14/0194/43

Levels and Mutagenic Activity of N-Nitrosodimethylamine in Fermented Fishes

ปริมาณและความสามารถในการก่อกลายพันธุ์ของสารเอ็น-ไนโตรโซไดเมธิลเอมีนในปลาร้า

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บทคัดย่อ : ประสงค์ คุณานุวัฒน์ชัยเดช ประหยัด พันธะศรี และ เทอดศักดิ์ ศุภสารัมภ์. 2543.ปริมาณและความสามารถในการก่อกลายพันธุ์ของสารเอ็น-ไนโตรโซไดเมธิลเอมีนในปลาร้า. วารสารวิจัยวิทยาศาสตร์การแพทย์ 14(2) : 65-75.

การตรวจวิเคราะห์หาปริมาณสารเอ็น-ไนโตรโซไดเมธิลเอมีน ที่เกิดขึ้นเนื่องจากกระบวนการหมักปลาร้า ใช้วิธีการแยกสกัดทำให้บริสุทธิ์และวัดการดูดกลืนแสง การศึกษาเชิงเปรียบเทียบตัวอย่างปลาร้าที่ได้มาจาก 3 ภูมิภาคในประเทศไทย รวมจำนวนทั้งสิ้น 88 ตัวอย่าง พบว่ามีเพียงร้อยละ 7.56 ของตัวอย่างทั้งหมด ที่ไม่สามารถตรวจสอบหาปริมาณของสารชนิดนี้ได้ สำหรับตัวอย่างปลาร้าที่สามารถวิเคราะห์ได้นั้น พบว่าปริมาณของสารชนิดนี้อยู่ในช่วงพิสัยที่แตกต่างกัน ปลาร้าจากภาคเหนือมีค่าระหว่าง 32-638 ส่วนในพันล้านส่วน ปลาร้าจากภาคกลางและภาคตะวันออกเฉียงเหนือมีค่าอยู่ระหว่าง 28-352 และ 42-1,228 ส่วนในพันล้านส่วน ตามลำดับ การตรวจสอบความสามารถในการก่อกลายพันธุ์ของสารชนิดนี้ โดยใช้บักเตรี Salmonella *typhimurium* สายพันธุ์ TA 98 และ TA 100 ภายใต้สภาวะการบ่มกับเอนไซม์จากตับหนู (S-9 Mix) พบว่าสารสกัดจากตัวอย่างปลาร้ามีความสามารถในการก่อกลายพันธุ์แบบ base-pair substitution ความเข้มข้นของสารสกัดมีความสัมพันธ์เชิงเส้นกับการก่อกลายพันธุ์ ความสามารถในการก่อกลายพันธุ์ของสารสกัดจากตัวอย่างปลาร้าทั้งสามภาคมีความสัมพันธ์กับปริมาณของสารเอ็น-ไนโตรโซไดเมธิลเอมีน

คำสำคัญ : สารเอ็น-ไนโตรโซไดเมธิลเอมีน ความสามารถในการก่อกลายพันธุ์ ปลาร้า

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Abstract : Prasong Koonanuwatchaidet, Prayad Pantasee and Terdsak Subhasaram. 2000. Levels and mutagenic activity of N-nitrosodimethylamine in fermented fishes. Thai J Hlth Resch 14(2) : 65-75.

Partially purified N-nitrosodimethylamine (NDMA) from 88 samples of uncooked fermented fish extracts obtaining from three different geographical regions of Thailand was quantitatively investigated using absorption spectrophotometric technique. Only about 7.56% of total specimens observed to be negative for this particular toxic compound. The NDMA levels of positive batches were in the range of 32 - 638 parts per billion (ppb), 28 - 352 ppb, and 42 - 1,228 ppb for the samples obtained from Northern, Central and Northeastern region of Thailand, respectively. The *in vitro* mutagenesis activity of chemically occurring NDMA in all positive batches were also detected by using two different strains of Salmonella *typhimurium* (TA 98 and TA 100) *via* preincubation with a rat liver microsomal enzyme system (S-9 Mix). The mutation was shown base-pair substitution. A linear dose-response relationship between mutagenicity and the extract concentration was investigated. The mutagenicity of fermented fish extracts obtained from 3 regions also shows correlation with the levels of NDMA.

Key words : N-nitrosodimethylamine, mutagenic activity, uncooked fermented fishes

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Introduction

It is now more than forty five years since the toxic effect of N-nitrosodimethylamine (NDMA) in various animal species was firstly demonstrated (Barnes and Magee, 1954), and following by the reports of hepatocarcinogenicity of this particular toxic compound in rat about two years later (Magee and Barnes, 1956). A large number of investigations have been appeared on the occurrence of N-nitroso compounds in foods and food products, mostly cured meat products, smoked fishes and Chinese salted fishes (Sen, 1972 ; Fong and Chan, 1976 ; Fong and Chan, 1977 ; Scanlan, 1983 ; Sugimura and Sato, 1983 ; Koonanuwatchaidet, 1984).

Since fermented fishes are used as a principal constituent of daily meal by over 85 per cent of the Thai households, especially in Northeastern part of Thailand (Koonanuwatchaidet and Srisuwan, 1985; Koonanuwatchaidet *et al.*, 1996), we felt that they were the most likely source of N-Nitrosodimethylamine ingestion for the local people. This study reports the levels of NDMA in uncooked fermented fishes from three different geographical regions of Thailand, and all of the positive specimens have been *in vitro* detected for the mutagenic activity using two different strains of Salmonella *typhimurium* in order to predict its carcinogenicity in higher animals including human.

Materials and Methods

Chemicals

Methylene chloride, chloroform, dimethylsulfoxide (DMSO) and polyamide resin were obtained from J.T. Baker Chemical Company, Phillipsburg, New Jersey, U.S.A.N-Nitrosodimethylamine, polychlorinated biphenyl (Arochlor 1254), nicotinamide adenine dinucleotide phosphate ($NADP^+$) and glucose-6-phosphate (G-6-P) were purchased from Sigma Chemical Company, Saint Louis, Missouri, U.S.A. Bacto agar and nutrient broth were from Difco Laboratories, Detroit, Michigan, U.S.A.

Biochemical assay for N-Nitrosodimethylamine

Fifteen samples of uncooked fermented fishes were obtained from local market retailers in three provinces of Northern part of Thailand. Thirteen and sixty batches purchased from local markets of Central and Northeastern Thailand, respecitively. In each experiment, the sample used was 100 grams. N-nitrosodimethylamine extraction was carried out by the methods of Fong and Chan (1977), and of Sen (1972). The sample

with solid component was chopped into small pieces, thoroughly homogenizes using Waring blender and then extracted with methylene chloride at pH 8.2 by GFL extraction-flask shaker for 100 min. The extraction was repeated once more, and after removal of methylene chloride by evaporation, NDMA occurred in crude extract was separated by steam distillation. The distillate was passed through a combined ion-exchange polyamide column which was topped with a layer of anhydrous sodium sulfate. The partially purified eluate was reextracted three 30 min-times with methylene chloride, and concentrated to 500-1,000 μl by evaporator. The final extract was determined semiquantitatively by thin-layer chromatography (TLC) (Cross *et al.*, 1978). Quantitative estimation of NDMA in the positive samples was carried out by scrapping the bands off TLC plates and eluting the silica gel with chloroform. Each eluent was made up to a standard volume and the definite determination was obtained by its absorbancy at 365 nm using Hitachi fluorescent absorption spectrophotometer, Model F-2000.

Preparation of samples for mutagenicity assay

Each sample of uncooked fermented fishes was minced and suspended into DMSO with proportion of 2 ml per gram. The suspension mixture was vigorously shaken by Vortex mixer for 150 min and then centrifuged at 2,000 x g for 15 minutes at room temperature. The supernatant was used for detection of mutagenic activity.

Bacterial strains

Tester strains of Salmonella *typhimurium* (TA 98 and TA 100) for assay of mutations, was kindly supplied by Professor Bruce N. Ames, University of California, Berkeley, CA, U.S.A.

Preparation of the S-9 mix

A 9,000 x g supernatant (S-9 fraction) of rat liver homogenate was prepared by the method of Ames *et al.*. (1975). A male Sprague-Dawley rat with body weight about 250 g which had been induced by a single intraperitoneal injection dose of polychlorinated biphenyl (Arochlor 1254) mixture (500 mg per kg body weight), and had been killed 5 days later. The one ml of S-9 mix was containing : S-9 fraction (0.15 ml), $MgCl_2$ (8 μmole), KCl (33 μmole), G-6-P (5 μmole), $NADP^+$ (4 μmole), and phosphate buffer with pH 7.4 (100 μmole).

Mutagenicity assay

The test was carried out by the method as described by Yahagi, *et al.*. (1977) and Maron and Ames (1983). S-9 mix (500 µl) and bacterial tester strain (100 µl) were added to a tube containing 200 µl of the testing substance in aqueous solution. This mixture was preincubated at 25° C for 20 min, then mixed well with 2 ml of molten top agar finally poured onto a minimal glucose agar plate containing 100 µl of 0.1 M L-histidine. After incubation at 37° C for 48 hours, the histidine prototrophy revertant colonies were counted.

Results

The levels of N-Nitrosodimethylamine (NDMA) in uncooked fermented fish extracts obtained from local markets of Northern, Central and Northeastern parts of Thailand are shown in Table 1, Table 2 and Table 3, respectively. The average amount of NDMA in the samples from Northern area was 252 ± 160 ppb, while in the samples from Central and Northeastern Thailand were 156 ± 92, and 430 ± 302 ppb, respectively. The results of *in vitro* mutagenesis assay on the dimethylsulfoxide extracts of uncooked fermented fish samples from three different areas are indicated in those Tables. NDMA levels and revertant colonies showed significantly correlation (p=0.000) with correlation coefficient of 0.955 for TA 98 and 0.972 for TA 100 respectively (Figure 1).

Table 1 The N-nitrosodimethylamine levels and *in vitro* S. *typhimurium* revertant colonies of fermented fish extracts from Northern part of Thailand

Sample Number	NDMA (ppb)	*S.typhimurium* Revertant Colonies TA 98	TA 100
1	179	108	486
2	258	132	580
3	92	80	324
4	208	130	488
5	ND	-	-
6	344	193	528
7	32	NS	NS
8	165	120	456
9	88	95	310
10	246	140	532
11	429	162	705
12	638	208	915
13	176	135	508
14	265	145	540
15	406	160	598
Min - Max	32 - 638	80 - 208	310 - 915
Mean ± SD	252 ± 160	139 ± 36	536 ± 154
N	14	13	13

ND = Not detectable NS = Not significant

Table 2 The N-nitrosodimethylamine levels and *in vitro* S. *typhimurium* revertant colonies of fermented fish extracts from Central part of Thailand

Sample Number	NDMA (ppb)	*S.typhimurium* Revertant Colonies TA 98	TA 100
1	105	96	360
2	219	140	515
3	96	100	345
4	68	NS	250
5	145	115	440
6	254	135	536
7	352	154	590
8	193	125	506
9	210	138	498
10	28	NS	NS
11	ND	-	-
12	126	110	475
13	78	NS	290
Min - Max	28 - 352	96 - 154	250 - 590
Mean ± SD	156 ± 92	124 ± 20	437 ± 110
N	12	9	11

Table 3 The N-nitrosodimethylamine levels and *in vitro* S. typhimurium revertant colonies of fermented fish extracts (some selected specimens) from Northeastern part of Thailand

Sample Number	NDMA (ppb)	*S.typhimurium* Revertant Colonies TA 98	TA 100
1	326	139	526
3	98	NS	150
5	120	60	185
7	366	135	536
9	ND	-	-
11	982	448	1,342
13	109	54	175
15	520	258	826
17	688	228	980
20	355	139	544
22	82	NS	182
24	1,048	398	1,432
25	536	202	804
26	421	166	632
30	486	190	689
31	317	144	569
33	235	148	520
35	602	247	915
37	402	218	830
39	284	122	557
41	1,228	532	1,627
43	511	190	712
45	386	170	678
47	ND	-	-
50	42	NS	NS
52	734	359	1,126
54	ND	-	-
56	73	NS	NS
58	359	176	549
60	306	140	539
Min - Max	42 - 1,228	54 - 532	150 - 1,627
Mean ± SD	430 ± 302	211 ± 119	705 ± 380
N	27	23	25

ND = Not detectable NS = Not significant

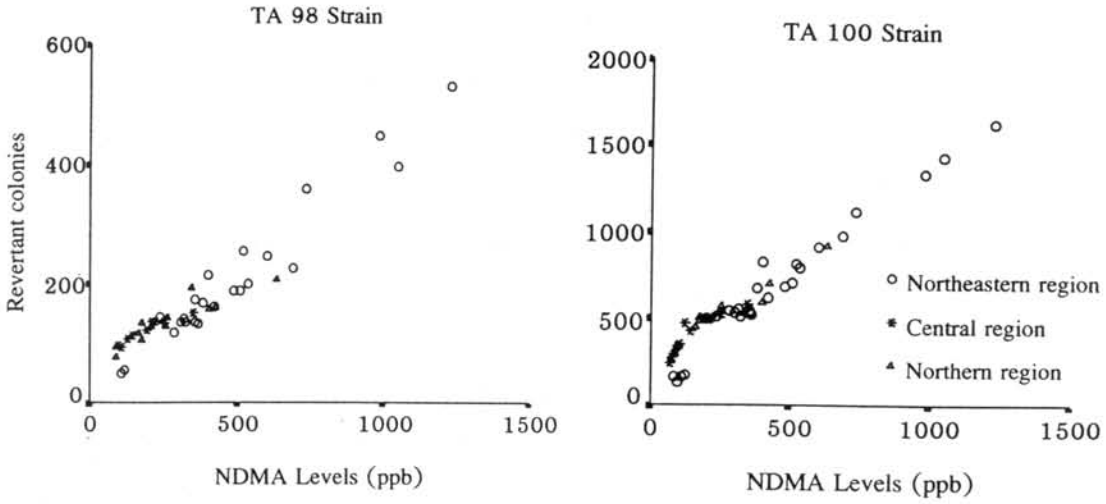


Figure 1 Correlation between NDMA and mutagenicity

We examplified only the samples which showed the high NDMA levels, No. 11 and 24 from Northeastern region (Table 3) for study the patterns on mutagenic activity. The mutagenicity dose-response curves of those two selected extracts are also illustrated in Figure 2.

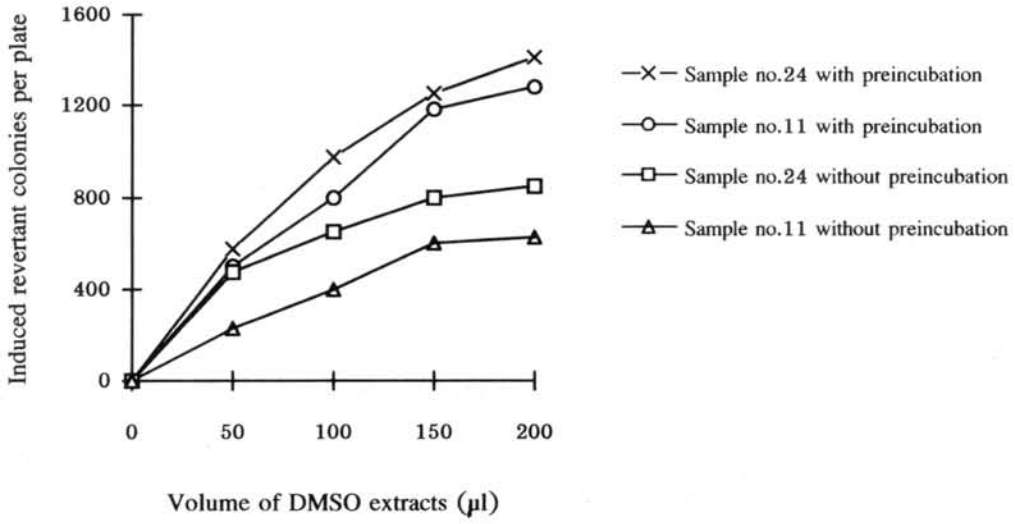


Figure 2 Mutagenicity dose-response curves of DMSO extracts from Northeastern region
(Tester strain used : Salmonella *typhimurium* TA 100)

Discussion

The levels of NDMA found in uncooked fermented fish samples analyzed, especially from Northeastern part of Thailand were approximately close by range to our previous findings (Koonanuwatchaidet *et al.*, 1987 ; Koonanuwatchaidet and Srisuwan, 1989) but the mean values of this nitrosamine produced in samples from each area in the present study were remarkedly higher than the amount found in our recent communication (Koonanuwatchaidet and Srisuwan, 1989), and some other investigations as reported elsewhere (Fong and Chan, 1976 ; Fong and Chan, 1977 ; Scanlan, 1983 ; Koonanuwatchaidet *et al.*, 1987). However, the NDMA levels of samples obtaining from Northern and Central areas are much lower than those collected from Northeastern Thailand. There would be several possible reasons for explanation, *e.g.* different species of fishes locally used for fermentation, duration, quality and quantity of some specific ingredients introduced in fermentation process. Traditionally, the general practice of processing fermented fishes in prepared under local village conditions without modern standards of hygiene. This would allow the contamination of some nitrate-reducing bacteria to be occurred in the system. Moreover, it is much interesting to notify that crude salt and rock salt which contain high content of nitrates are commonly used in fermentation of fermented fishes (Migasena *et al.*, 1980). During the storage of fermented fishes for several months nitrates are probably reduced to become nitrites by the bacterial reductase. The most important agent participating in nitrosamine formation *via* nitrosation reactions in the food system is nitrite, which can readily generates nitrous anhydride in aqueous acidic solution (Fong and Chan, 1977 ; Scanlan, 1983 ; Gray, 1981 ; Archer, 1984). Another group of precursors for nitrosamine production are the secondary and tertiary amines (nitrosatable amines) which are naturally occurred in the fishes themselves (Fong and Chan, 1976 ; Fong and Chan, 1977).

Using Salmonella *typhimurium* tester strain TA 98 and TA 100 to detect mutations after preincubation with a rat liver microsomal enzyme system. We have demonstrated the presence of mutagenicity at high levels in batches of uncooked fermented fishes clearly exhibit the base-pair substitution *via* TA 100 strain. The results for particular uncooked fermented fish extracts, give a fairly good agreement with our previous findings. In most cases, the mutagenicity was enhanced by rat liver microsomal activating enzyme and preincubation of the bacteria with S-9 mix at 25 $^{\circ}$ C for 20 minutes before proceeding to

the test. The procedure has been widely reported to show the enhancement in the case of such compounds.

A linear dose-response relationship was evident in the fermented fish extracts at low concentrations, but at high concentrations, the number of induced revertant colonies tended to be declined (Figure 1.), it was likely consistent to the works of other authors (Fong and Chan, 1977 ; Ho *et al.*, 1978 ; Fong *et al.*, 1979 ; Guttenplan, 1979) and of our previous communications (Koonanuwatchaidet *et al.*, 1987 ; Koonanuwatchaidet and Srisuwan, 1989). Since DMSO in the amount used in our experiments had no toxic and nonmutagenic effects on the tester strains, this lack of dose-response curve study at high concentrations demonstrated by the extracts was possibly reflected the toxic effect inherent in those extracts was possibly reflected the toxic effect inherent in those extracts manifesting at such doses.

In according with the findings of this observation, the consumption of uncooked fermented fishes which are rich in NDMA would be probably considered as one of the health risk factors for primary liver-cell carcinoma and cholangiocarcinoma, especially for Thai-northeastern inhabitants. It appears, therefore, that if the NDMA content in fermented fishes has probably to be minimized by direct boiling process for a appropriated period of time, this could be regarded as the carcinogenic hazard reduction. In addition, *in vitro* mutagenesis action assay of this particular nitrosamine and also newly registered mutagens (Ames *et al.*., 1975 ; Yahagi *et al.*, 1977 ; Maron and Ames, 1983 ; Guttenplan, 1979 ; Lee and Guttenplan, 1981 ; Sugimura, 1988) in the extracts by using Salmonella *typhimurium* with microsomal activation and preincubation systems provides a feasible tool for prediction and assessment of carcinogenicity in experimental animals including in the human.

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