CHAPTER III

MATERIAL AND METHODS

Reagents

Aminopyrene was obtained from Aldrich (St.Louis, U.S.A.). Sodium nitrite ($NaNO_2$), Hydrogen bromide (HBr), and N-(1-napthyl) ethylenediamine dihydrochloride ($C_{10}H_7NHCH_2CH_2NH_2 2HCl$) were purchased from BDH Chemicals Ltd. (Poole, England). Citric acid monohydrate GR ($C_6H_8O_7H_2O$), glacial acetic acid, L-histidine monohydrochloride, hydrochloric acid, magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), sodium chloride (NaCl), and sulfanilic acid ($C_6H_7NO_3S$) were obtained from E. Merck (Darmastadt, Germany). Bacto agar was a product of Difco Laboratories (Michigan, U.S.A.). D(+)-glucose monohydrate, dipotassium hydrogen phosphate anhydrous (K_2HPO_4), disodium hydrogen phosphate (Na_2HPO_4) and sodium ammonium hydrogen phosphate tetrahydrate ($NaNH_4HPO_4 \cdot 4H_2O$) were prepared by Fluka Chemica (Switzerland). Oxoid nutrient broth No.2 was furnished by Oxoid Ltd. (Basingstoke, Hants, England). Bovine serum albumin, d-biotin, sodium dihydrogen phosphate (NaH_2PO_4), and sodium thiocyanate (NaSCN) were obtained from Sigma Chemical Co. (St.Loius, U.S.A.).

Polysaccharides

Polysaccharides used in this study were soluble fiber except α-cellulose. Agar was obtained from Difco Laboratories (Michigan, U.S.A.). Alpha cellulose was a product of Sigma Chemical Co. (Missouri, U.S.A.). Carboxymethylcellulose was prepared by D.S.K. Internationals (Tokyo, Japan). Carrageenan was purchased from Systems Bioindustries (Baupte, France). Gum arabic and guar gum were obtained from Rickard Gum (England) and Jainson (Jodhiur, India) respectively. Locust bean gum was furnished by C.E.Roepera (GmbH and Co.) (Hamburg, Germany). Methylcellulose 25 cps, 1500 cps, and 4000 cps were obtained from Dow Chemical (Michigan, U.S.A.). Pectin was purchased from Hercules, Inc. (New York, U.S.A.). Sodium alginate was prepared by Went Cheme (Germany). Xanthan gum was obtained from C.N.I. (Paris, France). Ivy gourd was prepared by Laohavechvanich (1994). Their structure are shown in Appendix 1.

Nitrite Scavenging Study

Incubation Procedure Polysaccharide (0, 25, 50, 75, or 100 mg per tube) was added with 5 ml of simulated gastric mixture, containing 300 mg per 1,000 ml bovine serum albumin and imitated saliva consist of 0.3 mM sodium thiocyanate and 2 g of sodium chloride per 1,000 ml , and it was stored in a refrigerator overnight. Before incubation, 4.5 ml of 320 μM of sodium nitrite were added to reaction mixture

and pH was adjusted with 0.5 ml of 0.5 N hydrochloric acid to pH 1.5-2. The reaction was started by the addition of sodium nitrite and hydrochloric acid. Each reaction tube was incubated at 37°C for 120 min (Moller et al., 1988). The tube was then immersed in an ice bath for 10 min to stop the reaction. Then the reaction mixture was filtered through Whatman filter paper number 1.

Colorimetric Determination of Nitrite Remaining A modification of Takeda and Kanaya (1982) procedure was standardized and used to determine the content of nitrite. Filtrate of the reaction mixture (2 ml) was added to 2 ml of 1.5% hydrogen bromide in glacial acetic acid. The mixture was mixed and left to stand 15 min at room temperature. Then 4 ml of freshly prepared Griess reagent (0.5% sulfanilic acid, 0.05% N-(1-naphthyl)ethylenediamine dihydrochloride on 30% aqueous acetic acid) were added. The whole was mixed and allowed to stand for 15 min at room temperature, and the absorbance was determined at 550 nm. All steps in the nitrite scavenging evaluation are shown in Figure 3.1.

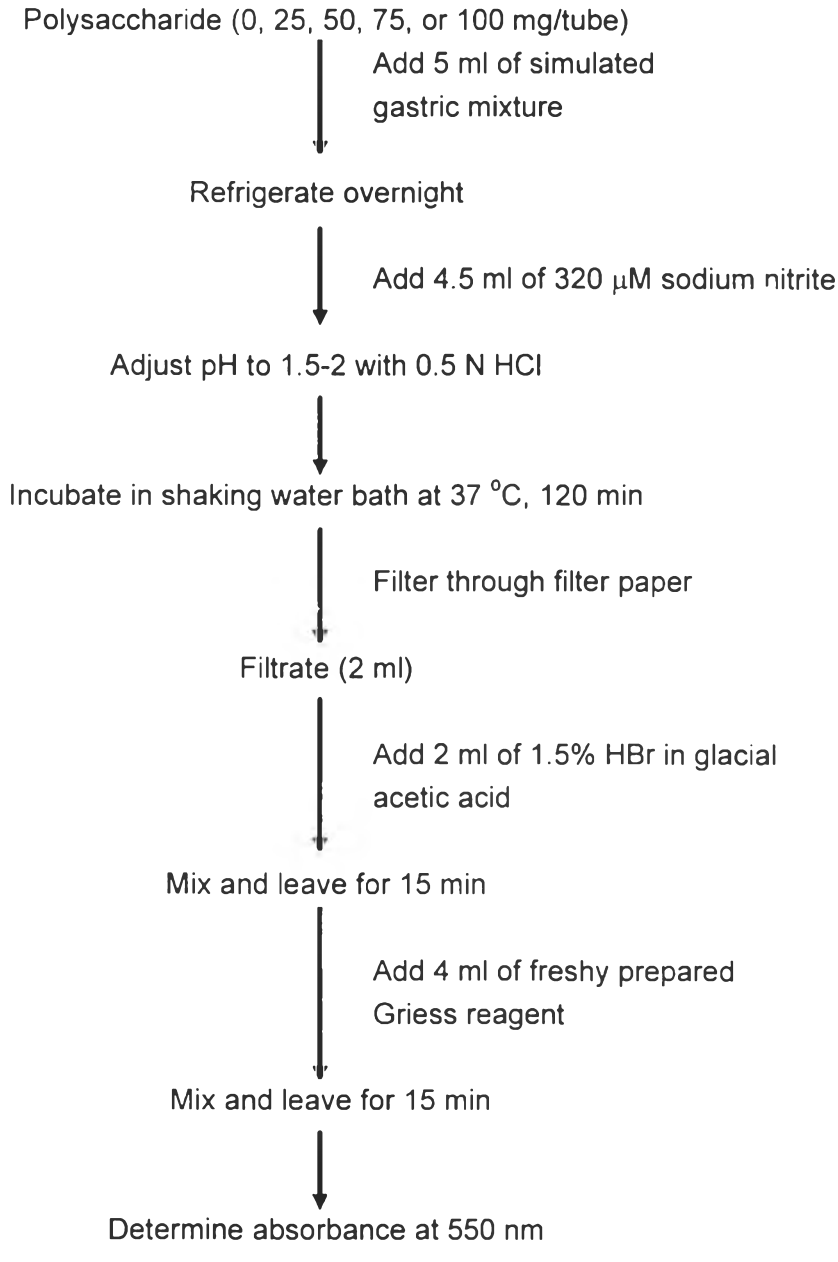

Figure 3.1 Steps in colorimetric determination nitrite scavenging evaluation

Mutagenesis assay

The bacterial tester strains *Salmonella typhimurium* strains TA 98 and TA 100 were provided by Dr. Wannee Kusamran of National Cancer Institute (Ministry of Public Health). Overnight cultures of strain TA 98 and TA 100 were

prepared in Oxoid nutrient broth No. 2 at 37°C with shaking. Manipulation of the cultures (Appendix 2) was done as suggested by Maron and Ames (1983).

Nutrient agar

Minimal agar plate. Each plate contained 30 ml of minimal glucose agar medium consisting of 1.5% Bacto-Difco agar and 2% glucose in Vogel-Bonner medium E (recipe in Appendix 2). A sterile petri dish was used.

Top agar. It contained 0.6% Difco agar and 0.5% sodium chloride (Appendix 2). It was autoclaved and kept warm in water bath (45°C). Before use, 10 ml of a sterile solution of 0.5 mM L-histidine and 0.5 mM biotin were added to each 100 ml of the molten agar and mixed throughly by swirling.

The pre-incubation method of Yahagi et al.(1977) was used throughout this study (Figure 3.2). For the determination of direct mutagenicity, an aliquot of 0.1 ml of each nitrite treated aminopyrene was mixed with 0.5 ml of Na_3PO_4-KCl buffer (pH 7.4) and 0.1 ml of fresh overnight culture of tester strain (*S. typhimurium* TA 98 or TA 100). The contents were mixed and incubated at 37°C for 20 min in a shaking water bath. After incubation, 2.0 ml of molten top agar (45°C) was added, mixed well and poured on to a minimal glucose agar plate. His^+ revertant colonies were counted after incubation at 37°C for 48 h. Duplicate tests were carried out for

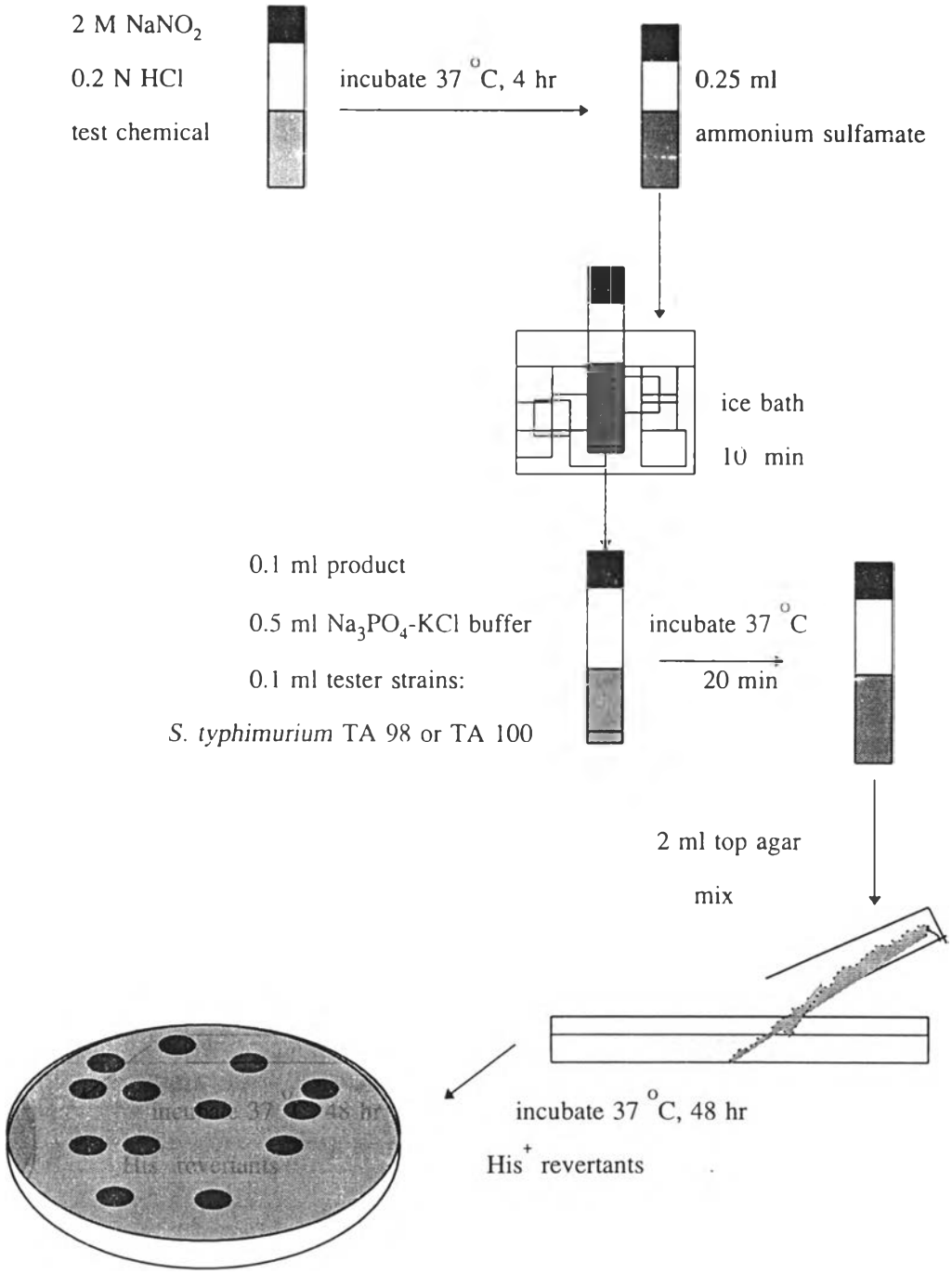

Figure 3.2 Steps in mutagenicity evaluation using the Ames test

(pre-incubation method)

each sample. Positive and negative control plates were included in each assay. Negative control containing the bacteria and solvent was required to establish spontaneous mutation of each tester strain. Aminopyrene (0.06, 0.12 or 0.24 μg/plate) treated with excess of nitrite in a gastric liked condition was used as positive control. The mutagenicity of each plate was presented as number of histidine (His^+) revertants per plate. The sample expressed its mutagenicity higher than 2 times of spontaneous revertants with a dose-response relationship was evaluated mutagenic (Brusich, 1982).

Antiformation of Mutagens of Polysaccharides Using Ames Test

Effect of Polysaccharides on the Mutagenicity of Aminopyrene Treated with Nitrite Polysaccharide sample (0, 5, 10, 15, or 20 mg per tube), was autoclaved at 121 °C for 15 min and added 1.75 ml of sterile water. The mixture was stored in a refrigerator overnight. Before incubation, the reaction mixture was added with 10 μl of aminopyrene (0.3 mg per ml), 250 μl of 2 M sodium nitrite and 740 μl of 0.2 N hydrochloric acid containing sufficient acid to acidify the reaction mixture to pH 3.0-3.5. The reaction was conducted at 37 °C with shaking for 4 h; then, it was added with 250 μl of 2 M ammonium sulfamate and was placed in an ice bath for 10 min. The reaction tube was centrifuged (3,500 rpm/min, 20 min) and the supernatant was collected and tested for its mutagenicity. The effect of polysaccharides on antiformation of mutagens of aminopyrene and nitrite model was presented as number of histidine (His^+) revertants per plate. The sample expressed its

mutagenicity lower than standard of aminopyrene treated with nitrite, it was evaluated antiformation of mutagen. All steps in this experiment are shown in Figure 3.3.

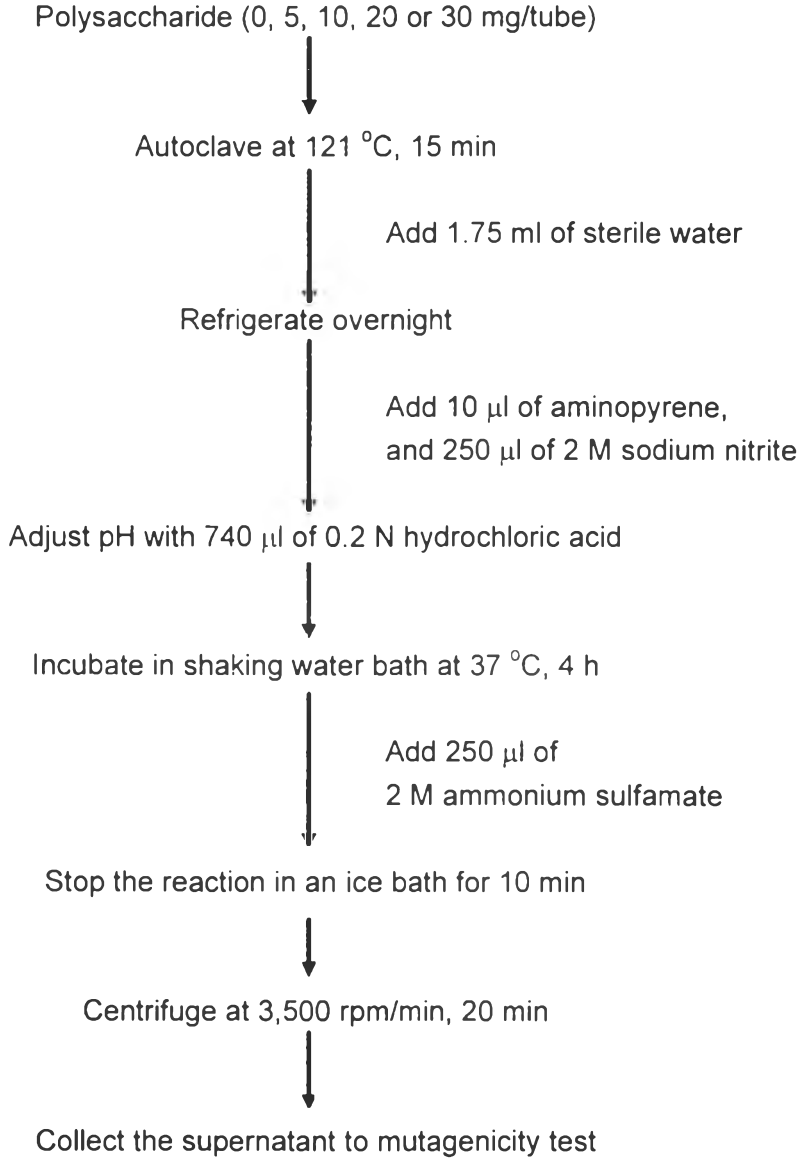

Figure 3.3 Steps in antiformation of mutagens study using Ames test

Aminopyrene Binding Strength of Different Fibers

Aminopyrene Binding Strength of Different Fibers Experiment was conducted (Figure 3.4) in duplicate and performed as described in effect of fibers on the mutagenicity of aminopyrene-nitrite model except the reaction mixture was shaken in the waterbath for 1 h and was centrifuged (3,500 rpm/min, for 20 min) afterwards. One ml of the supernatant of the first tube was transferred to a tube (Portion A). The remaining mixture in the first tube was added with 1 ml of sterile pH 3.0 distilled water and further incubation was carried for another 1 h. The mixture was centrifuged (3,500 rpm/min, for 20 min) and 1 ml of the supernatant was removed to another tube (Portion B). Both tubes containing Portion A and Portion B were added with 90 μl of 2 M sodium nitrite. In addition, the remaining second tube was incubated in a shaking water bath at 37 ^{o}C for 1 h and centrifuged (3,500 rpm/min, for 20 min); then, 1 ml of the supernatant (Portion C) was added with 90 μl of 2 M sodium nitrite. The three reaction mixtures were shaken in a water bath at 37 ^{o}C for another 2 h before they were mixed with 90 μl of 2 M ammonium sulfamate and placed in an ice bath for 10 min. Mutagenicity of each reaction mixture was determined as described earlier.

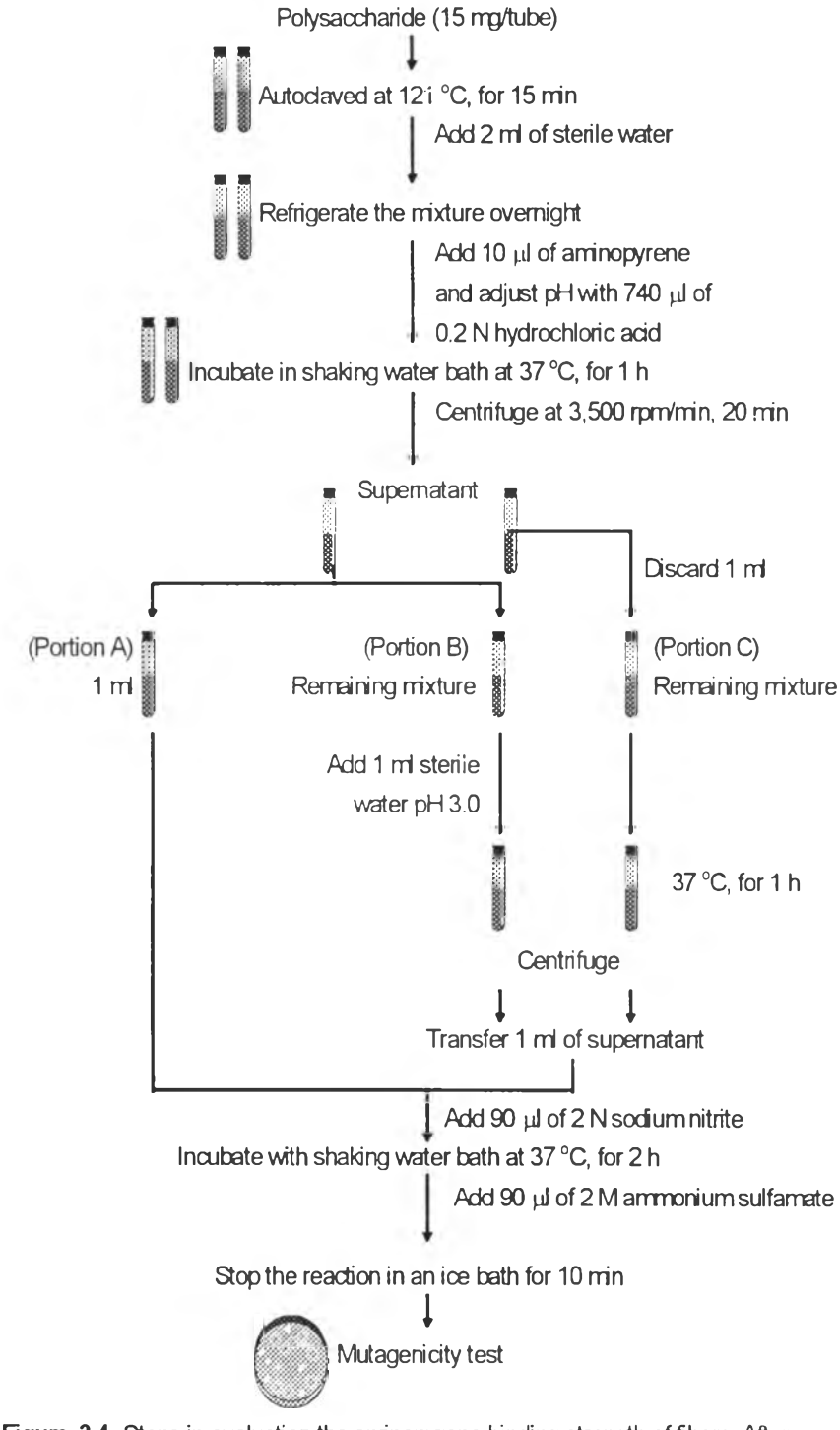

Figure 3.4 Steps in evaluation the aminopyrene binding strength of fibers. After an one-hour incubation, 1 ml of the mixture was remove (Portion A). The left over was either added with 1 ml sterile water (Portion B) or remained as it was (Portion C). All three portions were tested for their mutagenicity using Ames test.