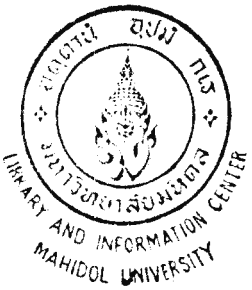

NUTRITIVE EVALUATION AND MUTAGENIC MODIFICATION ACTIVITY OF DIETARY SEAWEEDS

NATTINEE JITNARIN

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NATTINEE JITNARIN: NUTRITIVE EVALUATION AND MUTAGENIC MODIFICATION ACTIVITY OF DIETARY SEAWEEDS. THESIS ADVISORS : CHANIPHUN BUTRYEE M.Sc., KAEW KANGSADALAMPAI, Ph.D., PONGTORN SUNGPUAG, D.Sc. 109 P. ISBN 974-663-998-6.

This study a: made to determine: 1.) the nutritional value of, and 2.) the mutagenic modification activity of dietary seaweeds.

Some dietary seaweeds had possible antimutagenic activities probably associated with antitumor activity. Red seaweed, *Porphyra* spp., is sold in Thailand as seasoned and non-seasoned seaweeds, commonly used as healthy snacks or as an ingredient added to soup. Their potential health benefits in terms of nutritive value, iodine content and antimutagenic activities were evaluated. Nutritive value and iodine content of 6 seasoned seaweeds and 1 non-seasoned seaweed were determined according to AOAC and Moxon & Dixon method, respectively. The results, expressed per 100g dry seaweed, showed the following values: moisture content, 2.9-8.9 g; ashes, 9.5-13.2 g; protein, 10.4-39.9 g; fat, 1.7-3.3 g; carbohydrate, 43.0-74.3 g; dietary fiber, 29.2-41.4 g and iodine, 679-3617 μg. The highest values of protein, dietary fiber and iodine were in non-seasoned seaweed and it interestingly can be consumed as a source of iodine and fiber in one serving.

Antimutagenic effects of acetone, chloroform, methanol-chloroform (1:1), ether and hot water extracts of seaweed on the mutagenicity of nitrite treated- aminopyrene in gastric-like condition, were studied using *salmonella typhimurium* strains TA98 and TA100 in the absence of metabolic activation. The results demonstrated that acetone and chloroform extracts showed relatively strong suppressive activities for aminopyrene-nitrite model in both bacterial strains. For ether extract, the strong inhibitory activity to this model responded to TA98 whereas the weakly inhibition or no inhibition responded to TA100. The antimutagenic activity of methanol-chloroform (1:1) extracts to this model was different. This may be due to different ingredients in seasoned seaweed. On the other hand, no inhibitory effect occurred in hot water extracts in this model. However, these results suggested that *Porphyra* spp. has possible antimutagenic activities to nitrite treated 1-aminopyrene in Ames test.

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ณัฏฐิณี จิตนารินทร์ : การศึกษาคุณค่าทางโภชนาการและการเปลี่ยนแปลงฤทธิ์ก่อกลายพันธุ์ของสาหร่ายทะเล (NUTRITIVE EVALUATION AND MUTAGENIC MODIFICATION ACTIVITY OF DIETARY SEAWEEDS). คณะกรรมการควบคุมวิทยานิพนธ์ : ชนิพรรณ บุตรยี่, M.Sc., แก้ว กังสดาลอำไพ, Ph.D., พงศธร สังข์เผือก, D.Sc. 109 หน้า. ISBN 974-663-998-6.

สาหร่ายสีแดง *Porphyra* spp. ได้นำมาจำหน่ายในประเทศไทยทั้งชนิดที่ปรุงรสโดยนำมาเป็นขนมสำหรับเด็กและไม่ปรุงรสซึ่งนิยมนำมาประกอบอาหาร ทั้งอาหารไทยและจีนตลอดจนเพื่อเสริมโปรตีนในกลุ่มผู้นิยมบริโภคมังสวิรัติ มีการศึกษาพบว่าสาหร่ายทะเลบางชนิดสามารถแสดงฤทธิ์ยับยั้งการก่อกลายพันธุ์ได้โดยเฉพาะมีฤทธิ์ยับยั้งการเกิดเนื้องอกดังนั้นเพื่อประโยชน์ด้านสุขภาพจึงมีการศึกษาคุณค่าทางโภชนาการ ปริมาณไอโอดีนและฤทธิ์ต้านการกลายพันธุ์ โดยทำการศึกษาคุณค่าทางโภชนาการและปริมาณไอโอดีนของสาหร่ายทะเลแบบปรุงรส 6 ตัวอย่างและสาหร่ายทะเลแบบไม่ปรุงรส 1 ตัวอย่างโดยใช้วิธีของ AOAC และ MOXON & DIXON ตามลำดับ จากผลการศึกษาพบว่าในตัวอย่างสาหร่ายทะเล 100 กรัมมีปริมาณความชื้น 2.9-8.9 กรัม เถ้า 9.5-13.2 กรัม โปรตีน 10.4-39.9 กรัม ไขมัน 1.7-3.3 กรัม คาร์โบไฮเดรต 43.0-74.3 กรัม ใยอาหาร 29.2-41.4 กรัมและไอโอดีน 679-3617 ไมโครกรัม โดยพบว่าสาหร่ายแบบไม่ปรุงรสจะมีปริมาณโปรตีน ใยอาหาร และไอโอดีนสูงกว่าแบบปรุงรส เมื่อคิดเทียบปริมาณสาหร่ายต่อหนึ่งหน่วยบริโภคแล้วสรุปได้ว่าสามารถบริโภคสาหร่ายเพื่อเป็นแหล่งของไอโอดีนและใยอาหาร

ในการศึกษาฤทธิ์ต้านการก่อกลายพันธุ์ของส่วนสกัดอะซีโตน คลอโรฟอร์ม เมทานอล-คลอโรฟอร์ม (1:1) อีเทอร์ และน้ำร้อนของสาหร่ายทะเลทั้ง 7 ตัวอย่างต่อสารก่อกลายพันธุ์ที่เกิดระหว่างปฏิกิริยาของไนไตรท์และอะมิโนไพรีนในสภาวะที่เป็นกรดโดยทดสอบด้วยวิธีเอมส์เทสต์กับแบคทีเรีย *Salmonella typhimurium* สายพันธุ์ TA98 และ TA100 โดยไม่มีการเติมเอนไซม์กระตุ้นสารพิษพบว่าส่วนสกัดอะซีโตนและคลอโรฟอร์มแสดงฤทธิ์ยับยั้งการก่อกลายพันธุ์ต่อแบคทีเรียทั้งสองสายพันธุ์ ขณะที่ส่วนสกัดอีเทอร์แสดงฤทธิ์ยับยั้งการก่อกลายพันธุ์เฉพาะ TA98 และแสดงฤทธิ์ยับยั้งแบบอ่อนหรือไม่ยับยั้งเลยใน TA100 ในการแสดงฤทธิ์ต้านการก่อกลายพันธุ์ของส่วนสกัดเมทานอล-คลอโรฟอร์ม (1:1) พบว่าให้ผลที่แตกต่างกันไปในแต่ละตัวอย่างเนื่องมาจากเครื่องปรุงรสที่เติมลงไป ในส่วนสกัดน้ำร้อนพบว่าไม่แสดงฤทธิ์ยับยั้งการก่อกลายพันธุ์ของแบคทีเรียทั้งสองสายพันธุ์ อย่างไรก็ตามสามารถสรุปได้ว่าสาหร่ายทะเล *Porphyra* มีฤทธิ์ต้านการกลายพันธุ์ต่อสารก่อกลายพันธุ์ที่เกิดจากไนไตรท์และอะมิโนไพรีน

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LIST OF ABBREVIATIONS

AOAC	The Association of Official Analytical Chemists
AP	aminopyrene
°C	degree centigrade
DMSO	dimethylsulfoxide
HCl	hydrochloric acid
His^+	histidine prototrophy
His^-	histidine dependent
h	hour
kg	kilogram
mg	milligram
µg	microgram
ml	millilitre
µl	microlitre
min	minute
N	normality
No.	number
PK	partial killing effect
RDI	Recommended dietary Daily Intakes
S. typhimurium	*Salmonella typhimurium*

CHAPTER I

INTRODUCTION

Seaweed is rich in vitamins, minerals and many constituents that have been reported as cancer preventive agents. Eating more seaweed has more health benefits, including reduction of cancer risk. For example, one of epidemiological data indicated that ubiquitous consumption of seaweed in Japan may be a possible factor against breast cancer, because the incidence rate of breast cancer in Japanese women was only one sixth of the incidence rate of breast cancer in the USA (1). One group of Japanese investigators showed that the oral intake of seaweed powder reduced the rate of incidence of chemically induced mammary tumor in *in vivo* animal experiment (2). Seaweed also contained mammalian lignans such as enterolactone and enterodiol, which were produced in colon from precursor in food and have been suggested to a role in the cancer protection of vegetarian diet in amounts about 653-1147 micrograms per 100 grams seaweed. This data should be useful for the purpose of cancer risk reduction (3). A case-control study of colorectal cancer and esophageal cancer in Saitama prefecture of Japan indicated that the consumption of seaweed was inversely related to colon, rectal and esophageal cancer with a dose response relationship (4, 5).

Therefore, it is of interest to study the nutritive value of dried seaweeds consumed in Thailand as well as their antimutagenicity.

CHAPTER II

LITERATURE REVIEW

2.1 Antimutagenic Activity of Dietary Components

The significant differences in incidence of specific types of cancer in particular countries or regions of the world have directed attention to the possible influence of dietary components on the biological processes concerned with carcinogenesis. In many epidemiological studies, it has been implied that certain of these dietary factors may indeed be 'causative' with regard to various cancers, whereas others were seen as being implicated, to some extent, as 'protective' agents. Earlier estimates suggested that approximately 35% of cancer deaths may be attribute to dietary components or dietary habits (6, 7), with Doll and Peto (8) from their quote study provided some support for this estimate, but indicated that a range of 10-70% would probably be more appropriate.

Diets high in fat and low in fiber were associated with increased risk of cancer of the colon, breast, prostate, endometrium, pancreas and possibly other organs (9, 10, 11). In addition, foods also contain mutagens and/or carcinogens, some of which occur naturally and others that are introduced during the preparation of foods for consumption (12). Data obtained from animal studies (13, 14) as well as epidemiological studies (15,16) indicated that many types of cancer could be prevented by certain chemicals. These chemicals are classified as "chemopreventive agents" (17).

Chemopreventive agents are found in all categories of foods, fruits and vegetables (Table 1).

Table 1. Categories of foods with the most prominent chemopreventive agents.

Type of food	Chemopreventive agents
Fruits	Vitamins, flavonoids, polyphenolic acids, fiber, carotenes, monoterpenoids (d-limonene)
Vegetables	Vitamins, flavonoids, plant phenolics, chlorophyll, fiber, aliphaticsulfides, carotenes, aromatic isothiocyanates, dithiolthiones, phytic acid, selenium
Cereals	Fiber, α-tocopherol, phytic acid, selenium
Meat, fish, eggs and poultry	Conjugated isomers of linolenic acid, vitamins (A, E), selenites
Fat and oil	Fatty acids, vitamin E, tocotrienols
Milk	Fermented products, calcium, free fatty acids
Nut, beans and grains	Polyphenolics, fiber, vitamins E, phytic acid, coumarins, protein
Spices	Coumarins, curcumin, sesaminol
Tea	Plant phenolics, epigallocatechin
Coffee	Polyphenolic acids, diterpene alcohol esters, melanoidins
Wine	Flavonoids
Water	Selenium

The mechanism of action of the chemopreventive agents can be separated accordingly to Stavric (17) into two main categories and several subgroups according to the site or ambient at which they exert their influences and are illustrated in Table 2.

Table 2. Mechanisms of action of chemopreventive agents.

EXTRACELLULARY
During the preparation of foods, by:
• reducing (inhibiting) the formation of mutagens and/or carcinogens.
Effects in the intestine by:
• formation of non-mutagens and/or carcinogens complexes
• reducing bioavailability
• diluting with dietary fibers
• increasing adsorption or other food components
• accelerating intestinal transit
• protecting the mucosal barrier
• modifying intestinal microbial flora
• inhibiting the penetration of cells by mutagens and/or carcinogens
INTRACELLULARY
At cellular level, by:
• enhancing the activities of enzymes involved in detoxification of mutagens and/or carcinogens
• inhibiting the activities of enzymes involved in formation of mutagens and/or carcinogens metabolites
• trapping of electrophiles
• scavenging reactive oxygen species
• inhibiting metabolic activation
• protecting nucleophilic sites of DNA
• inhibiting the detrimental effect of pro-carcinogens on DNA

Although the mechanisms appear to be very heterogeneous (18), the antioxidative characteristic of chemopreventive agents seem to play the most significant part in their protecting activity (19, 20). In addition, Kada (21) discovered suppressive activity against heterocyclic amine mutagens such as 3-amino-1,4-dimethyl-5H-pyrido [4,3-b] indole (Trp-P-1) in various vegetable juices, including cabbage, broccoli and burdock. After investigation on the action mechanism of various agents suppressing mutagenicity, Kada (22) proposed that antimutagens be divided into two types; "desmutagens" and "bio-antimutagens". As shown in Figure 1, the former was concerned in various ways with the prevention of DNA damage in cells. The latter act in the processes of mutagenesis of the DNA-damaged cells, and the effect mostly involves enhancement of repair and suppression of repair error.

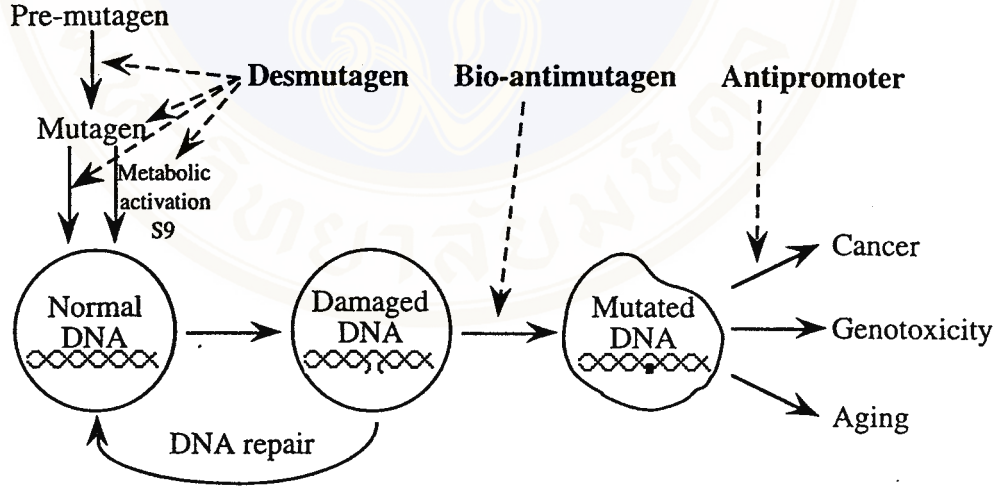

Figure 1. Schematic representation of antimutagenic and anticarcinogenic processes.

2.2 Antimutagens in plants

Chemical inactivation was observed on the metabolically active products of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and 3-amino-1,4-dimethyl-5H-pyrido

[4,3-b] indole (Trp-P-1) by green and black tea extracts as well as tea catechins (23). This result was of interest because of an epidemiological report showing that stomach cancer incidence in a tea producing area was lower than that in other areas in the Shizuoka prefecture of Japan (24). Oolong tea extract showed to suppress mutagenic activity induced by benzo(a)pyrene and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) in *Salmonella*/microsome assay (25, 26). The extracts of black and especially green tea were found to be antimutagenic against heterocyclic amine-induced colonic aberrant crypt foci (ACF) in the rat (27) and also had potentiating effects on the mutagenicity of eight heterocyclic aromatic amines (HAA) using the Ames *Salmonella typhimurium* TA98 and S-9 assay (28). As already mentioned, the main beneficial chemopreventive agents in green tea was (-)epigallocatechin-3-gallate (EGCG) (29). The anticarcinogenic potential of EGCG was demonstrated in several animal studies (30, 31). Japanese, Chinese and Ceylonese (now Sri Lankan) teas were able to prevent the formation of mutagenic nitrosated compounds found in salted fish (32) or human (33). These effects were attributed to the rich phenolic content of the teas including such substances as catechin, epicatechin derivatives, quercetin, kaempferol and myricetin (34, 35, 36).

A significant reduction in gastric cancer risk was found with increasing consumption of allium vegetables in the case-control studies in China and Italy. Protective effects on gastric cancer were seen for garlic, onions and other allium foods (37, 38). These findings were consisted with reports on tumor inhibition following administration of allium compounds or diallysulfide, using a variety of cancer inducing laboratory animal models (39, 40, 41). Diallyl sulfide and its analogous inhibited 1,2-

dimethylhydrazine (DMH)-induced colon tumorigenesis in mice and rats (42), and inhibited the formation of liver tumor (43). It also inhibited benzo(a)pyrene-induced formation of esophageal tumors in rats (41). In 1990, Hussain (44) found that diallyl sulfide inhibited 3-methylcholanthrene-induced uterine cervix tumors in mice. The beneficial effect of garlic extract in animal studies may be useful for the development of a chemopreventive agent for gastric cancer.

The effects of citrus fruit, orange and lemon oils, as well as monoterpenes d-limonene and d-carvone, on chemically induced formation of tumors in mice and rats were extensively studied by Wattenberg (45) and Gould (46). Gould and co-workers (47-50) reported that the monoterpenoid, d-limonene could prevent chemically induced mammary tumors in rats by the indirect/direct acting carcinogen.

Epidemiology and experimental evidence indicated that consumption of a diet high in cruciferous vegetables, such as broccoli, cabbage or brussels sprouts, was associated with a reduction in the incidence of cancer (51). Addition of cruciferous vegetables to animal diet inhibit tumorigenesis in experimental animals (52,53,54). Cruciferous vegetables contained several organosulfur compounds including isothiocyanated, aromatic thiocyanates and dithiolethions induced mammary gland tumors in rats (55). On the other hand, these compounds inhibited aflatoxin B_1 (AFB_1)-induced formation of liver tumor (52, 56) and also inhibit mammary gland, forestomach and lung tumorigenesis induced by polycyclic aromatic hydrocarbons in mice and rats (57,58, 59).

Important research in antimutagenesis was in *in vivo* studies on active oxygen damage; it was assumed that active oxygen species were important as direct and

indirect initiators as well as promoters of mutagenesis and carcinogenesis (60). In addition, it was showed that dietary antioxidants could act to reduce tumor incidence in animals, and as potential agents to prevent mutagenesis, carcinogenesis and aging (61, 62,63). Seeds of plants, beans and nuts including oil and crop seeds must be retain their germinating ability for long term preservation. Therefore, they usually contain effective antioxidants such as tocopherols for protection against oxidative damage (64). Among legumes, antioxidants are found in the beans and, interestingly, in the fermentation products of soybeans. These products such as miso, tempeh and natto were noted as foods having antioxidative potential (65).

2.3 Seaweed

Seaweed belongs to a rather ill-defined assemblage of plants known as the algae. The term 'seaweed' itself does not have any taxonomic values, but is rather a popular term used to described the common large attached (benthic) marine algae found in the groups Chlorophyceae, Rhodophyceae, Phaeophyceae or green, red and brown algae, respectively (66). The algae differ from the higher plants in that they do not possess true roots, stems or leaves. However, some of the larger species possess attachment organs, or hold-fasts, that have the appearance of roots, and there may also be a stem-like portion called a stipe, which flattens out into a broad leaf-like portion or lamina (e.g. *Laminaria*). Some species consisted simply of a flat plate of tissue (e.g. *Ulva*). In others the plant body, or thallus, is composed of a narrow, compressed or tubular axis with similar branches arising from it (e.g. *Gelidium*).

In primary classification, the algae are defined into 15 classes, excluding the Cyanophyceae (blue-green algae) which are true prokaryotes. Of these 15 classes, three are represented by macroscopic forms and presented in sufficient quantities in nature to have direct commercial importance in the terms of dietary seaweeds. These dietary seaweeds are the Chlorophyceae, Rhodophyceae and Phaeophyceae. The other classes are principally planktonic (unicellular or colonial), and with haptophytes (Haptophyceae), dinoflagellates (Dinophyceae) and planktonic (Chlorophyceae) have no commercial importance.

Seaweed is consumed in Japan and China as food for a very long time. Seaweed accounted for some 10% of the Japanese consumption in 1973, a 20% increase in 10 years (67). Most popularity is kombu (*Laminaria* spp.), wakame (*Undaria* spp.) and nori (*Porphyra* spp.). In the west, seaweed is largely regarded as a healthfood. *Palmaria palmata* (dulse or dillisk), a red seaweed, is consumed in Ireland and Scotland. *Chondrus cripus* (Irish moss) is recommended as a health remedy in Ireland (68). Various red algae are consumed in the Mediterranean as sources of dying agents or antihelmintic and other health remedies since pre-Christian times (69).

2.3.1 *Porphyra* spp. (Nori)

Nori is the Japanese name and Zicai is the Chinese term for a flat blade-like red seaweed belonging to the genus *Porphyra*. It is cultivated in the Inland Sea of Japan. There are about 20 species of *Porphyra* growing on the coast of Japan but only two of them, *Porphyra yezoensis* and *Porphyra tenera*, are cultivated by the Japanese (70).

Nori is sold as dried sheets. This is formerly carried out by a laborious, sun-drying process, but the job is now highly mechanized (71). The fresh nori is first washed with fresh water then fed into a shredding machine which reduce it to pieces 0.5 x 1.0 cm. in size. The cut nori/water mixture is then fed into a machine, resembling a paper maker. It is metered automatically on to wooden frames about 30 cm^2 on the outside, into which fits mats of split bamboo 20 x 18 cm. in size, and placed over a wire netting screen. About 600 ml of the mixture is fed into each frame and the water drains away through the mats and the screen. The frames then move slowly along a production line and the finally over a heated surface. The nori and the bamboo mats are then removed and the frames return in a circle for more nori/water mixture. The nori sheets are then piled up and put into an oven to reduce the moisture content to about 18%. In order to obtained a good-quality product, the temperature of the drying process should not exceed 50°C. The bamboo mats are then removed, the nori is put together in 10 and packed in bundle of 100. They are then shipped to a co-operative shipping point. Here they are carefully packed and sealed in cellophane so that moisture uptake would not reduced their quality.

2.3.2 Nutritive value of *Porphyra*

The principle components of *Porphyra* are carbohydrate, protein, fat (small quantities), and ash. Ash are largely composed of sodium and potassium and also rich in essential trace elements (66).

It is evident that *Porphyra* is really protein rich (25-35% of the dry matter) (72) and 75% of this material is said to be digestible (73). Miura (74) reported crude protein

of *Porphyra tenera* was 29-35.6 g per 100g dry matter. Konig and Bettelles (75) give some figures (Table 3) for the solubility of the nitrogen. The ratio of protein nitrogen to total nitrogen was 80-85% in *Porphyra yezoensis* (72). The protein quality and digestibility for 8 species of edible Japanese marine algae were studied. The E:T ratio (a measure of the sum of essential amino acids, in mg/g protein nitrogen) of the proteins was found to be 2326-3206 and was highest in *Porphyra tenera* (E:T ratio = 3206) which is similar to that of whole eggs (76). The E:T ratio of whole egg, human milk and provisional score in FAO/WHO (1973) are indicated as 3040, 2890 and 2250, respectively. It was interesting that the amino acid scores of the algal proteins were higher than those in the land plants, being rather more like animal proteins. It should be emphasized that the alkali soluble proteins in edible marine algae were of very good quality.

Table 3. Percentages of water soluble nitrogenous substances in various seaweeds.

Species	%water soluble nitrogenous substances
Undaria spp.	5.31
Hijikia fusiforme	3.89
Laminaria japonica	5.44
Porphyra tenera	21.85

Sato (77) demonstrated that the carbohydrate content of *Porphyra* was a mixture of sulphated galactans, made up of 3,6 anhydro-L-galactose, with D- and L-galactose units and β- 1,4 mannan. Other studies, Lerving (78) and Miura (74) determined starch and sugars content in *P. tenera*, they contained 40.1% and 39.1-40.5%, respectively.

Total carbohydrate found in *Porphyra yezoensis* and *Porphyra* spp. was 44.4 and 40.0 g per 100g dry matter, respectively (79). Human digests 75% of the protein and carbohydrate of *Porphyra* spp. (66) and in this respect it much better than other seaweeds. Kayama and his colleagues (80) reported that *Porphyra yezoensis* contained 2.5% of lipids. The main components of lipid were polar lipids, free fatty acids and triglycerides. The fatty acid composition was characterized by the content of eicosapentaenoic acid (EPA), which amounts to as much as half and of palmitic acid (PA), which may reach a quarter. Since EPA is one of the precursors of prostaglandins in animal (81), *Porphyra* has high nutritional value.

In general, red algae have higher content of B vitamins than brown algae (83). The amounts found in *Porphyra* spp. are typical for a red algae, although high level of vitamin B_2 (0.84-23.08 μg/g dry weight) (66) and vitamin B_{12} (1.5-3.5 μg/100g wet weight) (83) was reported. Other vitamins were detected in *Porphyra* spp. include pantothenic acid, folic acid, vitamin D and γ-tocopherol (78). In addition, Chapman (66) reported iodine level in *Porphyra tenera* was 18 ppm. of 100g dry materials, according to Lee, he determined iodine level in *Porphyra* spp., they contained 430 μg per 100g dry weight (84).

Because of *Porphyra* spp. has high nutritive value, so it is meet the human need in the purpose of health benefit.

2.4 Consumption of Seaweed as a Protective Factor in the Etiology of Cancer

Studies on the role of nutrition in cancer etiology focused on excess in fat intake (9), specific food additive (85), naturally occurring carcinogens (85) as well as trace elements deficiencies (86). The role of protective dietary factors which may act as anti-carcinogens were less investigated, although beta-sitosterols (87) and butylated hydroxytoluene (BHT) (88) have been shown to protect rats against chemically induced tumors.

To date, no food per se has been studied which may serve to protect a population from one or more kinds of cancer. On the other hand, Reddy and co-workers (9) reported the food eaten in Japan might have effect on the Japanese breast cancer rates. Breast cancer rates in Japan were only one sixth as high as in the United States. Migrant studies of Japanese showed that breast cancer mortality increased in the progeny of Japanese migrants to the United States. It was therefore unlikely the racial differences were the important variable. Dietary changes, in particular increases in dietary fat, were most often implicated. Although suggestive, this association does not appear sufficient. It was necessary to expand the hypothesis to include other foods that may acted as anticarcinogens and in understanding the lower breast cancer rates in Japan, to look at food that was eaten in Japan which was not eaten in United States. Seaweed was a logical choice, since it as a food commonly eaten by all people in Japan and rarely eaten by people in the United States (89).

Estimates of the frequency of seaweed eaten in Japan were 4.9 grams per capita per day (90). Another studies; seaweed consumption was 7.3 grams per person per day

(91). It was reported that 25% of the Japanese diet was seaweed (92). Hirayama (93) noted that the incidence of breast cancer was low in rural towns and villages where seaweed consumption increased and high incidence in cities where seaweed consumption decreased. When areas of low breast cancer rates within Japan were compared to the areas of seaweed production, there were some areas of similarity. In Sago prefecture, the area of lowest breast cancer rates, *Porphyra* was harvested (94, 95). Hokkaido had the next lowest breast cancer rates and was the prefecture where 65-80 % of the two most common brown seaweed, *Laminaria* and *Undaria*, were harvested. Hoyguard (96) noted that large amount of seaweed were eaten by Eskimos. Seaweed is a traditional food among the Eskimos, who have both a high fat diet and a low breast cancer rate (97).

Seaweed may affect on level of cholesterol via metabolism of steroids, bile acids and steroid hormones in the promotion of cancer. *Laminaria* demonstrated antilipemic action and lowering cholesterol levels in human study (98). Seaweed inhibited the enterohepatic absorption of bile acid and thereby influenced cholesterol levels (99). In support of this role of seaweed, Reddy *et al.*(100) reported that rats fed carrageenan, derivative of red algae, showed increased concentrations and daily excretion of fecal cholesterol, deoxycholic acid, lithocholic acid and total bile acids. Studies on the effects of edible seaweeds on cholesterol metabolism have been carried out by Abe (101) and Kimura (102). Both studies showed a hypocholesteremic effect of seaweed, particularly *Porphyra* was superior. In the first study, five rats were fed diet containing five percents seaweed for twenty-eight days, the total plasma cholesterol was reduced by 40 % and free cholesterol was reduced by more than 80 %. A possible mechanism in the

reduction of plasma cholesterol was related to fiber contained in seaweed (101). Mendelof (103) proposed that seaweed provided a good source of nondigestible dietary fiber that could increasing fecal bulk and possibly lowering cancer risk. Another study, the powder derivatives of *Laminaria*, the sodium and calcium salt of alginic acid, gave to 60 humans with colonic constipation. Mulinos (104) found that the sodium and calcium salts could effective to solve constipation problem. Although alginates swelled minimally in water or gastric juice, they could increase 25 to 65 times of original bulk in the alkalinity of intestinal secretions. Study on the comparative value of different fiber in reducing cholesterol, Story and Kritchevsky (105) reported the capacity for various types of fiber to bind bile acid or bile salts. Their capacities were quite variable and a high capacity seems to correspond with a hypocholesteremic effect. So, it seems likely that seaweed caused the effect by binding with bile acids. As an anti-carcinogen, fiber may work to dilute the effect of carcinogens in the alimentary canal. Therefore, seaweed would be an excellent diluter and hence be protector against carcinogens present in the gut (105).

Although large dietary fiber intake was thought to be partly responsible, numerous studies on the relationships between total dietary fiber intake and reduction colon cancer incidence showed conflicting results (9, 106). Presumable, not only the type of fiber but also other substances associated with the fiber-containing foods are determinants of health benefits of the diets. One of these suggested substances is lignans and their precursors (107, 108, 109). Many lignans have antitumor, antimitotic, antioxidant, weak estrogenic and antiestrogenic activities (107, 109-113). Some lignans were shown to prevent the growth of many tumor studies in the chemotherapy program

of the US National Cancer Institute (114). Thompson (3) reported that seaweed produced the concentration of lignans 900 ± 247, 998 ± 269 μg per 100 g seaweed on a wet basis and on a dry basis, respectively. He suggested that seaweed with high lignan-producing capability may reduce the risk for carcinogenesis.

A second aspect of seaweeds that may be important in lowering colon cancer depends on sterols contained in the seaweed. Using a 0.2% beta-sitosterols supplemented diet, rats fed a colon carcinogen, N-methyl-N-nitrosourea (MNU) significantly lowered the incidence of colon cancer than that of the control group. It was therefore possible that the amount of beta-sitosterols in seaweed could be of physiologic importance in protecting against colon cancer (87).

There was an indirect source of evidence for the role of *Porphyra* in protecting against breast cancer. Kaneda and Ando (115) analyzed the component lipids of *Porphyra* and tested for antioxygenic activities. They found that whole lipids and phospholipids had similar antioxygenic activities as butylated hydroxytoluene (BHT). When BHT was fed as a dietary supplement to rats which given with dimethylbenz(a) anthracene (DMBA), a mammary carcinogen. King and his colleagues (88) found a lower incidence and rate of tumor growth. In addition, seaweed has an antibiotic activity both *in vitro* and *in vivo* and this activity might selectively reduced or eliminated particular colonic bacteria that could produce carcinogenic substances.

Another property of seaweed was its ability to bind metal and radioactive pollutants. Alginate derived from brown seaweed can bind radioactive strontium, one of the most hazardous pollutants, effectively in the gastrointestinal tract. It also has been shown to bind other metal pollutants such as barium, cadmium and zinc (116).

Certain marine algae are used in traditional Chinese herbal medicine in the treatment of cancer, so, many scientists studied the antitumor activity of marine algae. Yamamoto et al. (117-119) and Nagumo (120) demonstrated that a non-dialyzable fraction of hot water extract obtained from *Laminaria* spp. showed marked inhibition of growth of sarcoma-180 cells subcutaneouly implanted into mice and 1,2-dimethylhydrazine (DMH)-induced intestinal cancer. Extracts from both *L.angustata* and *L.angustata var longissima* strongly inhibited the growth of tumor 70-76% and 83.6% in male ddY mice implanted Sarcoma-180 tumors, respectively (119).

In *in vitro* study, the acetone, chloroform, ether and methanol-chloroform extracts of *L. angustata* strongly inhibited the mutagenic activity of 7,12-dimethylbenz(a) anthracene and 3,2′-dimethyl-4-aminobiphenyl in *Salmonella typhimurium* TA98 and TA100 (121). Also, the hot water extract of *L. japonica* showed suppressive effect on *umu* C gene expression in the SOS response of *S. typhimurium* (TA1535/pSK 1002) induced by carcinogenic substances (122).

In another studies, a polysaccharide fraction obtained from *Sargassum,* a kind of dietary seaweed, manifested a prominent inhibition effect on the growth of Sarcoma-180 cells (120, 123-129), IMC ascites tumor cells (129) and L-1210 leukemia cells (130). Results from *in vitro* experiments for ddY mice and nude mice, crude fractions prepared from *Eisenia bicyclis* showed inhibitory effect on Sarcoma-180 cells in the rate of 84.6 and 68.9 %, respectively (131, 132). It also showed an antitumor activities in L-1210 bearing mice (133) and the DMH-induced intestinal cancer (118). The crude extract of *E. bicyclis* also had suppressive effect on *umu* C gene expression in the SOS

response of *S. typhimurium* (TA1535/pSK 1002) induced by carcinogenic substances (134).

The antitumor activities were also shown in other kind of dietary seaweed, *Hijikia fusiforme* and *Undaria pinnafida*. The extract of *Hijikia fusiforme* containing polysaccharides enhanced macrophage-dependent suppression against the growth of EL-4 tumor cells in an *in vitro* culture experiment (135). The extract of *Undaria pinnafida* had been shown strongly inhibited TPA-induced in mouse skin carcinoma (136). Kashiwagi and Mynderse (137,138) reported that thirty-seven of marine algae specimens from Pacific islands showed high antitumor activity in P-388 lymphocytic leukemia and Ehrlich ascites tumor in mice. The extract from *Ascophyllum nodosum* exhibited an inhibitory effect on *in vitro* NSCLC-NL cell cycle and *in vivo* for the growth inhibition of the implanted Sarcoma-180 cells (139).

Oral administration of polysaccharide or porphyran, extracted from *Porphyra* effectively inhibited the growth of Ehrlich carcinoma cells and Meth-A fibrosarcoma (140). Diet containing 2 % *Porphyra tenera* inhibited the development of spontaneous mammary tumors induced by 7,12-dimethylbenz(a)thracene (DMBA) with a tumor incidence of 30 % compared with 80 % of the control group (2). In addition, there was a significant decrease in the incidence of intestinal carcinoma in rats, induced by 1,2-dimethylhydrazine (DMH) (118). However, *Porphyra tenera* could showed suppressive effect in *in vitro* study, the extract of *Porphyra tenera* inhibited Trp-P-1 induced *umu* C gene expression in SOS response of *Salmonella typhimurium* (TA1535/pSK 1002) and TPA-dependent ornithine decarboxylase induction in BALB/C 3T3 fibroblast cells (141,142).

In conclusion, seaweed may be an important factor in preventing cancer risk. Proposed mechanisms of action were reduction of plasma cholesterol, inhibition of carcinogenic fecal flora, antioxygenic activity of phospholipids, binding of biliary steroids and binding of pollutants. In animal experiment, seaweed extracts were reported to have an antitumor effect. Thus, it was suggested that seaweed consumption may be able to prevent cancer. This dietary habit among Japanese was an important factor in understanding their lower cancer rates.

2.5 1-Aminopyrene and its Nitrosated products

1-Aminopyrene is a natural compound produced in human feces or in anaerobic incubation of 1-nitropyrene with fecal bacterial (143-145). 1-Nitropyrene is metabolized by human, rat and mouse intestinal microflora to 1-aminopyrene and others. The predominant metabolites produced by human, rat or mouse intestinal microflora following a 12-hr incubation with 1-nitropyrene is 1-aminopyrene. Data suggested that a similar mechanism exist in the biotransformation of 1-nitropyrene by intestinal microflora from all these three species.

1-Aminopyrene is known to be mutagenic after metabolic activation to *Salmonella typhimurium* TA98 and TA100, but less mutagenic without metabolic activation. The mutagenicity of this compound with S-9 was decreased on treatment with nitrite whereas the activity was increased without S-9. The number of histidine revertant colonies on *Salmonella typhimurium* TA98 without S-9 mix were 870 colonies / 0.022 micromole of 1-aminopyrene. The result indicated that this compound was transformed into potential direct-acting mutagens on treatment with nitrite (146).

Kato *et al.* (147) demonstrated that mutagenicity of 1-aminopyrene could be potentiated by nitrite. In their experiment, 1-aminopyrene (0.2 mmole) interacted with sodium nitrite (0.8 mmole) in 5 ml. acetonitrile-water (1:1 v/v), the mixture was adjusted to pH 3 and incubated at 37°C for 4 hr. Then 0.8 mole ammonium sulfamate was added. The mixture was extracted with 5ml. ethyl acetate. The mixture was analyzed with HPLC and found to contain 1-nitropyrene and unidentified nitro-compounds. The mutagenicity of 1-aminopyrene was one-tenth that of its nitro-introduced product, 1-nitropyrene, in the Ames test (148). However, 1-nitropyrene is converted back to 1-aminopyrene by microsomal enzyme in the living system (144).

The pathway for activation of 1-nitropyrene proceeds as illustrated in figure 2. The critical first step called a rate-limiting step, was enzymatic reduction by nitroreductase to 1-nitrosopyrene. This was followed by a subsequent reduction to the corresponding hydroxylamine, a species capable of undergoing acid-catalyzed decomposition to yield a covalent adduct at position 8 of guanine (149).

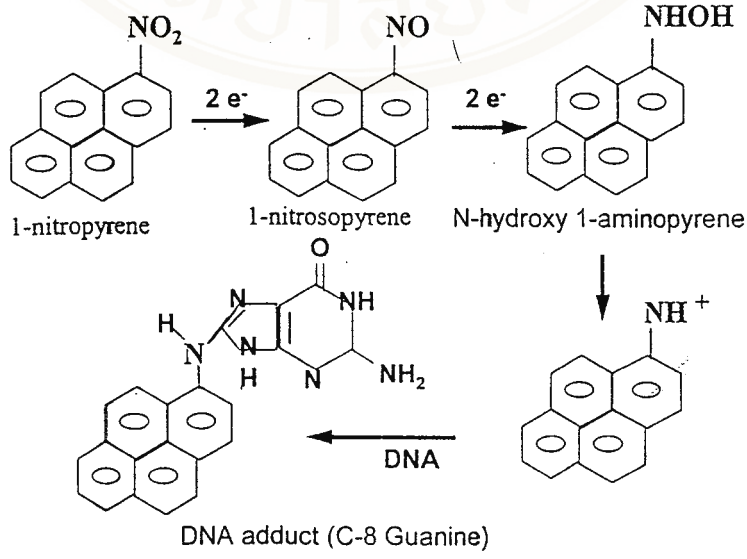

Figure 2. The pathway for activation of 1-nitropyrene.

The metabolism of 1-aminopyrene was studied in animals. Belisario *et al.* (150) characterized the induction of rat hepatic microsomal drug-metabolizing enzymes by 1-aminopyrene and the result suggested that the form of cytochrome P-450 induced by 1-aminopyrene resemble the one induced by 3-methylcholanthrene. A long-term study, the tumorigenicity was demonstrated in 1-aminopyrene administered by gavage to Sprague-Dawley rats. The experiment was terminated after 94 weeks; the approximate total dose per rat of 1-aminopyrene was 320 nmole (144).

Recently, 1-aminopyrene demonstrated its mutagenicity only on *Salmonella typhimurium* TA98; however, the treatment with nitrite had converted it to unknown mutagens for both strains. The result of the determination for possible N-nitroso compounds occurred during the treatment was negative. It was noted that the mutagenicity of 1-aminopyrene and of the product of nitrite treatment was greatly different e.g. the number of revertants of 0.12 μg/plate of untreated 1-aminopyrene on *Salmonella typhimurium* TA98 was 26±11 colonies but it was 1814±50 colonies after the compound was treated with nitrite (151).

2.6 The *Salmonella* Mutagenicity Test (Ames test)

The most generally used and validated bacterial reverse mutation test was devised by B.N. Ames and his colleagues (152). The Ames test was first validated in a study of 300 chemicals, most of which were known carcinogen (146, 153). It was subsequently validated in study by the Imperial Chemical Industries (154), the National Cancer Center Research Institute in Tokyo (155) and International Agency for research on Cancer (156). Nearly 90% of the carcinogens tested were mutagenic in these studies.

However, Ames and McCann estimated the correlation to be about 83%. All the validations showed that the test fails to detect a few classes of carcinogens such as polychlorinated pesticides (157-159)

Prior to the initial development of the *Salmonella* / microsome assay, there were several studies that employed bacterial systems to detect mutagenic agent (160). However, one of the problems with these earlier appearances was the use of screening techniques that did not employ bacterial strains designed to detect a broad range of mutagenic mechanism. Therefore, Ames *et al.* had developed a set of *Salmonella typhimurium* strains that are permeable to a wide range of chemicals and also are partially deficient in DNA repair (161).

2.6.1 The *Salmonella* Tester Strains

The reverse mutation system of *Salmonella typhimurium* uses that genetically well defined histidine-requiring mutants developed by Ames and his colleagues (161). The *Salmonella* histidine reverse mutation is based on the use of several *Salmonella typhimurium* strains that reversed from histidine dependence (auxotroph) to histidine independence (prototroph). Ames *et al.* had collected and characterized a large number of *Salmonella typhimurium* strains containing mutations in different gene of the histidine operon (Table 4). Later, they had been developed the newly *Salmonella* tester strains to make them more effective in detecting mutagens that were not previously detected with the original strains (162). The newly standard tester strains contain other mutations that greatly increase their ability to detect mutagens such as:

rfa mutation. The mutation causes partial loss of the lipopolysaccharide barriers that coat the surface of the bacteria and increase permeability of the cell wall to large molecules that do not penetrate the normal cell wall (162).

***uvr* B mutation**. The mutation is a deletion of a gene coding for the DNA excision repair system, resulting in greatly increased sensitivity in detecting many mutagens (161, 162).

R-factor plasmid (pKM 101). This plasmid exhibits a greatly enhanced response to mutagen and also gives clear positive response to chemical described as weak, borderline or nonmutagens with the original set of tester strains. Furthermore, Macphee implied that pKM 101 contains gene products associated with errorprone repair which may be responsible for the enhance sensitivity seen in these strains (163). Genotypes of the *Salmonella typhimurium* strains used for mutagenesis testing are shown in Table 4. The standard tester strains; TA97, TA98, TA100 and TA102 contain the R-factor parent strains (164, 165).

Therefore these standard tester strains are recommended for mutagenesis testing. TA98 was derived from TA1538 by which plasmid pKM 101 has been introduced. It can detect mutagens that cause frameshift mutation due to its DNA sequence (-CGCGCGCG-), which can be reverted to histidine independence by a variety of mutagens affected addition or deletion of the base pairs (166). TA100 containing R-factor plasmid derivative of TA1535 can detected mutagens that cause base-pair substitutions. The others *Salmonella* strains related to these 4 strains containing different characteristics in terms of DNA-repair capacity, cell permeability and presence of plasmid pKM101 are also available and have been described (164, 165).

However, some mutagens affect only one strain of frameshift mutation strain (TA1538 or TA98) or base-pair substitution strains (TA1535 or TA100), thus imparting a degree of mutagen specificity to the assay. But, many or even most mutagens can affect both types of strains at the different effective dose for each strain. Mutagen specificity, therefore, is frequently associated with quantitative rather than qualitative response (167).

2.6.2 Method used for Detecting Mutagens

There are three methods that have been used for testing mutagenicity of chemicals. These methods are spot test, plate incorporation test and preincubation method.

Spot test. The spot test is the simplest way to test compounds for mutagenicity and is useful for the initial rapid screening of large numbers of compounds. This test is primarily a qualitative test and has distinct limitations. It can be used only for testing chemicals that are diffusable in the agar. It will be confirmed by demonstrating a dose-response relationship using the standard plate incorporation test.

Plate incorporation test. The test is the standard method that has been used for the mutagenicity of chemicals. The test consists of combining the test compound and the bacterial tester strain in soft agar which is poured onto a minimal agar plate. After incubation at 37 °C for 48 hours, revertant colonies are counted (162, 168, 169). For initial screening, chemicals were tested in concentrations over a three-log dose range. A positive or questionable result should be confirmed by demonstrating a dose response relationship using a narrower range of concentrations. In a modification of the plate incorporation procedure, a preincubation step precedes addition of the top agar. This

modification is better for some compounds and appears to be at least as good for other compounds tested (168).

Table 4. Genotypes of the *Salmonella typhimurium* strains used for mutagenesis testing.

Histidine mutation				LPS	Repair	R-factor
his D6610 his D1242	his D3052	his G46	his G428 (pAQ 1)			
TA90	TA1538	TA1535	-	*rfa*	Δ*uvr*B	-R
(TA97)	(TA98)	(TA100)	-	*rfa*	Δ*uvr*B	+R
-	(TA1978)	TA1975	-	*rfa*	+	-R
TA110	TA94	TA92	-	+	+	+R
-	TA1534	TA1950	-	+	Δ*uvr*B	-R
-	-	TA2410	-	+	Δ*uvr*B	+R
TA89	TA1964	TA1530	-	*Δgal*	Δ*uvr*B	-R
-	TA2641	TA2631	-	*Δgal*	Δ*uvr*B	+R
-	-	-	(TA102)	*rfa*	+	+R

Tester strains in brackets are recommended for general mutagenesis testing. All strains were originally derived from *Salmonella typhimurium LT2.* Wild-type genes are indicated by a +. The deletion (Δ) through *uvr*B also includes the nitrate reductase (*chl*) and biotin (*bio*) genes. The Δ*gal* strains and the *rfa/uvrB* strains have a single deletion through *gal chl bio uvr*B. The *rfa* repair+ strains have a mutation in *gal E.* R represents for pKM 101. The tester strain TA1536, included in the original tester set (168), and all other strains containing the histidine mutation his *C207* have been discontinued due to the lack of specificity on few mutagens reversion and tester strains. TA97 replaces TA1537 and TA2637. Genotypes of these discontinued strains and of other derivatives of his C3076 were listed by Ames *et al.* (169).

Preincubation method. The significant finding of preincubation assay was of equal or greater sensitivity than the standard plate incorporation assay (170), when the mutagenic activity of aflatoxin B_1, benzodine, benzo(a)pyrene and methyl methanesulfonate have been determined by both plate incorporation and preincubation procedure. Some mutagens, such as dimethyl and diethylnitrosamine are poorly detected in the standard plate incorporation assay, a modification of standard procedure was recommended. The fact that the test compounds and bacteria were incubated 20-30 minutes at 37 °C at the higher concentrations before top agar were added to the mixture. This method provided more sensitivity of mutagenic detection. The preincubation assay was first described by Yahagi *et al.* in which carcinogenic azo dyes were found to be mutagenic (171).

The preincubation modification can be used routinely or when inconclusive results are obtained in the standard plate incorporation assay. This assay required an extra step and therefore involves more work than the standard test. However, many laboratories has been used to detect mutagenicity of 10 carcinogenic nitrosamines (172) and several carcinogenic alkaloids (173). Its use in screening assays has been recommended (174).

Positive control (diagnostic mutagens)

In each experiment, positive mutagenesis controls were used for diagnostic mutagens to confirm the reversion properties and specificity of each strain. The characteristic reversion patterns of the standard strains to some diagnostic mutagens are described. (175)

2.6.3 Antimutagenicity Test Using Ames Test

Considerable evidence indicated a strong association between the carcinogenicity and mutagenicity of chemicals (153, 176). Since the antimutagenic activity has also been correlated with anticarcinogenic activity of various compounds, it should be possible to use mutagen-testing procedure or Ames test for screening various compounds for antimutagenic and hence for potential anticarcinogenic effects (177-180).

2.6.4 Criteria of Antimutagenic Activity

The antimutagenic effects expressed as inhibition (%):

$$\% \text{ inhibition} = 100 - \left[\frac{\text{number of revertants with test compound}}{\text{number of revertants without test compound}} \times 100\right]$$

Interpretation of data: compounds were classified as positive antimutagen based on the % inhibition of the mutagenicity of tested mutagen. Data were qualitatively ranked according the following scheme:

% inhibition	Ranking for antimutagenicity
>60 %	strongly active
60-40 %	active
40-20 %	weakly active
20-0 %	not active

Positive antimutagens should be dose-responsive or positive dose-response relationship (181).

STATEMENT OF THE THESIS PROBLEM

Literature reviews indicate that edible seaweed has antitumor activity which might play an important role in the reduction of cancer risk. In addition, it is a good source of nutrients both macro- and micronutrients. Seaweed, *Porphyra* spp. is imported as raw and seasoned dried seaweed. It is very popular among consumer. For instance, the incidence of chemically induced tumorgenesis was decreased by administration of seaweed powder or extract in the diet (2, 118). Therefore, seaweed may be possible source of mutagen modifier. It is of great interest to evaluate nutritive values, and the effect of seaweed on the mutagenicity of direct mutagen occurring during the reaction of nitrite and amionopyrene in the acidic condition.

EXPERIMENTAL OBJECTIVE

1. To evaluate nutritive value namely proximate analysis and iodine content of some dried seaweed.

2. To study on the antimutagenic potential of various extracts of dried seaweed using Ames *Salmonella* assay.

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals

Aminopyrene (Aldrich, St.Louis) was used as standard mutagen in the Ames test. d-Biotin, ammonium sulfamate, termamyl (heat-stable, α-amylase), amyloglucosidase, protease and arsenic trioxide were purchased from Sigma Chemical Company (St. Louis, Missouri). L-Histidine monohydrochloride, sodium chloride, hydrochloric acid, magnesium sulfate heptahydrate, potassium carbonate, citric acid monohydrate GR, potassium chloride and di-sodium hydrogenphosphate were supplied by E. Merck (Darmstadt, Germany). D(+)-Glucose monohydrate, crystal violet indicator, di-sodium ammonium hydrogen phosphate tetrahydrate GR were bought from Fluka AG (Buch, Switzerland). Bacto agar was purchased from Difco Laboratory (Detroit, Michigan, U.S.A.). Oxoid nutrient broth No.2 was supplied by Oxoid Ltd., (Basingstoke, Hants, England). Sodium di-hydrogen phosphate was furnished by May & Baker Ltd., (Degenham, England). Ampicillin sodium was furnished by Verco pharmaceutical Ltd. (Bangkok, Thailand). Sodium hydroxide, di-potassium hydrogen phosphate anhydrous, zinc sulfate, sulfuric acid and sodium nitrite, were purchased from BDH Chemicals Ltd., (Poole, England). Acetone , chloroform, petroleum ether, ether, methanol and ceric ammonium sulfate were purchased from JT Baker Chemical Co., (Phillipsburg, New York).

3.2 Experimental Design

Six seasoned seaweeds and one non-seasoned seaweed in *Porphyra* spp. were studied. The study of each sample was followed to figure 3.

3.2.1 Survey on Consumption Behavior of Seaweed

A survey on consumption pattern of seaweed among people was study. The questionnaire (see appendix A) was designed to obtain an information on the popularity of seaweed, frequency of consumption. The survey was carried out during September and October 1998. Participants involved in this survey were working in Salaya campus and supermarket shoppers. The supermarket shoppers were randomly sampled from three supermarkets, namely Pata Pinklao, Central Pinklao and Tang Hua Seng Thonburi. Objectives of the study were explained to participants both males and females with various age before interviewing. Two hundreds and three participants were asked about the amount of seaweed consumed and estimated a serving portion.

3.2.2 Analytical Method

3.2.2.1 Moisture. Each sample was weighed approximately 0.3-0.4 g in aluminum dish. The dish was placed in a vacuum oven for 2 h. The dish was cooled in dessicator, weighed and returned to the oven. The sample was dried in oven until a constant weight was obtained. The equation used to calculate moisture content was as follow :

$$\text{Moisture} = \frac{b-c}{b-a} \times 100\ \%\ (w/w)$$

where a = weight of the aluminum dish;

b = weight of the aluminum dish and sample before the drying;

c = weight of the aluminum dish and sample after the drying.

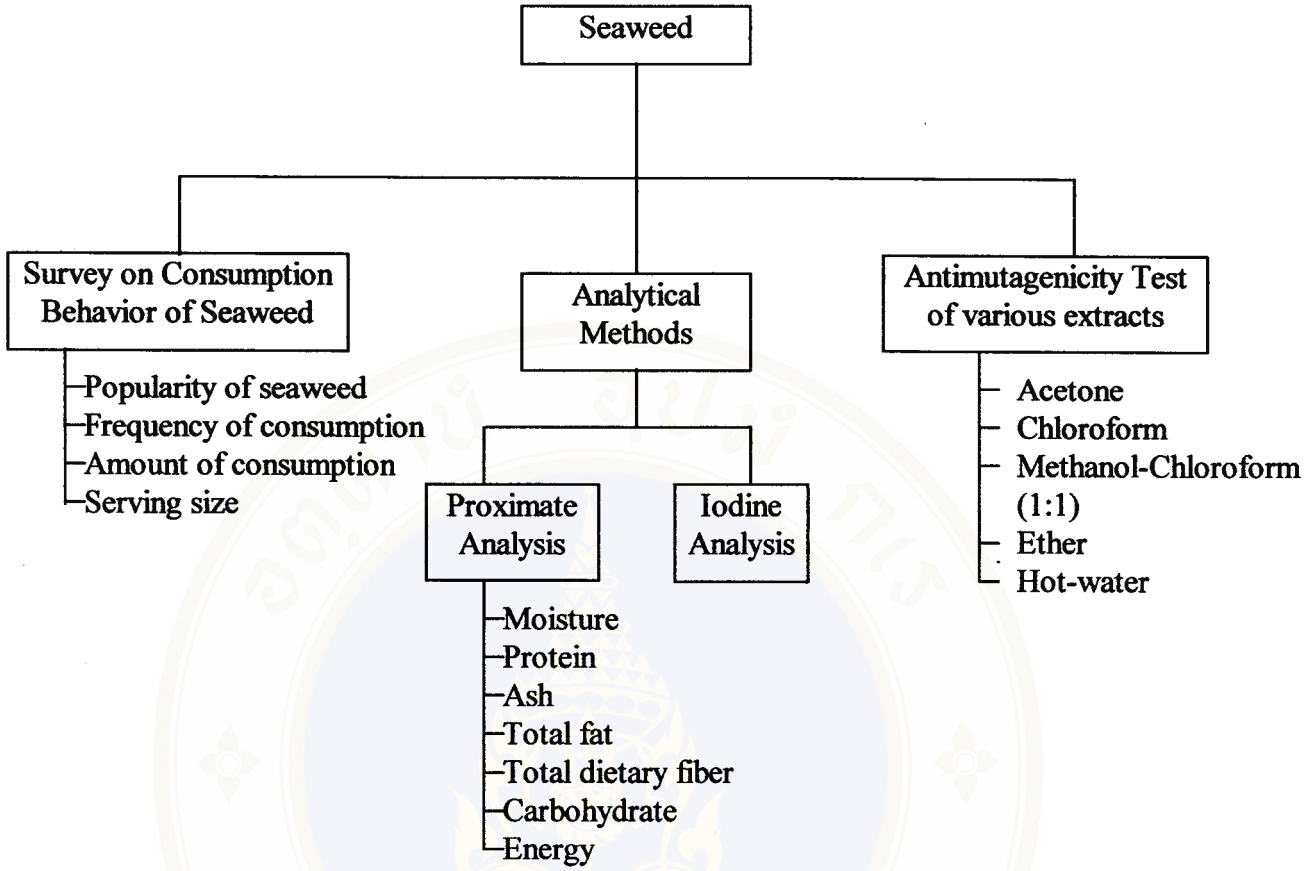

Figure 3. Schematic representation of experimental study.

3.2.2.2 Protein. Protein was determined by Kjeldahl method (182). Each sample was weighed as 0.2 g and transferred to 750 ml Kjeldahl digestion flask. Then the catalyst mixture of selenium, potassium (0.9 g) and 25-ml sulfuric acid were added into flask. The flask was placed on a digester for 1.5 h or until the solution became clear. The erlenmeyer flask which containing 100 ml boric acid, indicator and digested product was connected to condensing unit. The ammonia distillate was condensed into erlenmeyer flask and the product solution was titrated with standardized 0.1 N hydrochloric acid until the first appearance of purple. The titration determined the ammonia absorbed in the boric acid. Protein was calculated from total nitrogen using 6.25 as a conversion factor.

$$\% \text{ Nitrogen} = \frac{\text{titer (sample-blank)} \times \text{N of HCl} \times 1.4007}{\text{weight of sample}}$$

$$\% \text{ Protein} = \%\text{Nitrogen} \times 6.25$$

3.2.2.3 Ash. Ash was determined by drying method. The sample (1 g) in a porcelain crucible was put in an oven (100 °C) overnight. The crucible was heated over a flame burner or on an electric hot plate until became black and fumeless. The sample was put in a muffle furnace (450 °C) until it became white or gray ash and weighed. (182).

3.2.2.4 Total fat. The sample was weighed accurate to 2.5 g and transferred to erlenmeyer flask. Hydrochloric acid (50 ml) was added into flask and reflux with gentle boiling for 1 h. The solution was filtered and washed with hot water until the filtrate became free of acid by using pH paper. The filter paper containing digested sample was dried in an oven (60 °C) overnight and transferred it into an extracting. Petroleum ether (50 ml) was added into a round flat bottom flask and connected with soxhlet system HT (model 1043, tecator) and extracted the sample for 2 h. The extracted fat was dried in oven (100 °C) until a constant weight was obtained.

$$\% \text{ Fat} = \frac{w - w_o}{s} \times 100$$

where w = weight of container with fat;

w_o = weight of container without fat;

s = weight of sample.

3.2.2.5 Total dietary fiber. Total dietary fiber was determined by enzymatic-gravimetric method (183). The sample was weighed accurate to 0.3 g into 50 ml plastic tubes. The sample was digested with α-amylase, protease and amyloglucosidase. The

digested product was transferred to 250-ml beaker and washed with four 35 ml portions of 95% ethanol and left it for precipitating overnight. Alcohol-insoluble materials were collected on crucible by vacuum suction. Three 20 ml portions of 78% ethanol, two 10 ml portions of 95% ethanol and two 10 ml portions of acetone were used for washing the residues. The crucible containing residues were dried overnight in hot air oven (105 °C). The sample residues were analyzed for dietary fiber from remaining duplicate for crude protein and ash as described in section 3.2.2.2 and 3.2.2.3, respectively. The amount of total dietary fiber was calculated by equation as follow:

$$\% \text{ Total dietary fiber} = \frac{(\text{residue sample})-(\text{residue blank})-(\text{protein})-(\text{ash})}{\text{weight of sample}} \times 100$$

3.2.2.6 Carbohydrate. Carbohydrate was estimated by subtracting moisture, total fat, protein and ash (g/100 g) from 100 (182).

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

3.2.2.7 Energy. Energy was determined by calculation using the follow equation (182).

Energy (kcal) = (% protein x 4)+(% fat x 9)+(% carbohydrate x 4)

3.2.2.8 Iodine. The samples for iodine determination were prepared using the method described by Moxon and Dixon (184). Each sample was weighed (0.2 g) and transferred into a dry porcelain crucible. One ml of 30% potassium carbonate solution and one ml of 10% zinc sulphate solution were added in crucible and placed it on water bath until dry. The crucible was put into furnace 550 °C for 3 h and 1 ml of 10% zinc sulphate was added. The drying and ashing method was repeated. Distilled water was added to dissolve the residues and centrifuged at 3000 rpm for 5 min. The supernatant

was transferred into a polyethylene bottle; 1 ml of each sample solution was transferred into test tube containing 1 ml of arsenious acid solution and 1 ml of ceric ammonium sulphate solution. The solution was transferred into cuvette and recorded absorbance at 410 nm. The iodine content in sample was determined by compared with standard curve.

3.2.3 Antimutagenicity Test of Various Extracts of Seaweed

3.2.3.1 The Bacterial Tester Strain

Salmonella typhimurium tester strains used in this study were histidine-dependent strains (His^-) TA98 and TA100 which were capable of detecting frameshift mutation and base-pair substitution, respectively. Both strains were kindly provided by Dr. Wannee Kusamran, National Cancer Institute, Ministry of Public Health Thailand. The tester strains were manipulated as shown in appendix B. Overnight cultures of bacteria inoculated from frozen stock culture in oxoid nutrient broth No. 2 at 37 °C were used for mutagenesis assay within 24 hours.

3.2.3.2 Sample preparations

a) Seaweed. Six different brandnames of dried seaweed were studied. The details on producer, distributor and ingredients in seasoned or non-seasoned seaweed are shown in Table 5. The seasoned seaweed were bought from Had Yai market (Songkla) (the center of distributor in Thailand). Non-seasoned seaweed was bought from Yao Wa Rat market in Bangkok.

b) Extraction. Fifty grams of dried seaweed was blended and extracted with various organic solvents namely acetone, ether, chloroform and methanol-chloroform

(1:1) in a soxhlet apparatus for 6 h. Each solvent extraction was concentrated in a rotary evaporator under reduce pressure at 30 °C. Exception was made on ether extract; it was concentrated using N_2-stream. The dried residue of each extract was stored at 0°C before determination. Dimethylsulfoxide (DMSO) was used to bring each sample to_concentration ranging from 20 mg/ml to 400 mg/ml, except the chloroform extract was ranging from 1 mg/ml to 400 mg/ml .

The water extract of each dried seaweed was prepared by heating ten grams of sample with 2000 ml distilled water at 70°C with stirring; then it was filtered through the sieve and filter paper. The filtrates were concentrated by using freeze-drier (FTS systems, model TPS-3d-MP, New York) and stored at 0°C. The dried extract was mixed with DMSO and centrifuged at 1000 rpm for 10 min. The supernatant was stored at 0°C and made up to a range of 0.5 mg/ml to 2 mg/ml.

3.2.3.3 Direct Mutagenesis Assay

a) Preparation of a minimal agar plate

Minimal agar containing 1.5% Bacto-Difco agar was autoclaved and then it was mixed with 2% sterile glucose and Vogel-Bonner medium E (see in appendix B). About 30 ml of molten agar was poured on to the sterile petri dish. It was left until solidified and was stored at 37 °C in the incubator for 48 h.

b) Preparation of top agar

Top agar containing 0.6% Bacto-Difco agar and 0.5% Sodium chloride was autoclaved and was stored at 45°C. Before use, 10% (v/v) of a sterile solution of 0.5 mM. histidine and biotin was added to the molten top agar and then it was maintained at 45 °C in the water bath.

c) Standard Direct Mutagen

1-Aminopyrene treated with nitrite in acid solution was used as a standard mutagen since it has been shown to give direct-acting mutagenicity in the condition similar to that occurred in the stomach (185,186). Briefly explain, 20 µl (tested on TA98) or 80 µl (tested on TA100) 1-aminopyrene (0.0375 mg/ml) in a tube fitted with a plastic stopper was mixed with a suitable volume of 0.2N hydrochloric acid (sufficient to acidify the reaction mixture to pH 3.0-3.5) and 0.25 ml of 2M sodium nitrite. The final concentration of nitrite was 0.5M. The reaction tube was shaken at 37 °C in a water bath for 4 h and then it was stopped by placing the tube in an ice bath. In order to decompose the residual nitrite, 0.25 ml of 2M ammonium sulfamate was added to the reaction mixture and allowed to stand for 10 min in an ice bath. The mixtures (100 µl) was mixed with 0.5 ml phosphate buffer (pH 7.4), 100 µl of fresh overnight culture of tester strain and incubated at 37°C in a shaking waterbath for 20 min. After incubation, 2 ml of molten soft agar (45°C) was added. It was mixed well and poured onto a minimal glucose agar plate. The plate was rotated to achieve uniform colony distribution and incubated at 37°C in the dark for 48 h. After the incubation period, His^+ revertants colonies were counted. Based on the mutagenicity testing, aminopyrene (0.06 and 0.24 µg/plate) was used as positive control or positive mutagen with the treatment of excess of nitrite in a gastric like condition.

d) Effect of Various Extracts of Dried Seaweed (*Porphyra spp.*) on Mutagenic activity of 1-Aminopyrene treated with Nitrite

Effect of various extracts of dried seaweed on mutagenic activity of 1-aminopyrene treated with nitrite for 4 hr was determined. Extract of sample (0.1 ml)

was transferred into a sterile plastic stopper tube containing 0.1 ml of final 4 hr AP-nitrite mixture (as described above). And then 0.5 ml of 0.2 M sodium phosphate buffer (pH 7.4) and 0.1 ml of overnight bacterial culture were added. The reaction tube was mixed gently and incubated in a shaking waterbath at 37°C for 20 minutes. Then 2 ml of molten top agar containing 0.05 mM each of L-histidine and D-biotin were added, mixed gently and poured onto a minimal glucose agar plate. The His^+ revertant colonies were scored after incubation at 37°C for 48 h. Positive control containing 100 µl of the reaction mixtures of 1-aminopyrene treated with nitrite as described earlier. Negative control containing 100 µl DMSO was determined for spontaneous reversion.

Antimutagenicity was determined by comparing the number of colonies of the experimental plates (containing AP-nitrite reaction mixture and extract) to the number of colonies of the positive control plates (containing AP-nitrite reaction mixture). Percent inhibition was calculated by using the following formula (181) :

$$\% \text{ inhibition} = 100 - \left[\frac{\text{number of revertants with test compound}}{\text{number of revertants without test compound}} \right] \times 100$$

Antimutagenicity was ranked as follows:

Percent inhibition	activity
> 60%	strongly active
60-40 %	active
40-20 %	weakly active
20-0 %	not active

Table 5. Information of seasoned and non-seasoned seaweed

Sample code	Product	Size (cm.)	Producer	Distributor	Source in Thailand	Ingredients
sw 1	seasoned seaweed	8.5 x 3.0	Yamakawa Trading (PTE) Ltd., (Singapore)	Yamakawa (H.K.) Ltd. (Hong Kong)	Had Yai, Song Kla	Dried seaweed, soy sauce and sugar
sw 2	seasoned seaweed	8.5 x 3.0	Yamakawa Trading (PTE) Ltd., (Singapore)	Yamakawa (H.K.) Ltd. (Hong Kong) Yamakawa (M) SDN BHD. (Malaysia)	Had Yai, Song Kla	Dried seaweed, kelp extract, sugar, soy sauce, salt and liquorice spices
sw 3	seasoned seaweed	8.5 x 3.0	Not indicated	Soon Seng Huat (Singapore) PTE Ltd. (Singapore)	Had Yai, Song Kla	Dried seaweed, sugar, salt, soy sauce, red pepper and tangle
sw 4	seasoned seaweed	9.5 x 8.5	Not indicated	Yamakawa trading (PTE) Ltd. (Singapore) Yamakawa (H.K.) Ltd. (Hong Kong)	Had Yai, Song Kla	Dried seaweed, soy sauce, salt and seasoning
sw 5	seasoned seaweed	9.5 x 8.5	Not indicated	Yamakawa trading (PTE) Ltd. (Singapore) Yamakawa (H.K.) Ltd. (Hong Kong)	Had Yai, Song Kla	Dried seaweed, soy sauce, salt and seasoning
sw 6	seasoned seaweed	9.5 x 8.5	Product of Japan	Hwa Seng Confectionary PTE Ltd. (Singapore)	Had Yai, Song Kla	Dried seaweed, soy sauce, sugar, seasoning and amino acid
sw 7	Non-seasoned seaweed	22.0 *	Not indicated	Not indicated	Yao Wa Rat market, Bangkok	Dried seaweed

* Diameter is presented for circular form of seaweed number 7.

CHAPTER IV

RESULTS

4.1 Survey on Consumption Pattern of Seaweed

4.1.1 Characteristics of Surveyed consumer

The percentage of subject in relation to sex, age and seaweed consumption is shown in Table 6. The data indicated that the percentage of female (73.89%) was about three times more than males (26.11%). The highest percentage of the subjects' age was 21-30 years (52.22%). The percentage of the subjects who consumed seaweed was 87.19%.

Table 6. Characteristics of subjects separated by sex, age and seaweed consumption.

(N = 203)

Characteristics	Subjects	
	N	%
Sex		
Female	150	73.89
Male	53	26.11
Age (years)		
Under 10	6	2.96
11-20	29	14.29
21-30	106	52.22
31-40	44	21.67
41-50	16	7.88
Over 50	2	0.98
Seaweed Consumption		
Consumed	177	87.19
Not consumed	26	12.81

4.1.2 Characteristic of Seaweed

Each ready to eat seasoned dried seaweed was packed in plastic bag. Non-seasoned dried seaweed (number 7) was sold as a raw ingredient. Characteristic of dietary dried seaweed i.e. size (length and width) and weight per one sheet are shown in Table 7.

Table 7. Characteristics of Seaweed.

Seaweed number	Length (cm)	Width (cm)	Diametered (cm)	Weight (g)	Serving size on package (g)
1	8.5	3.0	-	0.3	12
2	8.5	3.0	-	0.3	12
3	8.5	3.0	-	0.3	5
4	9.5	8.5	-	0.8	12
5	9.5	8.5	-	0.9	12
6	9.5	8.5	-	0.8	-
7	-	-	22.0	30.0	-

4.2 Proximate Composition.

Table 8 shows data of proximate analyses of seaweed per 100 g. Because the serving size labeled on the package was different or the serving size was not labeled, so, the serving size of seaweed in the consumers was observed. One hundred and fifty females and fifty-three males aged 4-52 years old were interviewed. The questionnaire is shown in appendix A. The mean serving size from the surveyed consumer was calculated as 4.32 g for seasoned seaweed number 1-3 and 5.19 g for seasoned seaweed number 4-6, and the serving size of non-seasoned seaweed was 7.5 g. Proximate composition per serving and the percentages of Thai RDI of dietary dried seaweed in the surveyed consumers are shown in Table 9.

Table 8. Proximate composition of dietary dried seaweed (g per 100 g dry weight).

Seaweed number	Energy (Kcal)	Moisture	Protein	Total Fat	Carbohydrate	TDF	Ash
1	350	3.5	36.4	3.3	43.6	27.4	13.2
2	366	2.9	37.5	3.1	46.7	29.2	9.8
3	354	2.5	10.4	1.7	74.3	35.9	11.1
4	332	8.9	30.9	2.7	46.1	33.7	11.4
5	352	5.3	32.3	3.1	45.7	41.4	10.6
6	349	5.8	25.0	2.1	57.6	33.2	9.5
7	358	4.3	39.9	2.9	43.0	34.4	9.9

Protein. Protein content of seaweed is 10.4-39.9 g per 100 g. Seaweed number 7 had the highest amount of protein (39.9 g per 100 g) whereas seaweed number 3 had the lowest (10.4 g per 100 g). Average protein content per serving of seasoned seaweed number 6 was 1 g and 2 g was of seasoned seaweed number 1, 2, 4 and 5. Non-seasoned seaweed number 7 contained 3 g of protein per serving. Such, the protein content could provide 2-7 % Thai RDI for adult and 3-9 % Thai RDI for children aged 10-12 years

Total fat. The range of fat content of all seaweed is 1.7 g per 100 g of seasoned seaweed number 3 to the highest of 3.3 g per 100 g of seasoned seaweed number 1. Average fat content per serving was assumed to be 0 g per serving size of surveyed consumer. Therefore, seaweed is not a source of fat for daily consumption.

Carbohydrate. Carbohydrate content of dietary dried seaweed is 43.0-74.3 g per 100. For surveyed consumer, the average carbohydrate content of seaweed was 2-3 g per serving. It will provide 1-2 % Thai RDI for carbohydrate.

Total dietary fiber (TDF). TDF content of all seaweed is 27.4 g per 100 g of seasoned seaweed number 1 to the highest of 41.4 g per 100 g of seasoned seaweed number 5. In surveyed consumer, the seaweed will provide the amount of total dietary

fiber at 1,2 and 3 g. Most seasoned seaweed provided 5-8% Thai RDI whereas non-seasoned seaweed number 7 will provide 10% Thai RDI in dietary fiber. So, non-seasoned seaweed will be a good source of dietary fiber.

Energy. The range of energy content is 332 Kcal per 100 g in seasoned seaweed number 4 to 366 Kcal per 100g in seasoned seaweed number 2. The energy per serving in surveyed consumer was between 15-18 Kcal in seasoned seaweed and 27 Kcal in non-seasoned seaweed number 7. However, most of the calories in dietary dried seaweed were provided from carbohydrate.

Iodine value. Level of iodine in all dietary dried seaweed is ranged from 679 to 3,617 μg per 100 g. The highest iodine level was found in seasoned seaweed number 6. In surveyed consumer, the amount of iodine per serving was ranged from 29.3 to 63.89 μg in seasoned seaweed number 1, 2, 3, 4, and 5 but the highest amount (187.7 and 218.7 μg) were shown in seasoned seaweed number 6 and non-seasoned seaweed number 7. Both of them will provide 120% and 140% Thai RDI of iodine, respectively. These levels were more than Thai RDI, so, they will be a good source of iodine.

Table 9. Proximate composition per serving and the percentages of Thai RDI of dietary dried seaweed in the consumer .

Seaweed number	Serving size [a] (g)	Energy (Kcal)	Protein (g)	Total Fat		Carbohydrate		TDF	
				(g)	%Thai RDI	(g)	%Thai RDI	(g)	%Thai RDI
1	4.32	15	2	0	0	2	0	1	5
2	4.32	16	2	0	0	2	0	1	5
3	4.32	15	0	0	0	3	1	2	6
4	5.19	17	2	0	0	2	0	2	7
5	5.19	18	2	0	0	2	0	2	8
6	5.19	18	1	0	0	3	1	2	7
7	7.5	27	3	0	0	3	1	3	10

a = Serving size was determined from the questionnaire in surveyed consumer.

Table 10. Iodine content of dietary dried seaweeds per 100 g, per serving and the percentages of Thai RDI.

Seaweed number	Serving size of seaweed (g) [a]	Iodine (μg)		
		Per 100 g	Per serving	% Thai RDI[b]
1	4.32	1132	48.96	30
2	4.32	679	29.3	20
3	4.32	1479	63.9	40
4	5.19	1073	55.7	40
5	5.19	1025	53.2	30
6	5.19	3617	187.7	120
7	7.5	2916	218.7	140

a = The serving size determined from the questionnaires in the surveyed consumer.

b = %Thai RDI was calculated from the content of iodine in this experiment

4.3 Effect of Various Seaweed extracts on Mutagenicity of 1-Aminopyrene Treated with Nitrite towards *Salmonella typhimurium* TA98 and TA100.

Before testing for the antimutagenicity of the seaweed extracts, the mutagenicity of the extract was also tested. It was ascertained that most extracts of seaweed added to the bacteria did not influence their spontaneous mutation frequencies (data not shown).

All seaweed were extracted with acetone, chloroform, methanol-chloroform (1:1), ether and hot-water except seaweed number 6 was extracted with acetone, chloroform and methanol-chloroform (1:1). The extracted materials were dissolved in DMSO and investigated for the presence of antimutagenic activities with respect to mutagenicity induced by nitrite treated aminopyrene in *Salmonella typhimurium* TA98 and TA100. The results obtained in Table 11-17. The antimutagenicity of the acetone extracts at 2-

40 mg/plate in seaweed number 1-5 strongly inhibited nitrite treated aminopyrene induced mutagenesis in TA98. Percent inhibition was at 58-96% whereas the strongly inhibition was shown at 40 mg/plate in seaweed number 6. The strongly inhibition was shown in non-seasoned seaweed number 7 at dose level 10-40 mg/plate. The acetone extracts showed strongly active or active inhibition to aminopyrene-nitrite model in TA100, however, % inhibition of the extracts in TA100 was less than TA98 at the same concentrations.

In addition, the chloroform extracts at the concentration of 0.5-10 mg/plate showed more than 50% antimutagenic activity against product of aminopyrene treated nitrite in TA98 and TA100 except the chloroform extract of seaweed number 3 showed more than 50% inhibition at the concentration ranged 2-20 mg/plate. They produced the partial killing effect at the concentration of 20-40 mg/plate toward both strains, TA98 and TA100.

The methanol-chloroform (1:1) extracts of seaweed number 1 showed strongly inhibition at all dose level (2-40 mg/plate), whereas the extracts of seaweed number 2-3 showed active inhibition at the same concentrations. The extracts of seaweed number 4-5 showed weakly inhibition toward TA98 and TA 100. But there was no inhibitory effect of seaweed extracts number 6-7.

The ether extracts of all seaweed inhibited the mutagenicity of the reaction mixture of nitrite and aminopyrene in a dose-dependent manner on TA98 and the mutagenicity of the model was inhibited more than 50% at 2-40 mg/plate. However, the ether extract of seaweed number 3 showed more than 50% inhibition at 10-40 mg/plate. On the other hand, there was no inhibitory effect in the ether extract of

seaweed (number 1, 3-6) whereas the ether extract of seaweed number 2 and 7 showed weakly inhibition on TA100. The hot-water extracts of all seaweed did not show inhibitory effect towards both strains.

Table 11. Effect of various extracts of seaweed number 1 on mutagenic activity of 1-aminopyrene treated with nitrite towards *Salmonella typhimurium* TA98 and TA100.

Extracts of seaweed	Amount (mg/plate)	TA98		TA100	
		No. of His^+ revertants/plate	% Inhibition[a]	No. of His^+ revertants/plate	% Inhibition[a]
Acetone extract	0	726±190	0	1489±349	0
	2	108±8	85	438±73	70
	5	71±5	90	348±76	77
	10	64±4	91	355±60	76
	20	41±4	94	267±12	82
	40	29±10	96	244±14	84
Solvent control	0.2 ml	22±2	-	92±17	-
Chloroform extract	0	1006±68	0	1307±55	0
	0.1	938±49	7	660±10	50
	0.2	642±80	36	574±14	56
	0.5	332±10	67	333±27	74
	1	277±26	72	316±18	76
	2	176±3	82	277±3	79
	5	90±2	91	209±0	84
	10	79±1	92	182±14	86
	20	PK[b]	-	PK[b]	-
	40	PK[b]	-	PK[b]	-
Solvent control	0.2 ml	50±5	-	121±12	-

All data are expressed as mean±standard deviation of duplicate plates from two different experiments.

[a] % Inhibition is calculated by Calomme formula 1996.

[b] PK = Partial Killing effect

Table 11 (Continued). Effect of various extracts of seaweed number 1 on mutagenic activity of 1-aminopyrene treated with nitrite towards *Salmonella typhimurium* TA98 and TA100

Extracts of seaweed	Amount (mg/plate)	TA98		TA100	
		No. of His^+ revertants/plate	% Inhibition[a]	No. of His^+ revertants/plate	% Inhibition[a]
Methanol-chloroform extract (1:1)	0	465±15	0	1140±54	0
	2	98±0	79	354±20	69
	5	74±6	84	281±0	75
	10	60±2	87	152±10	87
	20	37±4	92	140±4	88
	40	22±1	95	134±27	88
Solvent control	0.2 ml	26±0	-	120±17	-
Ether extract	0	409±126	0	1174±141	0
	2	191±24	53	1208±75	0
	5	200±50	51	1131±76	4
	10	140±34	66	1183±116	0
	20	78±22	81	1134±117	3
	40	66±30	84	1208±106	0
Solvent control	0.2 ml	18±1	-	99±27	-
Hot-water extract	0	724±24	0	1212±85	0
	0.5	720±102	1	1149±55	5
	1	706±32	2	1087±48	10
	2	580±109	20	1052±41	13
Solvent control	0.2 ml	26±5	-	108±6	-

All data are expressed as mean±standard deviation of duplicate plates from two different experiments.
[a] % Inhibition is calculated by Calomme formula 1996.

Table 12. Effect of various extracts of seaweed number 2 on mutagenic activity of 1-aminopyrene treated with nitrite towards *Salmonella typhimurium* TA98 and TA100.

Extracts of seaweed	Amount (mg/plate)	TA98		TA100	
		No. of His^+ revertants/plate	% Inhibition[a]	No. of His^+ revertants/plate	% Inhibition[a]
Acetone extract	0	566±20	0	1062±130	0
	2	177±27	69	606±86	43
	5	203±26	64	375±28	65
	10	138±33	76	339±48	68
	20	121±34	79	339±104	68
	40	42±4	93	170±26	84
Solvent control	0.2 ml	24±1	-	78±5	-
Chloroform extract	0	1006±68	0	1006±68	0
	0.1	469±19	53	634±5	52
	0.2	346±7	66	332±52	75
	0.5	220±7	78	250±1	81
	1	130±2	87	192±32	85
	2	114±2	89	244±22	81
	5	88±3	91	174±10	87
	10	62±7	94	181±25	86
	20	PK[b]	-	PK[b]	-
	40	PK[b]	-	PK[b]	-
Solvent control	0.2 ml	50±5	-	121±12	-

All data are expressed as mean±standard deviation of duplicate plates from two different experiments.

[a] % Inhibition is calculated by Calomme formula 1996.

[b] PK = Partial Killing effect

Table 12 (Continued). Effect of various extracts of seaweed number 2 on mutagenic activity of 1-aminoopyrene treated with nitrite towards *Salmonella typhimurium* TA98 and TA100.

Extracts of seaweed	Amount (mg/plate)	TA98		TA100	
		No. of His^+ revertants/plate	% Inhibition[a]	No. of His^+ revertants/plate	% Inhibition[a]
Methanol-chloroform extract (1:1)	0	465±15	0	1312±30	0
	2	384±6	17	758±4	42
	5	236±8	49	710±2	46
	10	145±0	69	646±11	51
	20	102±4	78	651±28	50
	40	56±4	88	574±12	56
Solvent control	0.2 ml	26±0	-	95±15	-
Ether extract	0	584±85	0	1301±119	0
	2	112±35	81	887±5	32
	5	90±28	85	839±9	36
	10	71±17	88	714±22	45
	20	64±18	89	693±59	47
	40	44±8	92	613±55	53
Solvent control	0.2 ml	26±4	-	123±11	-
Hot-water extract	0	724±24	0	1212±85	0
	0.5	679±53	6	1167±73	4
	1	704±67	3	1140±82	6
	2	682±67	6	1186±43	2
Solvent control	0.2 ml	26±5	-	108±6	-

All data are expressed as mean±standard deviation of duplicate plates from two different experiments.

[a] % Inhibition is calculated by Calomme formula (1996).

Table 13. Effect of various extracts of seaweed number 3 on mutagenic activity of 1-aminopyrene treated with nitrite towards *Salmonella typhimurium* TA98 and TA100.

Extracts of seaweed	Amount (mg/plate)	TA98		TA100	
		No. of His^+ revertants/plate	% Inhibition[a]	No. of His^+ revertants/plate	% Inhibition[a]
Acetone extract	0	691±26	0	1418±20	0
	2	293±9	58	780±18	45
	5	246±45	64	686±8	52
	10	178±33	74	528±16	63
	20	120±18	83	362±17	74
	40	90±7	87	338±14	76
Solvent control	0.2 ml	25±2	-	103±5	-
Chloroform extract	0	1041±54	0	1593±296	0
	0.1	2023±26	0	1899±59	0
	0.2	1584±1	0	1741±56	0
	0.5	1058±2	0	1469±1	0
	1	650±18	38	805±64	49
	2	390±3	62	622±29	61
	5	148±24	86	468±10	71
	10	116±18	89	270±49	83
	20	76±16	93	150±10	91
	40	PK[b]	-	PK[b]	-
Solvent control	0.2 ml	49±3	-	169±14	-

All data are expressed as mean±standard deviation of duplicate plates from two different experiments.

[a] % Inhibition is calculated by Calomme formula 1996.

[b] PK = Partial Killing effect

Table 13 (Continued). Effect of various extracts of seaweed number 3 on mutagenic activity of 1-aminopyrene treated with nitrite towards *Salmonella typhimurium* TA98 and TA100

Extracts of seaweed	Amount (mg/plate)	TA98		TA100	
		No. of His^+ revertants/plate	% Inhibition[a]	No. of His^+ revertants/plate	% Inhibition[a]
Methanol-chloroform extract (1:1)	0	616±27	0	1313±30	0
	2	518±10	16	704±4	46
	5	374±38	39	663±17	50
	10	334±10	46	640±12	51
	20	272±16	56	609±18	54
	40	200±16	68	562±2	57
Solvent control	0.2 ml	26±1	-	95±15	-
Ether extract	0	440±27	0	1232±205	0
	2	396±29	10	1225±31	1
	5	336±33	24	1180±14	4
	10	209±39	52	1121±54	9
	20	170±37	61	1005±70	18
	40	64±13	85	914±82	26
Solvent control	0.2 ml	15±2	-	86±27	-
Hot-water extract	0	724±24	0	1212±85	0
	0.5	623±55	14	1116±25	8
	1	577±108	20	1094±27	10
	2	514±78	29	1139±88	6
Solvent control	0.2 ml	26±5	-	108±6	-

All data are expressed as mean±standard deviation of duplicate plates from two different experiments.

[a] % Inhibition is calculated by Calomme formula 1996.

Table 14. Effect of various extracts of seaweed number 4 on mutagenic activity of 1-aminopyrene treated with nitrite towards *Salmonella typhimurium* TA98 and TA100.

Extracts of seaweed	Amount (mg/plate)	TA98		TA100	
		No. of His^+ revertants/plate	% Inhibition[a]	No. of His^+ revertants/plate	% Inhibition[a]
Acetone extract	0	567±23	0	932±51	0
	2	189±16	67	516±104	45
	5	128±3	77	392±71	58
	10	102±7	82	364±14	61
	20	62±5	89	330±22	65
	40	28±2	95	323±0	65
Solvent control	0.2 ml	24±1	-	77±6	-
Chloroform extract	0	1041±54	0	1593±296	0
	0.1	1436±32	0	1456±1	9
	0.2	959±1	8	1126±59	29
	0.5	690±1	34	745±22	53
	1	360±26	65	540±42	66
	2	178±8	83	369±1	77
	5	58±22	94	300±19	81
	10	54±9	95	201±36	87
	20	PK[b]	-	PK[b]	-
	40	PK[b]	-	PK[b]	-
Solvent control	0.2 ml	49±3	-	169±14	-

All data are expressed as mean±standard deviation of duplicate plates from two different experiments.

[a] % Inhibition is calculated by Calomme formula 1996.

[b] PK = Partial Killing effect

Table 14 (Continued). Effect of various extracts of seaweed number 4 on mutagenic activity of 1-aminopyrene treated with nitrite towards *Salmonella typhimurium* TA98 and TA100

Extracts of seaweed	Amount (mg/plate)	TA98		TA100	
		No. of His^+ revertants/plate	% Inhibition[a]	No. of His^+ revertants/plate	% Inhibition[a]
Methanol-chloroform extract (1:1)	0	616±27	0	1313±30	0
	2	848±8	0	1154±56	12
	5	804±4	0	1124±22	14
	10	654±54	0	1002±26	24
	20	536±24	13	920±2	30
	40	474±10	23	846±46	36
Solvent control	0.2 ml	26±1	-	95±15	-
Ether extract	0	440±27	0	1232±205	0
	2	40±8	91	1234±92	0
	5	36±6	92	1134±175	8
	10	30±8	93	1087±136	11
	20	PK[b]	-	990±134	20
	40	PK[b]	-	798±104	35
Solvent control	0.2 ml	15±2	-	86±27	-
Hot-water extract	0	701±44	0	1336±109	0
	0.5	433±27	38	1420±196	0
	1	415±77	41	1378±165	0
	2	354±29	50	1352±162	0
Solvent control	0.2 ml	27±4	-	114±10	-

All data are expressed as mean±standard deviation of duplicate plates from two different experiments.

[a] % Inhibition is calculated by Calomme formula 1996.

[b] PK = Partial Killing effect

Table 15. Effect of various extracts of seaweed number 5 on mutagenic activity of 1-aminopyrene treated with nitrite towards *Salmonella typhimurium* TA98 and TA100.

Extracts of seaweed	Amount (mg/plate)	TA98		TA100	
		No. of His^+ revertants/plate	% Inhibition[a]	No. of His^+ revertants/plate	% Inhibition[a]
Acetone extract	0	567±23	0	932±51	0
	2	94±10	83	375±8	60
	5	58±2	90	248±2	73
	10	49±2	91	292±10	69
	20	42±10	93	208±2	78
	40	28±0	95	180±0	98
Solvent control	0.2 ml	24±1	-	77±6	-
Chloroform extract	0	660±89	0	1229±30	0
	0.1	582±1	12	936±95	24
	0.2	454±14	31	639±1	48
	0.5	246±14	63	350±12	72
	1	154±23	77	252±10	79
	2	82±1	88	244±4	80
	5	61±4	91	204±28	83
	10	50±4	92	154±2	87
	20	38±8	94	144±1	88
	40	PK[b]	-	PK[b]	-
Solvent control	0.2 ml	54±3	-	104±8	-

All data are expressed as mean±standard deviation of duplicate plates from two different experiments.

[a] % Inhibition is calculated by Calomme formula 1996.

[b] PK = Partial Killing effect

Table 15 (Continued). Effect of various extracts of seaweed number 5 on mutagenic activity of 1-aminopyrene treated with nitrite towards *Salmonella typhimurium* TA98 and TA100

Extracts of seaweed	Amount (mg/plate)	TA98		TA100	
		No. of His^+ revertants/plate	% Inhibition[a]	No. of His^+ revertants/plate	% Inhibition[a]
Methanol-chloroform extract (1:1)	0	566±11	0	1302±119	0
	2	574±10	0	675±5	48
	5	554±23	2	774±20	41
	10	469±18	17	816±0	37
	20	414±0	27	900±10	31
	40	314±26	44	783±87	40
Solvent control	0.2 ml	26±2	-	124±11	-
Ether extract	0	440±27	0	1232±204	0
	2	96±20	78	1220±51	1
	5	80±21	82	1154±29	6
	10	50±11	89	1090±12	12
	20	34±6	92	1010±18	18
	40	30±7	93	906±40	26
Solvent control	0.2 ml	15±2	-	86±27	-
Hot-water extract	0	764±12	0	1624±76	0
	0.5	759±20	1	1533±7	6
	1	710±2	7	1471±35	9
	2	690±4	10	1567±57	4
Solvent control	0.2 ml	25±0	-	112±2	-

All data are expressed as mean±standard deviation of duplicate plates from two different experiments.
[a] % Inhibition is calculated by Calomme formula 1996.

Table 16. Effect of various extracts of seaweed number 6 on mutagenic activity of 1-aminopyrene treated with nitrite towards *Salmonella typhimurium* TA98 and TA100.

Extracts of seaweed	Amount (mg/plate)	TA98		TA100	
		No. of His^+ revertants/plate	% Inhibition[a]	No. of His^+ revertants/plate	% Inhibition[a]
Acetone extract	0	567±23	0	932±51	0
	2	480±14	15	1177±1	0
	5	497±57	12	1301±81	0
	10	468±46	17	1005±43	0
	20	356±38	37	758±48	19
	40	230±14	60	488±7	48
Solvent control	0.2 ml	24±1	-	77±6	-
Chloroform extract	0	582±8	0	1086±57	0
	0.5	256±41	56	584±12	46
	1	154±20	74	434±14	60
	2	106±2	82	300±12	72
	5	83±0	86	159±18	85
	10	36±12	94	PK[b]	-
	20	PK[b]	-	PK[b]	-
	40	PK[b]	-	PK[b]	-
Solvent control	0.2 ml	34±4	-	110±10	-

All data are expressed as mean±standard deviation of duplicate plates from two different experiments.

[a] % Inhibition is calculated by Calomme formula 1996.

[b] PK = Partial Killing effect

Table 16 (Continued). Effect of various extracts of seaweed number 6 on mutagenic activity of 1-aminopyrene treated with nitrite towards *Salmonella typhimurium* TA98 and TA100.

Extracts of seaweed	Amount (mg/plate)	TA98		TA100	
		No. of His^+ revertants/plate	% Inhibition[a]	No. of His^+ revertants/plate	% Inhibition[a]
Methanol-chloroform extract (1:1)	0	409±126	0	1110±103	0
	2	413±147	0	1194±79	0
	5	400±111	2	1276±57	0
	10	434±127	0	1330±155	0
	20	472±88	0	1366±78	0
	40	487±129	0	1492±153	0
Solvent control	0.2 ml	18±1	-	88±25	0

All data are expressed as mean±standard deviation of duplicate plates from two different experiments.
[a] % Inhibition is calculated by Calomme formula 1996.

Table 17. Effect of various extracts of seaweed number 7 on mutagenic activity of 1-aminopyrene treated with nitrite towards *Salmonella typhimurium* TA98 and TA100.

Extracts of seaweed	Amount (mg/plate)	TA98		TA100	
		No. of His^+ revertants/plate	% Inhibition[a]	No. of His^+ revertants/plate	% Inhibition[a]
Acetone extract	0	926±91	0	878±81	0
	2	965±61	0	984±122	0
	5	652±16	30	654±12	26
	10	332±10	64	423±5	52
	20	203±0	78	348±35	60
	40	128±6	86	156±8	82
Solvent control	0.2 ml	19±1	-	110±19	-
Chloroform extract	0	1007±24	0	1212±26	0
	0.1	822±38	18	1078±1	11
	0.2	598±48	41	870±64	28
	0.5	204±2	80	685±19	43
	1	190±12	81	455±12	62
	2	56±6	94	328±9	73
	5	PK[b]	-	179±17	80
	10	PK[b]	-	PK[b]	-
	20	PK[b]	-	PK[b]	-
	40	PK[b]	-	PK[b]	-
Solvent control	0.2 ml	50±2	-	122±7	-

All data are expressed as mean±standard deviation of duplicate plates from two different experiments.

[a] % Inhibition is calculated by Calomme formula 1996.

[b] PK = Partial Killing effect

Table 17 (Continued). Effect of various extracts of seaweed number 7 on mutagenic activity of 1-aminopyrene treated with nitrite towards *Salmonella typhimurium* TA98 and TA100

Extracts of seaweed	Amount (mg/plate)	TA98		TA100	
		No. of His^+ revertants/plate	% Inhibition[a]	No. of His^+ revertants/plate	% Inhibition[a]
Methanol-chloroform extract (1:1)	0	409±126	0	1110±103	0
	2	314±65	23	1240±106	0
	5	372±88	9	1260±64	0
	10	456±174	0	1346±118	0
	20	528±166	0	1476±111	0
	40	636±167	0	1578±162	0
Solvent control	0.2 ml	18±1	-	88±25	-
Ether extract	0	714±17	0	1418±20	-
	2	133±9	81	1135±25	20
	5	102±0	86	1005±25	29
	10	63±1	91	933±29	34
	20	52±4	93	712±8	50
	40	40±0	94	502±30	65
Solvent control	0.1 ml	24±1	-	103±5	-
Hot-water extract	0	701±44	0	1336±109	0
	0.5	525±65	25	1335±118	0
	1	449±17	36	1209±129	10
	2	350±45	50	1117±17	16
Solvent control	0.1 ml	27±4	-	114±10	-

All data are expressed as mean±standard deviation of duplicate plates from two different experiments.

[a] % Inhibition is calculated by Calomme formula 1996.

CHAPTER V

DISCUSSION

5.1 Proximate and Iodine Analysis

The result of proximate analysis of dietary dried seaweed demonstrated that the protein content of *Porphyra spp.* in this study was 10.4 – 39.9 g/100g dry weight. Comparing with Miura's study (74), the protein content of *Porphyra* spp. was 29.0 – 35.6 % and with many other high protein foods such as roast beef (25%), canned salmon (20%) and soybean (30%) (187). Interestingly the amino acid scores of *Porphyra* proteins were higher than the land plants and being rather more like animal proteins (76). Human can digest 75% of the protein and carbohydrate in *Porphyra* spp. (66). It should be emphasized that the protein in *Porphyra* spp is of very good quality. However, the serving size of seasoned seaweed and non-seasoned seaweed are 5 g and 7 g, respectively. So, the protein content in each serving are varied between 0-3 g (Table 9). It can not be used as the main source of protein in the diet but it can be used as a supplementary protein in the diet.

The low fat content of seaweed renders it as unimportant source, however, the lipids are easily assimilated by man (188). Non-seasoned seaweed number 7 contained 43 g of carbohydrate per 100 g dry matter accordingly with Araki (79) found that total carbohydrate in *Porphyra* spp. was 40 g per 100 g dry matter. Total carbohydrate of seasoned seaweed number 1-6 was ranged from 44-58 g per 100 g dry matter, this

result may due to sugar that added in these products (The ingredients of seasoned seaweed were shown in Table 5). Both of seasoned and non-seasoned seaweeds contain carbohydrate and total dietary fiber that provided 1% Thai RDI and 5-10% Thai RDI, respectively. Total dietary fiber content of seasoned seaweed (number 1-6) was 1-2 g (5-8 %Thai RDI) while non-seasoned seaweed (number 7) was 3 g (10 %Thai RDI).

Iodine content of all seaweed ranged from 29.3-218.7 μg per serving. Seaweed numbers 1-5 provided 20-40% of the Thai RDI for iodine requirement, whereas seaweed number 6-7 provided more than 100% of the iodine requirement per day. Therefore, the seasoned seaweed number 6 and non-seasoned seaweed number 7 are the good source of iodine compared with the other samples. Consumption of 6 sheets of seasoned seaweed number 6 or 1/5 sheets of non-seasoned seaweed number 7 can provide iodine more than 100% Thai RDI. Hou (189) reported that concentrations of elements such as vitamins or minerals were also different for algae that grew in different places and the collecting seasons, these factors could affect to their constituents, particularly the iodine content. In addition, Fellows (190) suggested that the industrial process could reduce nutritive value of seaweed. These factors may affect to the iodine content among seasoned and non-seasoned seaweed in this study.

In conclusion, all dietary dried seaweeds can provide dietary fiber, protein and iodine. They can be used as a good source of iodine and fiber in a serving. Interestingly, the content of protein, dietary fiber and iodine of non-seasoned seaweed (seaweed number 7) in a serving was higher than all seasoned seaweed.

5.2 Effect of various seaweed extracts on mutagenicity of 1-aminopyrene treated with nitrite towards *Salmonella typhimurium* TA98 and TA100

As outlined above, strong and consistent associations have been detected between a consumption of seaweed and a cancer reduction (191). Two of the main mechanisms involved including the inhibition of carcinogenic fecal flora and antitumor effect. Epidemiological data has shown a possible correlation between the intake of nitrite and human gastric cancer suggesting that nitrosation is its possible cause (192- 194).

Kato and co-workers (147) demonstrated that aminopyrene treated with 4 equivalent amounts of nitrite in acid condition (pH 3) at 37°C was shown mutagenic activity to *Salmonella typhimurium* TA 98 and TA100 strain without metabolic activation. The results agreed with the work of Kangsadalampai *et al.*(186) which stated that nitrite treated aminopyrene exhibited stronger mutagenicity than the authentic aminopyrene toward *Salmonella typhimurium* in both strains, TA98 and TA100 in the absence of metabolic activation. Thus, the mutagenicity of aminopyrene treated with nitrite in acid condition has been established as a model for antimutagenicity study. The following information will confirm such an idea that the precursor of toxic substance which produced mutagen can be inhibited by seaweed.

In this study, the addition of acetone and chloroform extracts of all seaweeds could inhibit the mutagenicity of nitrite treated aminopyrene; this is the important factor for prevention *in situ* gastric mutagenic nitroso compound. These extracts inhibited frameshift and base-pair substitution mutagenesis but the results showed a genotoxic induction at high dose of chloroform extract (20-40 mg extract). According to the

study of Okai and his colleagues (141), they found a relatively strong antimutagenic activity of *Porphyra tenera* which showed a suppressive effect on mutagen-induced umu C gene expression in *Salmonella typhimurium* (TA1535/pSK 1002). In 1996, they found that the active principles for the antimutagenic activity in the extracts of *Porphyra tenera* were three major pigments which seemed to be β-carotene, chlorophyll a and lutein. All of them and the combination of these pigments caused significant suppressive activities against mutagen-induced umu C gene expression (142). Because of β-carotene can be extracted by acetone (142) and also extracted by chloroform (195). Acetone and chloroform extract of *P. tenera* in this study may exhibit suppressive activities of 1-aminopyrene treated with nitrite in various number of seaweeds (Table 11-17).

Yamamoto (2,118) found that *Porphyra* spp. strongly inhibited on chemically induced mammary tumorigenesis and DMH-induced intestinal carcinogenesis. This effect was thought to be due to an antioxidant contained in large quantities in *Porphyra* spp, possibly β-carotene. The considerable amounts of β-carotene in *Porphyra tenera* when it was extracted with methanol were 181-219 μg/ml extract (141). In addition, *Porphyra* was effective against both Ehrlich ascites carcinoma and Meth-A fibrosarcoma (140), and its extracts were able to stimulate both *in vitro* and *in vivo* macrophage function (196,197). Furthermore, β-carotene was shown to cause significant suppressive effects on the mutagenesis in *Salmonella typhimurium* test by cyclophosphamide (198), nitroarenes (199), nitro-aromatic compounds (200) and benzo(a)pyrene (201). Not only β-carotene, the antimutagenic activity of chlorophyll in different biological sources was reported by other assay systems (202,203). Chlorophyll

suggested to be an active factor responsible for the antimutagenic effects following *Salmonella typhimurium* microsome assay (204,205). Chlorophyll also reduced the mutagenicity action of a number of direct-acting carcinogens like *N*-methyl-*N*'-nitro-*N*-nitroguanidine (MNNG) and *N*-methyl nitrosourea and indirect-acting carcinogens like 7,12-dimethylbenzanthracene (206). Mutagenicity of 3-hydroxyamine-1-methyl-5-H-pyrido [4,3-b] indole, the metabolically active form of Trp-P-2 was suppressed effectively with chlorophyll in Somatic Mutation And Recombination Test (SMART) and Ames test (202). Because chlorophyll a is a major pigment contained in *P. tenera* (142) and is soluble in acetone, chloroform and ether (195). Therefore, acetone and chloroform extracts could inhibit the mutagenicity of nitrite treated aminopyrene in this study.

For ether extract of seaweed, the strong inhibitory activity to aminopyrene treated with nitrite responsed in TA98 whereas the weakly inhibition or no inhibition responsed to TA100. This result may due to compound that soluble in ether such as chlorophyll a was active inhibitor of frameshift mutation. In addition, lutein was found to be an active principle for the antimutagenicity in *P. tenera* (142) and it also soluble in ether (195). Lutein has a potent inhibitory activity for the mutagenicity of 1-nitropyrene, a compound which its structure related to 1-aminopyrene, in Ames *Salmonella*/microsome assay (207). So, it was possible that lutein contained in *P. tenera* was also shown inhibitory activity to aminopyrene treated nitrite and it was active inhibitor of frameshift mutation in this study.

It is significant that chlorophyll, β-carotene and lutein exhibit antimutagenic activities. Negishi et al. (202) reported on the antimutagenic activity of chlorophyll

against Trp-P-2 in *Drosophila* and provided preliminary data suggesting that the inhibitory mechanism may involve complex formation between chlorophyll and the heterocyclic amine, thereby reducing the bioavailability of the carcinogen. In addition, the chlorophyll probably act by blocking activation by microsomal enzymes of promutagens to mutagens and by forming inactive complexes with mutagenic derivatives (208). The chlorophyll also appears to be useful in reacting with and in activating aromatic mutagens within the gastrointestinal tract (209).

β-Carotene is an important fat-soluble antioxidant (210), it can scavenged reactive oxyradicals through their binding to polyene chain atoms (211). Another possible role for β-carotene was affect in blocking reaction with DNA, the compound must intercepted the proximal carcinogen, probably within milliseconds of formation, and must compete with available tissue nucleophiles for covalent binding (212). In addition, the possible mechanism of lutien was to form and extracellular complexes between mutagen and lutein, which could limit the bioavailability of mutagen (207). Thus, trapping by complex formation plays an important role in the antimutagenic action of chlorophyll, β-carotene and lutein.

All hot-water extracts of seasoned seaweeds did not show antimutagenic activity on nitrite treated aminopyrene. It was probably because of the solubility of the extracts was low. Reddy (121) found that the inhibitory effect to 7,12-dimethylbenz(a) anthracene (DMBA) and 3,2'-dimethyl-4-aminobiphenyl (DMAB) by the hot-water extracts of *Laminaria angustata* (brown seaweed) was less marked when studied using the *Salmonella typhimurium* strains TA98 and TA100. Whereas, other extracts such as acetone, ether, chloroform, methanol-chloroform (1:1) showed greatly inhibit DMAB-

induced mutagenicity in both strains and inhibited DMBA-induced mutagenicity in TA100.

Differencies among the results of the methanol-chloroform extracts may be due to the marinade used in seasoning that added in each number of seasoned seaweed. When they were extracted by methanol-chloroform (1:1), different active ingredients of seasoning were dissolved. However, non-seasoned seaweed in the extract of methanol-chloroform (1:1) did not inhibit mutagenic activity induced by nitrite treated with aminopyrene. On the other hand, it induced framshift and base-pair substitution mutagenesis of nitrite treated aminopyrene. However, the mixtures of methanol and chloroform extract of non-seasoned seaweed also showed simultaneously mutagenesis induce frameshift mutation in this study (Data was not shown). So, the active ingredients soluble in methanol-chloroform (1:1) that can induce mutagenesis should be studied. In addition, quercetin was found to be the principal compound in the methanol-chloroform (1:1) extracts of Japanese pickles (213). So, it is possible that the methanol-chloroform (1:1) can extract quercetin from dietary dried seaweed in this study. Quercetin induced point mutations in strains TA97, TA98, TA100 and TA102 in *Salmonella typhimurium* (214,215). On the other hand, in several *in vitro* experiments quercetin showed growth-inhibitory effects on various human cancer cell lined such as colon (216), breast (217) and gastrointestinal (218). In *in vivo* study, quercetin showed anticarcinogenic effect against 7,12-dimethybenz(a)thracene (219), 20-methylcholanthracene (220) and *N*-nitrosomethyurea (221). Robak (222) and Nakayama (223) demonstrated that quercetin scavenged oxygen free radical. So, quercetin acts as both mutagen and anticancer agent.

In conclusion, the antimutagenic activity observes on the seaweed extracts upon the reaction between aminopyrene and sodium nitrite with in stomach simulation in this study demonstrated the inverse relationship between the amount of seaweed extracts and number of revertants per plate. These results implied that the precursor of direct mutagen could be inhibited by seaweed in *in vitro* model. Because of *in vitro* bacterial mutagenesis was used to primary screening mutagenicity testing. The *in vivo* mutagenicity and antimutagenicity testing should be elucidated for determination of antimutagenic substances contained in *Porphyra* spp.

CHAPTER VI

CONCLUSION

Seaweed, P*orphyra* spp., can provide protein, dietary fiber and iodine. It is a potentially source of iodine and dietary fiber in a serving. It should be recommended to serve as snack for children or as food for people who lived in the iodine deficient area or the vegan groups. Interestingly, the content of protein, dietary fiber and iodine of non-seasoned seaweed was higher than in seasoned seaweed. This result may due to the industrial process or the different place of origin and collecting season can reduce nutritive value of seaweed. So, the non-seasoned seaweed can be a complementary source of food for human nutrition.

Using the Ames *Salmonella* mutagenicity assay in the absence of activating system, it was suggested that the acetone, chloroform and ether extracts of all seaweeds contained substance act as mutagenic inhibitor to aminopyrene treated nitrite. The antimutagenicity of the extracts may due to β-carotene, chlorophyll a and lutein. Because of these compounds can be extracted by acetone, chloroform and ether; it was suggested that these components were partially responsible for the antimutagenic properties of seaweed extracts in this study. On the other hand, there was no inhibitory effect of all hot-water extracts. It is probably due to low solubility of the extracts, so, the compounds in the extract did not show their activity.

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APPENDIX A

แบบสอบถามโครงการวิจัย

เรื่องการบริโภคสาหร่ายทะเลแบบแผ่น

แบบสอบถามนี้เป็นส่วนหนึ่งของการวิจัยซึ่งได้รับทุนจากสำนักงานคณะกรรมการวิจัยแห่งชาติ ประจำปีงบประมาณ 2540 ข้อมูลจากท่านจะเป็นประโยชน์อย่างยิ่งต่อการวิจัย คณะผู้วิจัยขอขอบคุณในความร่วมมือที่ท่านได้สละเวลาในการให้ข้อมูล

<u>ข้อมูลเกี่ยวกับผู้บริโภค</u>

1. อายุ ..... ปี

2. เพศ ( ) 1. ชาย ( ) 2. หญิง

3. สถานภาพสมรส ( ) 1. โสด ( ) 2. คู่ ( ) 3. หย่า/ม่าย

4. ระดับการศึกษา ( ) 1. ต่ำกว่าปริญญาตรี ( ) 2. สูงกว่าปริญญาตรี

5. อาชีพ ( ) 1. รับจ้าง รับราชการ รัฐวิสาหกิจ ( ) 2. ค้าขาย ธุรกิจส่วนตัว
( ) 3. นักเรียน นักศึกษา ( ) 4. แม่บ้าน ( ) 5. อื่น ๆ ระบุ .....

6. รายได้เฉลี่ยต่อเดือน (บาท) ( ) 0 = ไม่ได้ทำงาน ยังเรียนอยู่
( ) 1. น้อยกว่า 6,000 ( ) 2. 6,001-10,000 ( ) 3. มากกว่า 10,000

7. จำนวนสมาชิกในครอบครัว (รวมทั้งตัวท่าน) ..... คน

8. จำนวนบุตรที่อาศัยอยู่กับท่าน.....คน (ระบุอายุ).....ปี

<u>ข้อมูลเกี่ยวกับการบริโภค</u>

9. ท่านรับประทานสาหร่ายทะเลแบบแผ่นหรือไม่
( ) รับประทาน กรุณาตอบคำถามข้อ 11
( ) ไม่รับประทาน กรุณาตอบคำถามข้อ 10

10. หากท่านไม่รับประทาน สมาชิกในครอบครัวของท่านรับประทานหรือไม่
( ) รับประทาน โดยรับประทานทั้งหมด.....คน กรุณาตอบคำถามข้อ 11
( ) ไม่รับประทาน (ไม่ต้องตอบคำถามข้อ 10-16)

11. ท่านหรือสมาชิกในครอบครัวรับประทานสาหร่ายทะเลแบบแผ่นด้วยวิธีใด

( ) นำมารับประทานเป็นอาหารว่าง กรุณาตอบคำถามข้อ 12

( ) นำมาปรุงเป็นอาหาร กรุณาตอบคำถามข้อ 13

12. ท่านหรือสมาชิกในครอบครัว รับประทานสาหร่ายทะเลแบบแผ่นชนิดปรุงรสเป็นอาหารว่าง ครั้งละ .....แผ่น (คิดเฉลี่ยต่อ 1 คนใน 1 ครั้ง) กรุณาตอบคำถามข้อ 15

13. ท่านนำสาหร่ายทะเลแบบแผ่นชนิดใดมาปรุงเป็นอาหาร (ตอบได้มากกว่า 1 ข้อ)

( ) สาหร่ายทะเลแบบแผ่นปรุงรส ชนิดสี่เหลี่ยมผืนผ้า

( ) สาหร่ายทะเลแบบแผ่นไม่ปรุงรส ชนิดวงกลมโดยนำมาแช่น้ำให้อ่อนตัวก่อนนำไปประกอบอาหารเช่นแกงจืดต่าง ๆ

14. ในการปรุงอาหารแต่ละครั้ง ท่านใช้สาหร่ายทะเลแบบแผ่น .....แผ่น และ จำนวนสมาชิกที่รับประทานอาหารที่มีส่วนประกอบของสาหร่ายมื้อนั้น.....คน

15. ท่านคิดว่าสาหร่ายทะเลแบบแผ่นมีประโยชน์หรือไม่

( ) มีประโยชน์

คือ.....

( ) ไม่มี แต่รับประทานเพราะ .....

16.ใน 1 สัปดาห์ท่านได้รับประทานสาหร่ายทะเลทั้งชนิดปรุงรสเป็นอาหารว่างและชนิดไม่ปรุงรสมาประกอบ อาหารบ่อยเท่าใด

( ) น้อยกว่า 1 ครั้ง

( ) 1-2 ครั้ง

( ) 3-4 ครั้ง

( ) มากกว่า 4 ครั้ง (ระบุ.....ครั้ง)

APPENDIX B

MANIPULATION OF THE TESTING STRAINS

1. Preparation of Stock Solution and Media

1.1 Vogel-Bonner medium E stock salt solution (VB salt)

Ingredient	1 liter	2 liter
Distilled H_2O	670 ml	1,340 ml
Magnesium sulfate ($MgSO_4.7H_2O$)	10 g	20 g
Citric acid monohydrate	100 g	200 g
Potassium phosphate, dibasic (anhydrous) (K_2HPO_4)	500 g	1,000 g
Sodium ammonium phosphate ($NaNH_4HPO_4.4H_2O$)	175 g	350 g

Add salts in the order indicated to water and allowed each salt to dissolve completely before adding the next. Filter the solutions and then autoclave at 121 °C for 20 min.

1.2 Minimal glucose agar plate

Ingredient	300 ml	350 ml
Bacto agar	4.5 g	5.25 g
Distilled H_2O	280 ml	330 ml
VB salts	6 ml	7 ml
40% glucose	15	17.5 ml

Add agar to distilled water in a glass bottle. Autoclave at 121°C for 20 min. When the solution has cooled slightly, add sterile VB salts and sterile 40% glucose. Mix and pour 30 ml into each sterile petri plate. Minimal glucose agar plates were kept in incubator at 37°C before using.

1.3 Oxoid nutrient broth No.2

Dissolve 2.5 g of nutrient broth No.2 in 100 ml distilled H_2O. Transfer 12 ml of nutrient broth for each flask (covered with sterile gauze). Autoclave at 121°C for 20 min.

1.4 Top agar

Ingredient	200 ml	300 ml
Bacto agar	1.2 g	1.8 g
Sodium chloride (NaCl)	1.0 g	1.5 g
Distilled H_2O	200 ml	300 ml

Dissolve ingredients in water. Store in a glass bottle. Autoclave for 20 min at 121°C and then add 20 ml and 30 ml of 0.5 mM histidine HCl-0.5 mM biotin for 200 ml and 300 ml of top agar respectively.

1.5 0.1 M L-histidine HCl stock

Ingredient	100 ml
L-histidine HCl	2.096 g
Distilled H_2O	100 ml

Dissolve 2.096 g of L-histidine HCl (MW 209.63) in 100 ml distilled water. Autoclave at 121°C for 20 min.

1.6 1mM L-histidine HCL stock

Ingredient	100 ml
0.1 M L-histidine HCl	1 ml
Distilled H_2O	99 ml

Dilute 1 ml of 0.1 M L-histidine HCl in 99 ml of distilled water. Autoclave at 121°C for 20 min.

1.7 1mM biotin stock

Ingredient	100 ml
Biotin	24.43 mg
Distilled H_2O	100 ml

Dissolve biotin (MW 244.3) in distilled water. Warm it until dissolve completely. Autoclave at 121°C for 20 min.

1.8 0.5 mM L-histidine HCl-0.5 mM biotin

Ingredient	200 ml
1 mM L-histidine HCl	100 ml
1 mM biotin	100 ml

Mix and autoclave at 121°C for 20 min.

1.9 $NaPO_4$-KCl buffer

Ingredient	330 ml
0.5 M $NaPO_4$ pH 7.4	100 ml
1 M KCl	16.5 ml
Distilled H_2O	213.5 ml

Mix and autoclave at 121°C for 20 min.

1.10 1 M KCl

Ingredient	1,000 ml
Potassium chloride	74.56 g
Distilled H_2O	1,000 ml

Mix and autoclave at 121°C for 20 min.

1.11 8 mg/ml ampicillin solution

Ingredient	10 ml
Ampicillin (sodium)	800 mg
Distilled H_2O	10 ml

Dissolve and store in glass bottle with screw cap at 0°C.

1.12 0.1% crystal violet

Ingredient	10 ml
Distilled H_2O	10 ml
Crystal violet	10 mg

Mix and store at 0°C in glass bottle with screw cap.

2. Procedure for Reisolation and Growing Culture.

Tester strains, TA98 and TA100 are grown in Oxoid nutrient broth No.2 and incubated overnight in a 37°C in shaking water bath. The growth period should not exceed 16 hours. These cultures are reisolation by streaking on minimal glucose agar plates which the surface were spread with 0.1 ml of 8 mg/ml ampicillin, 0.3 ml of 0.1 M histidine HCl and 0.1 ml of 1mM biotin. These plates are incubated at 37°C for 48 hours. After incubation, the 5 single colonies per strain TA98 and TA100 are picked up

and grown in Oxoid nutrient broth No.2 overnight at 37°C in shaking water bath. Each culture is confirmed genotypes of the strains and kept the cultures as the source of bacteria for mutagenicity testing. For each 1 ml of culture, 0.09 ml of spectrophotometric grade DMSO were added. Combine the culture and DMSO in a sterile tube and distribute 200µl of the culture aseptically into sterile cryotube (Nunc) and then transfer to a -80°C freezer.

3. Confirming Genotype of Tester Strains

The broth cultures of TA98 and TA100 are used to confirm genotypes in the following ways.

3.1 Histidine requirement

The his^- character of the strains is confirmed by demonstrating the histidine requirement for growth on the minimal glucose agar plates enriched with histidine and biotin.

Procedure:

plate a no histidine and biotin

plate b 0.1 ml of 1mM biotin

plate c 0.3 ml of 0.1 M his-HCl

plate d 0.3 ml of 0.1 M his-HCl + 0.1 ml of 1 mM biotin

Four minimal glucose agar plates are required for each tester strains. Each of the plates is applied on the surface with 0.1 ml of 1 mM biotin, 0.3 ml of 0.1 M his-HCl, 0.3 ml of 0.1 M his-HCl plus 0.1 ml of 1 mM biotin and no application (plate b, c, d, a respectively). Make a single streak of each strain across these plates. Five strains could

be tested on the same plate. Incubated at 37°C for 24 hours. The growing of bacteria on histidine plus biotin plate is the result of histidine requirement.

3.2 R Factor

The R-factor strains (TA97, TA98, TA100 and TA102) should be tested routinely for the presence of the ampicillin resistance factor because the plasmid is somewhat unstable and can be lost from the bacteria.

Procedure: For each tester strain (TA98 and TA100), 0.3 ml of fresh overnight culture was added to a tube containing 0.1 ml of 0.1 M histidine-HCl. And then 2 ml of molten top agar containing 0.5 mM histidine-HCl and 0.5 mM biotin were added, mixed and poured on a minimal agar plate. Rotate the plate to distribute the mixtures and allow several minutes for agar to become firm. R-factor and rfa mutation (see the next section) are performed in the same plate by dividing the plate into 2 areas, one for R-factor and the other for rfa mutation. For R-factor, filter paper disc containing 8 mg/ml ampicillin is applied on the surface of the agar by using sterile forceps. The disc is pressed lightly to embed in the overlay. The plates are incubated at 37°C for 24 hours. The absence of the clear zones of inhibition around the disc indicates resistance to ampicillin.

3.3 rfa mutation

Strains having the deep rough (rfa) character should be tested for crystal violet sensivity.

Procedure: Pipette 0.1% solution of crystal violet to the sterile filter paper disc (1/4 inch) and transfer the disc to plates, seed with bacteria (the procedure is similar to R-factor). Incubated at 37°C for 48 hours. The clear zone appeared around the disc

indicated the presence of the rfa mutation (the permitted crystal violet to enter and kill bacteria).

4. Spontaneous Reversion

Spontaneous reversion of the tester strains to histidine independence is measured routinely in mutagenicity experiments and is expressed as the number of spontaneous revertants per plate. The revertant colonies are clearly visible in a uniform background lawn of auxotrophic bacteria. Each tester strain reverts spontaneously at a frequency that is characteristic of the strain. Nevertheless, there is variability in the number of spontaneous revertants from one experiment to another and from one plate to another, and it is advisable to include at least 2-3 spontaneous mutation control plates for each strain in a mutagenicity assay.

Procedure: 0.1 ml of DMSO is added to capped culture tube. Add 0.5 ml of $NaPO_4$-KCl buffer pH 7.4, 0.1 ml of fresh overnight culture of TA98 or TA100, followed by 2.0 ml of molten top agar. Mix and then pour on minimal glucose agar plate. Rotated plates and left it to become hardens. Incubate at 37°C for 48 hours and the his^+ revertants colonies are counted.

5. The Response to Standard Carcinogen

Standard mutagens or positive mutagens are used routinely in mutagenicity experiments to confirm the reversion property and specificity of each strain. The standard mutagen, which used in this experiment, is nitrosoaminopyrene. Tester strain which highly response to positive mutagens be collected.

Procedure: 0.01, 0.02, 0.04 and 0.08 ml of 0.0375 mg aminopyrene /ml of acetonitrile are pipetted to sterile capped tube. Add 0.74, 0.73, 0.71 and 0.67 ml of 0.2 N HCl, respectively and 0.25 ml of 2 M $NaNO_2$ are added for each tubes. The final concentrations of aminopyrene were 0.3, 0.6, 1.2 and 2.4 mg, respectively and the final concentration of nitrite was 0.5 M. Mix and shake in water bath at 37°C for 4 hours. Place the tube in an ice bath and add 0.25ml of 2 M $NH_2SO_3NH_4$. Stand for 10 min. Pipette 0.1 ml of each mixture to capped culture tube for testing the stock culture TA98 (0.03 and 0.06 mg aminopyrene/plate) and TA100 (0.12 and 0.24 mg aminopyrene/plate). Then evaluate their mutagenicity as described in spontaneous reversion. The characteristic of the stock culture for TA98 and TA100 as the source of bacteria for mutagenicity is

a. contained R-factor (pKM 101) and rfa mutation.

b. His+ requirement.

c. Low spontaneous reversion.

d. Highly response to standard carcinogen.

After the characteristic of the culture is tested, the mutagenicity test is started.