibility with a multienzyme system (pepsin, trypsin, and chymotrypsin) of Romero and Ryan (1978) and Nutjira Imanothai (1986) was followed with certain modifications as described below.

Homogenized sample contained 0.12 g protein was homogenized with 25 ml 0.5 M sodium chloride. A standard bovine serum albumin (BSA) was simultaneously

prepared. The pH was adjusted to 1.5, the optimum pH for pepsin, with 0.08 M hydrochloric acid in 0.5 M sodium chloride. The slurry was maintained at 37°C in a constant temperature waterbath. Then, 12 ml of pepsin (1 mg/ml, containing 100 mM calcium chloride) was added to the slurry, keeping the enzyme : protein ratio to 1 : 10. The slurry would be digested enzymatically at 37°C for 4 h. Then to each digest, 0.8 M sodium barbital (pH 10.3) buffer was added and the pH adjusted to 8 for subsequent digestion with trypsin and chymotrypsin. A mixture of equal volume (6 ml) of enzyme trypsin and chymotrypsin (1 mg/ml, containing 100 mM calcium chloride) was added to the slurry (enzyme to protein ratio 1 : 10) maintained at 37°C and digestion was continued for 4 h at 37°C. The slurry was then heated in a boiling waterbath 5 min to arrest the enzyme reaction and volume was made to 200 ml with deionized distilled water. An aliquot of this digest was filtrated and supernatant was diluted 6.25 folds with deionized distilled water. For each digestibility determination : a substrate with enzyme, substrate without enzyme, and control(an enzyme only) were prepared.

The determination of alpha-amino groups with TNBS method of Field (1972) was applied. Diluted supernatant (0.5 ml) was added to 0.5 ml of borate buffer (solution C). Then, 22 µl of 1.0 M TNBS solution was added and the solution was rapidly mixed. After 5.0 min, the reaction was stopped by adding 2.0 ml of 0.1 M sodium dihydrogen phosphate containing 1.5 mM sulfite (solution D), and the absorbance at 420 nm was determined against appropriate blank.

Calculation

Value of optical density (OD) for substrate without enzyme and enzyme only were substracted from OD value of a substrate with enzyme. Calculation of per cent peptide bond hydrolysis from changes in absorbance involved determining the ratio of the number of new amino acid groups in the digestion to the total number of peptide bonds in the digestion mixture and express this ratio as a percentage. The number of new amino groups was calculated using 22000 as the molar extinction coefficient for TNP-alpha-amino groups at 420 nm (Fields, 1972), and the total number of peptide bonds was calculated by dividing the

grams of substrate in the digestion mixture by 113 g/mol, the average residue molecular weight of amino acids.

Per cent related peptide bond hydrolysis which shown in this study was calculated as described below.

$$\% \text{ related peptide bond hydrolysis} = \frac{\% \text{ peptide bond hydrolysis of sample}}{\% \text{ peptide bond hydrolysis of BSA}} \times 100$$

5.4. Vitamin analysis

5.4.1. Vitamin B_1 (thiamin)
5.4.2. Vitamin B_2 (riboflavin)
5.4.3. Niacin
5.4.4. Vitamin B_6 (pyridoxine)
5.4.5. Vitamin B_{12} (cobalamine)
5.4.6. Pantothenic acid

The above vitamins were assayed by microbiological methods (the analysis procedures are in appendix I).