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Reduction in *Salmonella* mutagenicity of mainstream cigarette smoke condensate by cation exchange chromatography

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ABSTRACT : Mutagenicity of cigarette smoke is one of the major health concerns related to smoking. Reduction of the components comprising mutagenic activity in cigarette mainstream smoke can be expected to bring about reduced risk of smoking. The purpose of this study is to isolate mutagenic compounds and to investigate the relative contribution to allover mutagenicity of smoke to find clues for the effective elimination of the components.

Cigarette smoke condensate (CSC) was obtained from total particulate matter (TPM) of mainstream smoke, and several fractions fractionated from CSC were made by combination of cation exchange chromatograph and reverse-phase chromatography. The mutagenic activity of these fractions was assessed using *Salmonella* mutagenicity assay with *S. typimurium* TA98 strain in the presence of metabolic activation system (S-9). The fractions isolated by cation exchange and reverse-phase column showed relatively high mutagenic activity. The basic and hydrophilic fraction 9 showed approximately 33% of mutagenic activity of CSC and its specific activity was 2,459 revertants/mg TPM. These results suggest that hydrophilic cation exchanger and/or other adsorbents possessing similar properties may be used to remove the mutagenic compounds from mainstream smoke.

***Key words** : Mutagenicity, total particulate matter, cation exchange chromatography*

In vitro toxicology assays are short-term tests for evaluating potential damage to DNA or cells. The Ames assay, which is the most widely used genetic toxicology test worldwide, is generally the first step in a standard *in vitro* toxicity test battery.

The mutagenicity of cigarette smoke condensate (CSC), which is the particulate fraction of cigarette smoke as measured in the Ames *Salmonella* assay was first reported in 1974 (Kier *et al.*,) and 1975 (Hutton and Hachey). Following these initial reports, several laboratories have determined that CSC is mutagenic in a variety of Ames assay bacterial strains following metabolic activation by rat liver S9 metabolic activation, and particularly so in TA98, a strain that measure frame shift mutations (DeMarini, 2004). The mutagenicity of CSC is influenced by many factors: tobacco type (for example, burley vs. flue-cured), stalk

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position, age, sugar and nitrogen content of tobacco leaves (Sato *et al*., 1977). Mutagenicity may also be affected by the draw resistance, filters, and other physical characteristics (Steele *et al*., 1995).

CSC is a complex mixture consisting of over 4000 identified chemical components (Dube and Gtreen, 1982). The chemicals reported in the CSC include mutagenic classes such as polynuclear aromatic hydrocarbons(PAH), nitrosamines and heterocyclic aromatic amines (Manabe and Wada, 1990; Rodgman and Green, 2003). Among them, the Ames mutagenicity of heterocyclic amines formed during cooking and other heat processing of protein rich foods, is very much higher than comparable measurements for other classes of CSC mutagen (Tewes *et al*., 2003; Shin *et al*., 2007). Since cigarette smoke is a complex aerosol composed of several thousand compounds (Hoffman *et al*., 2001; Lauterbach, 2002), the relative contribution made by the small amount of heterocyclic amines to the over all mutagenicity of CSC is unknown. In the present report, we have described the use of ion exchange chromatography, and reverse-phase column chromatography for separation and characterization of the large array of mutagenic components present in the CSC. Also we described sub-fractionation of this characteristic mutagen and the relative activity of each fraction.

MATERIALS AND METHODS

Chemicals

Dimethysulfoxide (DMSO), mitomycin C, sodium dodecyl sulfate (SDS), cyclophosphamide, 2-aminoanthracene (2AA), agar, glucose, histidine/biotin and ampicillin were purchased from Sigma-Aldrich Company (St Louis, USA). Ion exchange column (Oasis WCX and WAX, 6 cc/500 mg) and reverse phase column (Oasis HLB, 6 cc/500 mg) were purchased from Waters. The S-9 fraction of aroclor 1254-induced rat liver and co-factors for the NADH regenerating system were purchased from Mol-Tox (Annapolis, USA) and Wako (Osaka, Japan), respectively. All other chemicals and reagents used were of the best available grade.

Bacteria and incubation

Salmonella typhimurium tester strains were obtained by Mol-Tox (Annapolis, USA), and were grown with 2.5% nutrient liquid in a spinner flask at 37℃ in a shaking incubator maintained at a speed of 90 rpm. *Salmonella* bacteria were selected for this assay due to their demonstrated sensitivity in previous genotoxicity assays designed to evaluate the activity of cigarette smoke.

Preparation of cigarette mainstream smoke

Total particulate matter (TPM) was prepared by smoking burley, flue-cured, and 2R4F Kentucky reference cigarettes (Tobacco and Health Research Institute, Lexington, KY), on a smoking machine under Standard Federal Trade Commission condition (35 ml puff volume, 2-sec puff duration, once per min) using a RM20/CS smoking machine (Heinr Borgwaldt, Germany). TPMs from 2R4F cigarettes were collected on Cambridge filter pad. The pads were extracted with H_2O, 5% HCl, 0.5% HCl, 50% Methanol (MeOH) and DMSO at a concentration of 5 mg TPM/ml of solvents, separatively. The cigarette smoke condensate (CSC) was filtrated using 0.8 um syringe filter and then stored at -70 ℃ until use.

Fractionation procedure for CSC by column chromatography technique

Mutagenic active component fractions were fractionated by a combination of anion exchange chromatography and reverse-phase chromatography. The aqueous extract (20 mL) with DMSO was loaded onto a cation exchange chromatography column (Oasis WCX, 6 cc/500 mg) equilibrated with methanol. The non-interacting solutes were

washed from the column with 10 ml of 1% HCl at a flow rate of 1 mL/min. The bound fractions were eluted using 10 mL of methanol. The basic compound fraction was eluted with 10 mL of 5% NH_4OH solution. Fractions collected were assayed for mutagenic activity as described below, and the remainder was stored at 4℃.

The active basic fraction (Fr. 4) was further fractionated on hydrophilic-liphophilic-balanced reverse phase column chromatography (Oasis HLB, 6 cc/500 mg) equilibrated with 5% of NH_4OH. The non-interacting solutes were washed from the column with 10 mL of 0.05 N NaOH at a flow rate of 1 mL/min. The bound fractions were eluted using stepwise gradient from 30% to 80% methanol solved with 0, 2, 4, and 8% acetic acid. Active fractions were concentrated by vacuum evaporation in a Speed Vac apparatus under a stream of N_2.

Mutagenicity assay

Mutagenicity was evaluated in strains of *Salmonella typhimurium* strain TA98 according to the method described by Maron and Ames (1983), via the plate incorporation method. For plating, approximately 10^8 bacteria suspended in 100 μL of culture medium, 100 μL of TPM dissolved in solvent or solvent alone, and 500 μL of S-9 mix (4%, v/v) were added to 2 mL of top agar supplemented with histidine and biotin (0.05 mM each). The components were mixed and evenly spread on minimal glucose agar plates. After the top agar had hardened, the plates were incubated for 48 hours in darkness at 37℃. The number of revertant colonies were then counted using an automatic colony counter. Concurrent negative and positive controls were utilized in all experiments. All testing was conducted using triplicate plates at each of the experimental concentrations. The mutagenicities of CSC and all fractions were determined from linear dose-response relationship (triplicate plates for each of 4-6 different amounts). Revertants/cigarette values were calculated by multiplying the revertants/mg TPM by mg TPM/cigarette.

Statistical analysis

Data obtained from the toxicological experiments were expressed as means±SD. One-way analysis of variance was employed in order to compare the results obtained from each of the cigarette. In cases in which this overall comparison revealed a significant statistical difference between the cigarettes, the Duncan test for pairwise comparison was applied.

RESULTS AND DISCUSSION

Preparation of mutagenic active CSC

The particulate phase of mainstream smoke was collected on Cambridge filter pad. The pad

Table 1. Mutagenicity of cigarette smoke condensate prepared by various extraction solution

Dose (μL/plate)	Mutagenicity (revertants/plate)				
	H_2O	0.5% HCl	5% HCl	50% MeOH	DMSO
0	28±6	28±2	34±5	28±3	28±3
10	44±4	60±6*	53±2*	51±7*	123±4*
40	46±6	131±12*	128±14*	177±8*	267±13*

2R4F cigarette was smoked in accordance with FTC condition. TPMs from mainstream smoke of 10 cigarettes were collected on Cambridge filter pad and then, the pad were extracted with above solutions at concentration of 5 mg TPM/mL for 20 min. Respective solvents were used for solvent control (0 μL/plate). *significantly different from solvent control at $p<0.01$.

was combined with H_20, 0.5% HCl, 5% HCl, 50% MeOH, and DMSO, separatively. The efficiency of extraction of mutagens as a function of extraction solvent was tested by subjecting the extract of 2R4F cigarette to Ames mutagenicity assay using tester strain TA98 in the presence of S9 metabolic activation, as this strain has been shown to respond to cigarette smoke (Doolittle *et al*., 1990). The CSC extracted with 5 kinds of solvents was tested at dose of 0, 10, 20, 40 μL per plate. The number of revertants per plate for each extract are depicted in Table 1. For 0.5% HCl, 5% HCl, 50% MeOH, and DMSO extracts, an increase in the revertant colony was observed in a dose dependent manner and at almost all dose tested. The differences were statistically significant between treated groups and respective solvent control (dose of 0 μL/plate). Among them, DMSO extract showed the greatest mutagenic activity.

Fractionation by cation exchange chromatography

Scheme 1 illustrates separation of the basic fraction from CSC by cation exchange chromatography. CSC extracted with DMSO from TPM on a Cambridge filter pad (20 mL) was loaded onto a cation exchange chromatography column equilibrated with methanol and then the basic compound fraction was eluted with 10 mL of 5% NH_4OH solution. The mutagenic activities for CSC, fraction 1, 2, 3, and 4 were tested at dose of 0, 10, 20, and 40 μL per plate (Fig. 1). The CSC and fraction 4 induced dose-dependent

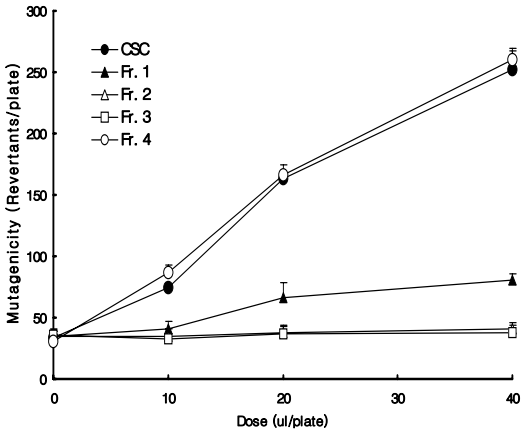


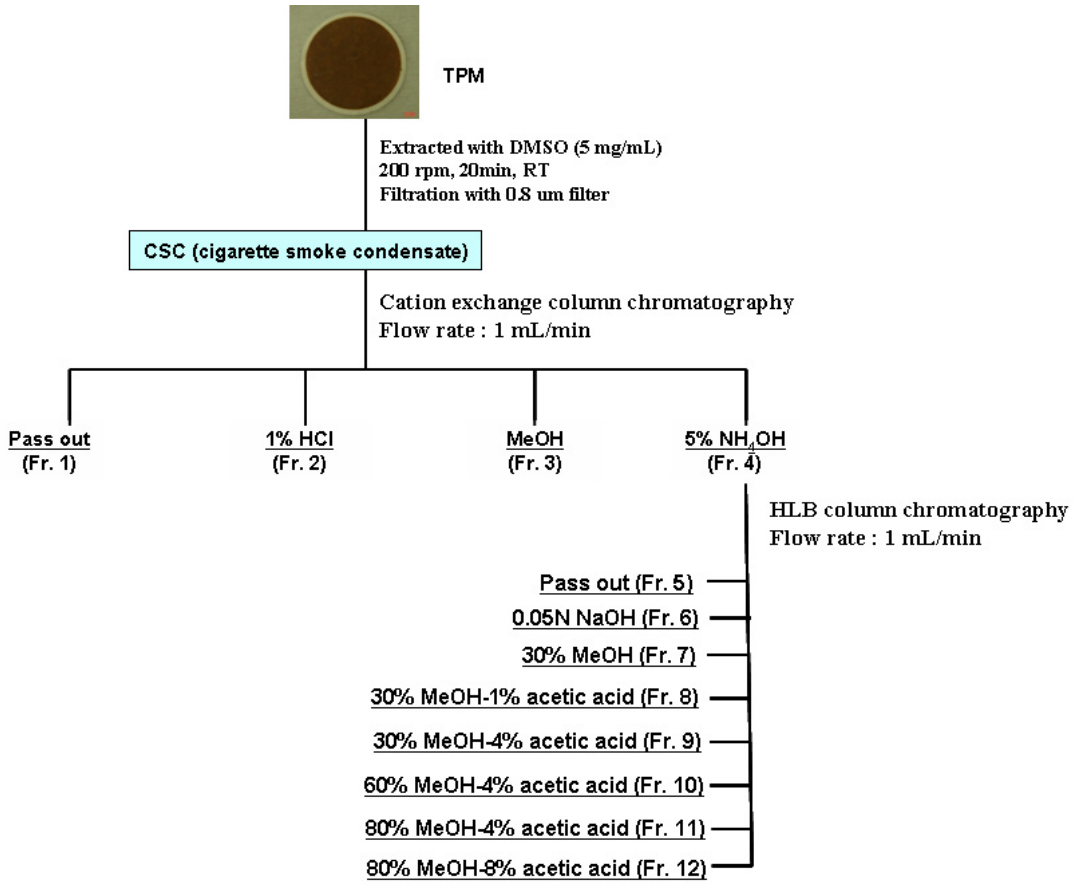
Fig. 1. Mutagenicity of cation exchange column chromatography fractions.

The *Salmonella typhimuriem* strain TA98 with an S9 metabolic activation system was utilized to examine the potential of the each fraction to induce mutation. The number of revertant colonies in solvent-treated (40 μL/plate) group and 2-AA-treated (2 ㎍/plate) group were 28-35 and 1646±117, respectively.

increase with similar number of revertant colonies. These results are provided in Table 2 with data expressed as both specific activity (revertants per mL) and total mutagenicity (revertants per total amount). No statistically significant differences in specific mutagenic activity were detected between CSC and fraction 4. Fraction 1 and 4 contained approximately 25% and 51% of the mutagenic activity of CSC.

Table 2. Contribution of basic fractions to the overall mutagenicity of the CSC of 2R4F cigarette

Smoke fraction	Specific activity (revertants/mL)	Yield (mL)	Total mutagenicity (revertants)
CSC	5,022±305	20 mL	100,440
Fr. 1	1,231±186	20 mL	24,620
Fr. 2	95±27	10 mL	1,210
Fr. 3	105±15	10 mL	1,350
Fr. 4	5,125±643	10 mL	51,250



Scheme 1. Isolation procedure of mutagenic compounds from TPM

Fractionation by reverse phase chromatography

The active basic fraction (Fraction 4) was further isolated on hydrophilic-liphophilic-balanced reverse-phase column chromatography equilibrated with 5% of NH_4OH (Scheme 1). Dose-response curves for fraction 4 - 12 were determined. The fraction 4, 9, 10, and 11 induced dose-dependent increase with similar number of revertant colonies (Fig. 2). The mutagenicity of fraction 9 and 10 eluted from column with 30-60% MeOH containing 4% acetic acid contained approximately 95% and 51% of those of CSC (Table 3). The results of the isolation procedure are summarized in Table 4. The final mutagenicity of fraction 9 was 33 % of mutagenic activity of CSC and specific activity was 2,459 revertants/mg. To compare the contribution of the fractions eluted through chromatographys to the overall mutagenicity of the CSC, we used burley and flue-cured cigarettes. No difference in the contribution pattern of each fraction to the mutagenicity of the CSC was observed for among 3 kinds of cigarettes (Fig. 3). This means that high mutagenicity of burley cigarette was deduced from not quality of mutagen but a quantity of mutagenic components compared to flue-cured

Table 3. Contribution of hydrophobic fractions to the overall mutagenicity of the CSC basic fraction (Fr. 4)

Smoke fraction	Specific activity (revertant/mL)	Yield (mL)	Total mutagenicity (revertants)
Fr. 4	5,125±643	10 mL	51,250
Fr. 5	52±10	26 mL	1,352
Fr. 6	62±10	8.1 mL	502
Fr. 7	55±10	7.5 mL	412
Fr. 8	71±8	7.5 mL	532
Fr. 9	4,917±245	6.8 mL	33,436
Fr. 10	1,941±248	7.0 mL	13,587
Fr. 11	1,454±262	6.5 mL	9,451
Fr. 12	796±89	6.5 mL	5,651

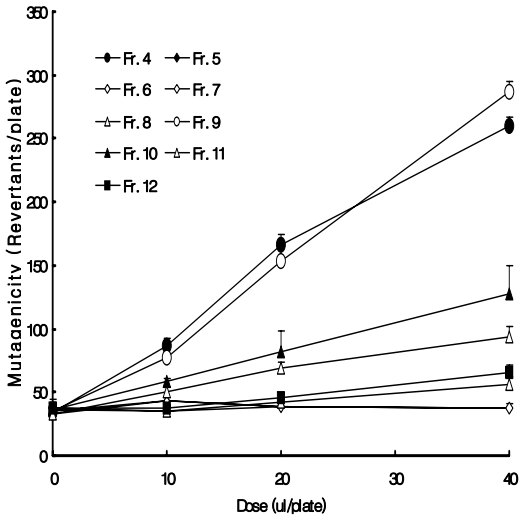


Fig. 2. Mutagenicity of HLB column chromatography fractions.

The *Salmonella typhimuriem* strain TA98 with an S9 metabolic activation system was utilized to examine the potential of the each fraction to induce mutation. The number of revertant colonies in solvent-treated (40 μL/plate) group and 2-AA-treated (2 ㎍/plate) group were 31-47 and 1785±127, respectively.

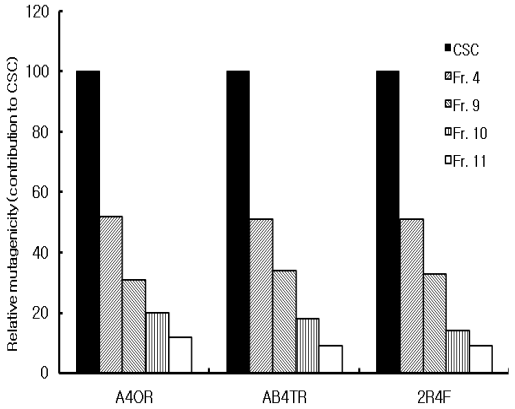


Fig. 3. Contribution of the each fraction to the overall mutagenicity of the CSC from three kinds of cigarettes

The *Salmonella typhimuriem* strain TA98 with an S9 metabolic activation system was utilized to examine the potential of the each fraction to induce mutation. The number of revertant colonies in solvent-treated (40 μL/plate) group and 2-AA-treated (2 ㎍/plate) group were 25-33 and 1523±99, respectively. The total mutagenicity of CSC-treated groups (100 mg TPM) from A4OR, AB4TR and 2R4F cigarettes were 62,180, 119,420 and 100,440, respectively.

Table 4. Typical purification of mutagenic compounds and contribution of the fractions to the overall mutagenicity of CSC from 2R4F

Smoke fraction	Total Weight	Total mutagenicity	Contribution To CSC	Specific activity (revertants/mg)	Purification fold
CSC	100 mg	100,440	100 %	1,004	1
Fr. 4	20 mg	51,250	51 %	1,231	1.2
Fr. 9	13.6 mg	33,436	33 %	2,459	2.5
Fr. 10	7.0 mg	13,587	14 %	1,941	1.9
Fr. 11	10.4 mg	9,451	9 %	909	0.9

cigarette. These results suggest that hydrophobic-cation exchanger and/or other adsorbents possessing similar properties may be used to remove the mutagenic compounds from mainstream smoke.

결 론

담배 주류연의 고체상(TPM) 부분에는 *in vitro* 독성이 있다고 알려진 여러 화학성분들이 포함되어 있다고 알려져 있다. 본 논문에서는 담배 주류연의 생물학적 활성을 감소시키기 위한 연구의 일환으로, 담배연기 응축물로부터 유전독성을 나타내는 성분의 분리 및 특성 파악을 위한 연구를 수행하였다. 2R4F 표준담배를 이용하여 양이온 교환수지 및 역상 수지 chromatogrphy등을 이용해서 TPM으로부터 유전독성 활성 분획들을 분리하였다. 유전독성 측정은 *Salmonella typhimurium* TA98을 이용한 대사활성법으로 돌연변이 유발성을 조사하였다. 담배연기 TPM으로부터 돌연변이성 성분 분리를 위한 최적의 용매로서 DMSO가 선정되었고, 양이온 및 역상 수지 column등을 이용해서 높은 돌연변이 활성을 나타내는 성분을 분리하였다. 그 중 염기성-소수성 특성을 나타내는 분획9는 전체 담배연기응축물에 대하여 33%의 돌연변이 유발성을 보였으며, specific activity는 2.459 revertansw/mg TPM으로 나타났다. 이와 같은 결과로부터, 담배 주류연의 유전독성 활성 성분을 제거하는데 hydrophobic-양이온 필터를 이용할 수 있을 것으로 사료된다.

REFERENCES

DeMarini, D. M. (2004) Genotoxicity of tobacco smoke and tobacco smoke condensate. *Mutat. Res*. 567: 447-474.

Dube, M. F. and Green, C. R. (1982) Method of collection of smoke for analytical purpose. *Rec. Adv. Sci*. 8: 42-102.

Doolittle, D. J., Lee, C. K., Ivett, J. L., Mirsalis, J. C., Riccio, E., Rudd, C. J., Burger,G. T. and Hayes A. W. (1990) Comparative studies on the genotixic activity of smoke condendsate from cigarettes which burn or only heat tobacco. *Enviromental and Molecular Mutagenesis* 15: 93-105.

Hoffmann, D., Hoffmann, I. and EI-Bayoumy, K. (2001) The less harmful cigarette: a controversial issue. *Chem. Res. Toxicol.* 14: 767-790.

Hutton, J. J. and Hackney, C. (1975) Metabolism of cigarette smoke condensates by human and rat homogenates to form mutagens detectable by *Salmonella typhimurium* TA1538. *Cancer Res.* 35: 2461-2468,

Kier, L. D., Yamasaki, E. and Ames, B. N.(1974) Detection of mutagenic activity in cigarette smoke condensates. *Proc. Nat. Acad. Sci. USA* 71: 4159-4163.

Lauterbach, J. H. (2002) Smoke chemistry: A useful predictor of smoke toxicology. *Rec. Adv. Tob. Sci.* 28: 6-68.

Maron, D. M. and Ames, B. N. (1983): Revised methods for the *Salmonella. Mutat. Res.* 113: 173-215.

Manabe, S. and Wada, O. (1990) Carcinogenic tryptophan pyrolysis products insmoke conensate and cigarette smoke-polluted indoor air. *Environ. Pollute.* 64: 121-132.

Rodgman, A. and Green, C. R. (2003) Toxic chemicals in cigarette mainstream smoke-hazard and hoopla. *Beitragezur Tabakforschung* 20: 481-545.

Sato, S., Seino, Y., Ohka, T., Yahagi, T., Nagao, M., Matsushima, T. and Sugimura, T. (1977) Mutagenicity of smoke condensates from cigarettes, cigars and pipe tobacco. *Cancer Lett.* 3: 1-8,

Shin, H. J., Sohn, H. O., Park, C. H., Lee, H. S., Min,Y. K. and Hyun, H. C. (2007) Evaluation of the *in vitro* biological activity of selected 35 chemicals. *J. of the Korean. Soc. of Tobacco Sci*. 29: 30-40.

Steele, RH, Payne, V. M., Fulp, C. W., Rees, D. C., Lee, C. K.and Doolittle, D. J. (1995) A comparison of the mutagenicity of mainstream cigarette smoke condensates from a representative sample of the U.S. cigarette market with a Kentucky reference cigarette (1R4F). *Mutat. Res.* 342: 179-190.

Tewes, F. J., Meisgen, T. J., Veltel, D. J., Roemer, E. and Patskan, G. (2003) Toxicological evaluation of electrically heated cigarette. Part 3: genotoxicity and cytotoxicity of mainstream smoke. *J. Appl. Toxicol.* 23: 341-348.