Characterization of *N*-(Nitrosomethyl)urea in Nitrosated Fermented Fish Products

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To characterize chemical carcinogens in acidic-nitrosated fish sauce sample, *N*-nitrosamides in the sample were separated by two kinds of reversed-phase HPLC columns, and with detection by photolysis−pyrolysis−thermal energy analyzer. A strong chromatographic peak at t_R 12 or 4.5 min, same as that for *N*-(nitrosomethyl)urea (NMU), was obtained on PRP-1 or C_{18} HPLC column from fish sauce sample with 10 mM trifluoroacetic acid as the basic mobile; acetonitrile, as organic modifier after the sample was nitrosated by 5 mmol/L of sodium nitrite (final concentration) at 37 °C and pH 2.0 for 1 h. No response above t_R could be observed from the nitrosated sample in the detection system without photolysis. Such a peak could not be obtained from the unnitrosated fish sauce either. These results indicated that the component was NMU. Furthermore, this component, NMU, could also be detected in the nitrosated human gastric juice sample spiked with fish sauce. The formation of NMU in the sample was pH- and nitrite-dependent. This paper provides direct evidence that NMU formation could occur in fish sauce from the high-risk area for stomach cancer and in the fish sauce spiked human gastric juice during nitrosation under simulated gastric conditions.

Keywords: *Nitrosomethylurea; fish products; nitrosation; human; gastric juice; stomach cancer; nitrosamide; nitroso compounds; nitrite*

INTRODUCTION

Stomach carcinoma is still the leading cause of cancer death in China and the second one in the world. Since the intragastric *N*-nitrosamide formation may play an important role in the etiology of gastric carcinomas (Mirvish, 1983), much attention has been paid to the characterization of natural *N*-nitrosamides in human environment. Many *N*-nitrosamines can be sensitively analyzed by the thermal energy analyzer (TEA) detector relying on thermal cleavage of the N−N bond to produce a nitrogen oxide (NO) radical. Unlike *N*-nitrosamines, *N*-nitrosamides and related compunds typically rearrange on pyrolysis to yield molecular nitrogen instead of nitrogen oxide. A photolysis device was assembled by Shuker and Tannembaum (1983) in which *N*-nitrosamides were cleaved photolytically by UV irradiation to produce nitrogen oxide. HPLC−photolytic interface−TEA methods were established to detect precisely *N*-nitrosamides and other nonvolatile NOC (Conboy and Hotchkiss, 1989). We set up a selective and sensitive HPLC−PHPS−chemiluminescence method for *N*-nitrosamides (Chen et al., 1991; Li and Deng, 1995). The establishment of sensitive methods for determining of *N*-nitrosamides makes it practicable to detect the trace amount of *N*-nitrosamides in the environment. Some progress was made in the investigation of the natural *N*-nitrosamides in the past years. It was reported that the level of total *N*-nitrosamides in human fasting gastric juice samples from high-risk areas correlated with the severity of gastritis and was significantly higher than that from low-risk areas (Zhang et al., 1991; Deng et al., 1995, 1997). However, little is known about the detailed chemical structures of the natural *N*-nitrosamides, although Mende et al. (1994) reported that Indian nasal snuff contained low amounts of *N*-nitrosopyrrolidin-2-one. Fish sauce is a liquid product of small marine fish and sodium chloride (7:3). The main species of fishes are *Sardinella aurita* (Val.) and *Decapterus maruadsi* (T. & S.). The fishes are completely liquidized after fermentation for 1−2 years. It is consumed daily (about 30 mL/capita) by residents as a traditional seasoning in the Chinese southeast coast, the highest risk area for stomach cancer in China. It is direct mutagenic in prokaryocytes and mammalian cells and human gastric mucosal cells, and carcinogenic to glandular stomach of Wistar rats after being nitrosated in the simulated gastric condition (Deng et al., 1991; Zhang et al., 1991; Chen et al., 1992). Recent epidemiological studies showed further that the intake of fish sauce is a high-risk factor for gastric carcinogenesis for the local residents (Cai et al., 1993; Ye et al., 1994). However, the specific chemicals ultimately responsible for the carcinogenicity of nitrosated fish sauce are still unknown, although nitrosable amines can be detected in fish sauce (Zhang et al., 1993). In this paper, NMU in nitrosated fish sauce was identified by two HPLC methods connected with a postcolumn PHPS device and TEA.

MATERIALS AND METHODS

Chemicals and Samples. Standard *N*-nitrosamide NMU was purchased from Sigma Chemical Co. (N-4766); acetonitrile, from BDH, England. Both acetone and dichloromethane were analytical reagents and purified by redistillation. Fish sauce samples were collected from Changle County, Fujian Province, the area with the highest mortality for stomach

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cancer (more than $120/10^5$ in males after age-adjusted) in China (Zhang et al., 1984). Human fasting gastric juice samples were collected by gastric tube or fibroendoscope and analyzed for the total amounts of *N*-nitrosamides (Deng et al., 1997). The gastric juice samples without *N*-nitrosamides were pooled and stored at −20 °C for further study of *N*-nitrosamide formation from fish sauce.

Nitrosation and Purification of Fish Sauce and Gastric Juice Samples (Deng et al., 1991; Zhang et al., 1991). A 10 mL fish sauce sample or mixture of gastric juice sample and fish sauce (1/1, v/v) was adjusted to pH 2.0 with 6 N HCl, nitrosated with 5 mmol/L of sodium nitrite (the final concentration) at 37 °C for 1 h and then stopped by 50 mmol/L of sulfamic acid (the final concentration). Different concentration of nitrite and pH values were also used in the experiments to study relationships between the nitrosamide formation and concentration of nitrite or hydrogen ion. Samples were treated with crystal sodium chloride and tungstophosphoric acid, centrifuged, filtrated to separate proteins, and further extracted by acetone/dichloromethane (1:5). Then, the extracts were evaporated, and dry residues were dissolved in 2 mL of 10 mM trifluoroacetic acid just before use. The absolute recovery of the procedures for NMU was about 50%.

Chromatography. Reversed-phase HPLC was used to separate NOC. Trifluoroacetic acid (10 mM) was used as the basic mobile; acetonitrile, as organic modifier with gradient elution. The flow rate was 1.0 mL/min. Columns were a Hamilton PRP-1 polymeric analytical (150 × 4.1 mm i.d., 10 mm particle size) and guard columns. The Waters HPLC system consisted of one 680 automated gradient controller, two 510 pumps, and two Rheodyne injectors (7125 before and 7012 after column, the latter one was used to monitor the working status of detector rapidly) (Li and Deng, 1995). In addition, a Chrompac chromsphere C18 analytical (100 × 3 mm i.d., 5 mm particle size) column was used.

***N*-Nitrosamide Detector (Chen et al., 1991; Deng et al., 1995).** A Hitachi 056 recorder was used. The detector was composed of a photolysis device, a pyrolysis apparatus, a series of cold traps (two ice−water ones, two dry ice−acetone ones, and a liquid nitrogen−ethanol one), and a Model 510 TEA. This detector could detect *N*-nitrosamides and distinguish *N*-nitrosamides from other kinds of NOC by controlling the working status of photolysis device. With the postcolumn photolysis, both *N*-nitrosamides and other NOC could be detected with high sensitivity (1−10 ng). Without photolysis, the response for *N*-nitrosamides decreased from 100 to 84%; for other kinds of NOC, only 0−32%.

RESULTS

A strong chromatographic peak (19.8 μmol/L) was obtained at t_R 12 min from nitrosated fish sauce when it was separated by PRP-1 columns (Figure 1A-b). A stronger peak could be seen with nitrosated fish sauce spiked with NMU at the same t_R (Figure 1A-c). The height of the peak in the unspiked nitrosated fish sauce decreased by 92% when the photolysis device was switched off (Figure 1A-a). The peak was not detected in unnitrosated fish sauce. A peak at t_R 4.5 min and another at t_R 5.5 min were observed on chromsphere C18 column from the concentrated chromatographic fraction of nitrosated fish sauce collected from t_R 11 min to t_R 13 min on PRP-1 column (Figure 1B-b). A stronger peak at t_R 4.5 min could also obtain from the fraction spiked with NMU at the same position (Figure 1B-c). No peak at t_R 4.5 or 5.5 min could be detected in the unspiked fraction without photolysis (Figure 1B-a).

The same peak at t_R 12 min could also be detected on a PRP-1 column from the nitrosated mixture of human fasting gastric juice samples and fish sauce (1/1, v/v). The formation of the peak (NMU) was pH-dependent in two independent experiments (Table 1). Of NMU, 3.04, 1.57, and 0.33 μmol/L was detected in the mixture treated with 10.0, 5.0, and 2.5 mmol/L of sodium nitrite.

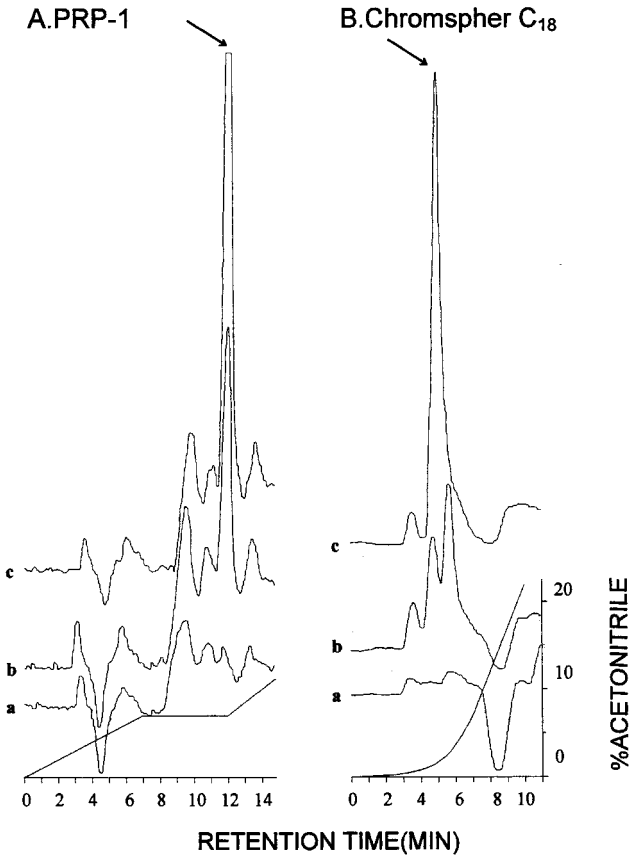

Figure 1. Chromatograms obtained by HPLC−photolysis−pyrolysis−TEA from fish sauce. Fish sauce was nitrosated by 5 mmol/L of sodium nitrite at 37 °C, pH 2.0, for 1 h and extracted with acetone/dichloromethane and concentrated five times. NMU and other NOC were fractionated on PRP-1 or C18 column using an acetonitrile gradient, as indicated: (a) without photolysis; (b and c) with photolysis; (A-a and A-b) from nitrosated fish sauce; (A-c) from NMU (40 ng)-spiked nitrosated fish sauce; (B-a and B-b) from the condensed fraction between t_R 11 min and t_R 13 min of nitrosated fish sauce on PRP-1 column; (B-c) the NMU-spiked fraction. The chemical marked by arrow was NMU, 20 μL injection.

Table 1. Formation of NMU in Human Fasting Gastric Juice Spiked by Fish Sauce (1:1) after Nitrosation with 8 mg per 10 mL of Sodium Nitrite at Various pH Values

	concentration (μM)[a] of NMU formed at various pH values						
	1.0	2.0	3.0	4.0	5.0	6.0	7.0
expt 1	3.68	5.22	3.35	0.86	BDL[b]	BDL	ND[b]
expt 2	5.16	4.09	3.44	BDL	BDL	ND	BDL

[a] Concentrations of NMU were calculated from chromatogram on PRP-1 and corrected with the recovery (50%). [b] BDL, below detection limits; ND not determined.

DISCUSSION

Weisburger et al. (1980) reported that several fish consumed predominantly in Japan were mutagenic in *S. typhimurium*, and carcinogenic for the glandular stomach of Wistar rats after incubation with nitrite. It was reported that 16−31 mg/kg of methylurea could be detected in dried, salted bonito fish after nitrosation and denitrosation, though no methylurea could be detected in the fish directly (Mirvish et al., 1978). Further studies showed that methylurea was synthesized through

5-oxocreatinine 5-oxime and 1-methyl-5-oxohydrantoin 5-oxime during nitrosation of creatinine (Mirvish et al., 1982, 1993). Piacek-Llanes and Tannenbaum (1982) also reported that the treatment of fresh and dried fava beans with low concentrations of nitrite under simulated gastric conditions results in the production of a powerful activated mutagen, which was strongly suggested to be a *N*-nitrosourea. Fish sauce, the fermented fish product consumed traditionally in the highest risk area for stomach cancer in China, was suggested to be one of the major causal factors of gastric cancer in the local population (Deng et al., 1991; Zhang et al., 1991, 1993; Cai et al., 1993; Ye et al., 1994). Creatinine and other nitrosable amines can be detected in fish sauce (Zhang et al., 1993). Here, we reported that a strong peak with the same t_R as NMU (12 min by PRP-1 and 4.5 min by C18 columns) was observed on the chromatogram from nitrosated fish sauce by two different reversed HPLC columns, connected with a sensitive and selective detector for *N*-nitrosamides. In addition, like *N*-nitrosamides, its response in the detector decreased almost completely when the photolysis device was switched off. These results indicated that the component was NMU. The chemical structure of the product peak at t_R 5.5 min obtained on C18 column is unknown. Like standard *N*-nitrosamides, the peak at t_R 5.5 min did not appear without photolysis. This suggests it is another *N*-nitrosamide.

NMU is directly carcinogenic to the glandular stomach of experimental animals (Tatematsu et al., 1993). It can be synthesized chemically from nitrite and methylurea both in acidic condition in vitro (pH 1−3) and in the stomach of rats, guinea pig in vivo (Yamamoto et al., 1987). We found that the formation of NMU in human fasting gastric juice mixed with fish sauce (1:1) was pH-dependent as well. It was reported that the synthesis of NMU at pH 6−7 could be catalyzed by some bacteria isolated from gastric juice of subjects at the high-risk area for gastric cancer (Pan et al., 1995). The residents in the studied high-risk area, Changle County, consumed fish sauce daily and were exposed to high levels of nitrate and nitrite. Chronic atrophic gastritis, achlorhydria, and intragastric colonization of bacteria were common among the residents (Zhang et al., 1984; Cai et al., 1993; Ye et al., 1994). Pignatelli et al. (1993) reported that the level of nitrite in fasting gastric juice in Columbia was up to 472 μmol/L. Maragos et al. (1991) reported the level of nitrite in pig gastric juice was increased to 2.l mmol/L with the combined treatment of 6 mmol of nitrate and cimetidine. The level of nitrite in fasting gastric juice in Changle County was up to 100 μmol/L, and the content of nitrate in the vegetables consumed by the local residents was up to 16 mmol/kg (Zhang et al., 1984). We observed that 3.04, 1.57, and 0.33 μmol/L of NMU were detected in the human fasting gastric juice samples mixed with the same volume of fish sauce after treatment with 10.0, 5.0, and 2.5 mmol/L of sodium nitrite, respectively. Thus, NMU may also be synthesized in their stomachs and be responsible for the high risk for gastric cancer.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; NMU, *N*-nitrosomethylurea; NOC, *N*-nitroso compounds; PHPS, photolysis−pyrolysis; TEA, thermal energy analyzer; t_R, retention time.

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