A Chemical Approach to Searching for Bioactive Ingredients in Cigarette Smoke

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Cigarette smoke, a collection of many toxic chemicals, contributes to the pathogenesis of smoking-related diseases such as chronic obstructive pulmonary disease and cancer. Much work has been done on the chemical analysis of ingredients in cigarette smoke, but there are few reports on the active ingredients that can modify biomolecules. We used a sensitive liquid chromatography-mass spectrometry (LC/MS) and LC/MS/MS method to show that L-tyrosine (Tyr), an amino acid with a highly reactive hydroxyl group, readily reacts with cigarette smoke extract (CSE) at body temperature (37°C) to form various Tyr derivatives. Among these derivatives were *N*-(3-oxobutyl)-Tyr and two acetylated compounds, *N*-acetyl-Tyr and *O*-acetyl-Tyr, which were synthesized by reaction of Tyr with methyl vinyl ketone and acetic anhydride, respectively, at 37°C. The presence of methyl vinyl ketone and acetic anhydride in CSE was confirmed by gas chromatography-mass spectrometry (GC/MS). These results indicate that Tyr can easily react with active ingredients in CSE. The present analytical methods should aid the search for active ingredients in cigarette smoke.

Key words cigarette smoke extract; L-tyrosine; LC/MS; LC/MS/MS; GC/MS

Cigarette smoking is the major risk factor for cardiovascular disease, atherosclerosis, lung disease and cancer.[1)] Cigarette smoke is an extremely complex mixture of over 4800 chemicals.[2)] Its gas phase components are considered to penetrate the small airways and alveoli, and play a significant role in the development of pulmonary parenchymal and systemic diseases.[3,4)] Cigarette smoke extract (CSE) is known to induce cytotoxicity in various cells.[5–8)] However, what is not well understood is whether or not the ingredients in cigarette smoke can act on biomolecules and lead to the development of smoking-related diseases.

The chemical analysis of ingredients in cigarette smoke is one of the most challenging tasks for analysts, and extensive work has been done using many analytical techniques. GC and GC/MS are usually used for the analysis of volatile compounds such as reactive carbonyl compounds.[9,10)] Despite such work, there have been no reports regarding the analysis of reaction products from the compounds in cigarette smoke and functional biomolecules.

To find a clue as to whether biomolecules can be modified by active ingredients in cigarette smoke, we tried to identify the products formed by reaction of nicotine- and tar-removed CSE, *i.e.*, the gas-phase extract of cigarette smoke, with chemically reactive amino acids that make up biomolecules. In this study, the amino acid chosen for study was L-tyrosine (Tyr) because it has a hydroxyl group and its reaction products with CSE are easy to analyze. The reaction temperature was set at 37°C (average normal human body temperature). A highly sensitive LC/MS/MS system was utilized to detect and identify trace amounts of the reaction products. We also tried to identify the active ingredients in CSE.

The authors declare no conflict of interest.

Experimental

Chemicals Frontier Lights (cigarette brand name) was purchased from Japan Tobacco Inc. (Tokyo, Japan), Cambridge filters from Borgwaldt (Germany). Commercially purchased Tyr was purified by HPLC (Shimadzu Co., Kyoto, Japan). *N*-Acetyl-Tyr was purchased from Sigma-Aldrich Inc. (Tokyo, Japan), *O*-acetyl-Tyr from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.), methyl vinyl ketone and crotonaldehyde from Tokyo Chemical Industry Co., Ltd., (Tokyo, Japan). LC/MS grade H_2O, CH_3OH and guaranteed grade acetic acid were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and LC/MS grade formic acid and guaranteed grade acetic anhydride from Nacalai Tesque, Inc. (Kyoto, Japan). An ODS column (Cosmosil 5C_{18}-AR-II 4.6 mm×150 mm) was purchased from Nacalai Tesque, Inc.

Preparation of CSE CSE was prepared by a modification of the technique described in a previous report.[11)] Briefly, CSE was prepared by bubbling into phosphate-buffered saline (PBS) (1 mL per three cigarettes) the mainstream of smoke (gas phase) from which the particulate phase, including tars and nicotine, had been almost completely removed by passage through a Cambridge filter using an aspiration pump. The pump flow rate was kept constant (1 L/min). Smoke was bubbled only for 1 min after lighting the cigarettes. The CSE solution was immediately filtered through a 0.22-μm filter. The resulting solution was designated the 100% CSE, and stored at −80°C. CSE solutions were reacted with 2 mM Tyr solution at 37°C for 24 h, except for the experiment examining the reactive time course. The products in the reaction solutions were analyzed by LC/MS and LC/MS/MS.

Triple-Quadrupole Mass Spectrometer and HPLC Conditions A Quattro Premier triple-quadrupole LC/MS (Micromass, Manchester, U.K.) with an electrospray ionization

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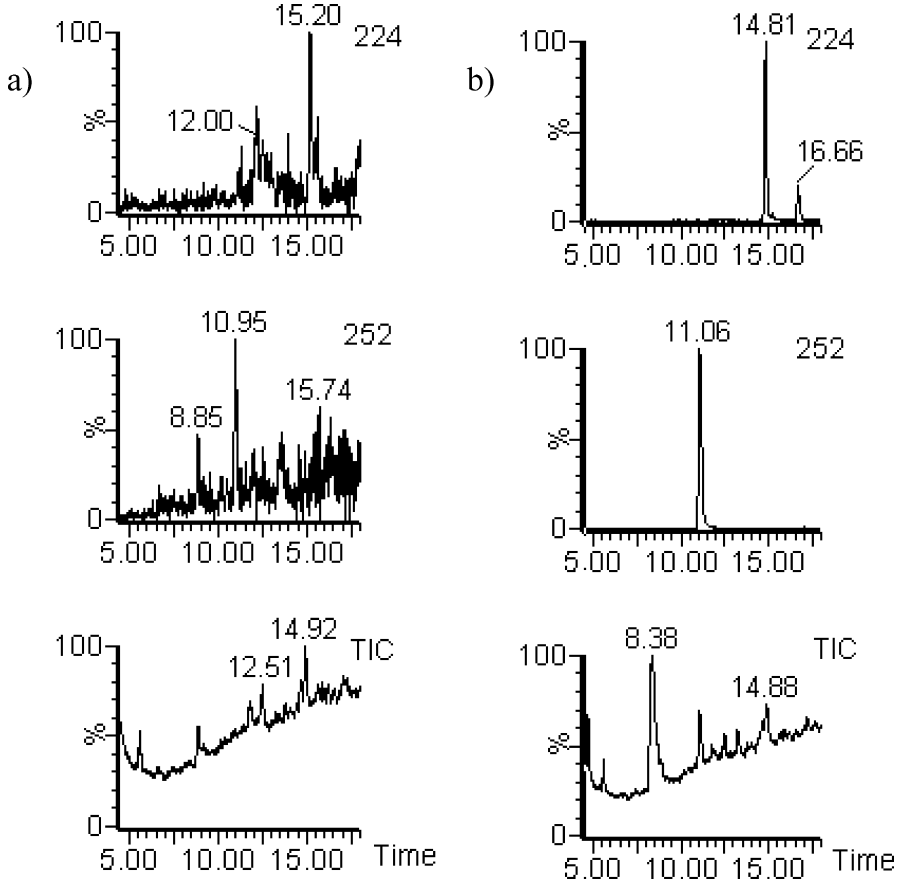

Fig. 1. Mass Chromatogram of the CSE Solutions before (a) and after (b) Reaction with the Tyr Solution for 24 h at 37°C

For LC condition, a binary mobile phase consisting of 0.05% HCOOH (solvent A) and CH_3OH (solvent B) was used in the following program: 2 min, 5% B; 12–15 min, 40% B; 16 min 5% B.

(ESI) source was used for the positive and negative ion mode Q1 scan and MS/MS analysis coupled to the Alliance HT 2795 Separations Module (Waters Co., Milford, MA, U.S.A.). The mass spectrometer was operated at low resolution for both Q1 and Q3 in the selected reaction monitoring (SRM) mode. The optimized conditions were as follows: source temperature, 120°C; desolvation temperature, 350°C; flow rate of cone nitrogen, 50 L/h and flow rate of desolvation nitrogen, 800 L/h. Capillary and cone voltages were 3.0 kV and 15–25 V, respectively. The flow rate of argon collision gas for fragmentation in the SRM mode was 0.3 mL/min ($3.37–3.39\times10^{-3}$ mbar) by which the collisional energy was optimized for the fragment ions of Tyr and other analytes (10–13 eV). All chromatographic separations were performed using a Cosmosil $5C_{18}$-AR-II column (4.6 mm×150 mm). The mobile phase was composed of water containing 0.05% formic acid as solvent A and methanol as solvent B, and the flow rate was set at 0.4 mL/min. A linear gradient analysis was used for LC conditions in the separation. Main gradient analysis was follows: the initial elute solvent consisted of 5% solvent B until t=2 min, and using a linear gradient, it was raised to 40% solvent B at t=12 min, and then held for 3 min until t=15 min, and using a linear gradient, it was raised further to 95% solvent B at t=18 min and then held at 95% solvent B for 5 min until t=23 min. Finally, the gradient solvent was lowered to 5% solvent B by 24 min and held for 2 min. Other conditions of gradient analysis are given in the figure caption (Fig. 1). The LC oven temperature was set at 27°C and the injection volume was 5 μL.

High-Resolution (HR) MS Conditions A HR MS matrix-assisted laser desorption/ionization (MALDI)-Spiral time-of-flight (TOF)/TOF (JEOL Ltd., Tokyo, Japan) was used for elemental analysis. *N*-Acetyl-Tyr and its Na and K adduct ions as well as platelet-activating factors were used as internal standards for mass calibration.

Gas Chromatography (GC)-MS Conditions A mass spectrometer (Automass SUN, JEOL Ltd., Tokyo, Japan) equipped with a GC (6890N, Agilent Technology Inc., Santa Clara, CA, U.S.A.) was used for analysis of active compounds that can react with Tyr in CSE. GC/MS conditions were as follows: ionization energy, 70 eV; current, 300 μA; PM voltage, 500 V; source temperature, 250°C; interface temperature, 250°C; inlet temperature, 250°C; He gas, 1.0 mL/min (at constant flow); splitless mode. Total ion current (TIC) chromatography and selected ion monitoring (SIM) chromatography GC/MS were used to identify and quantify the search compounds. Chromatographic separations were performed using a Zebron capillary GC column ZB-WAX (Phenomenex Inc., Torrance, CA, U.S.A.), 30 m long×0.25 mm i.d.×1.00 μm film thickness, phase: 100% polyethylene glycol. GC conditions to separate active compounds in CSE were as follows: initial oven temperature, 40°C; fold time, 2 min; rate temperature 4°C/min rise to 100°C; fold time, 2 min; rate temperature 15°C/min rise to 220°C, hold time, 3 min; rate temperature 30°C/min down to 40°C, hold time, 1 min.

Table 1. Comparison of Retention Time t_R and SRM Analyte Peak Area Ratio between Tyr Derivatives (*m/z* 224, t_R 15.2 and 16.8 min) Produced by CSE and the Respective Authentic *O*-Acetyl-Tyr and *N*-Acetyl-Tyr

Compound	t_R (min)	Peak area ratio of SRM chromatogram	
		222>180/224>178	222>205/224>178
M_r 223 (Tyr+42)	15.2	0.006	0.0023
O-Acetyl-Tyr	15.2	0.0061	0.0025
		224>178/224>182	222>180/224>182
M_r 223 (Tyr+42)	16.8	1.431	0.095
N-Acetyl-Tyr	16.8	1.485	0.093

HPLC conditions: flow rate, 0.4 mL/min; column oven temperature, 27°C; linear gradient analysis system. Mass transition pattern: negative ion mode, *m/z* 222>180, 222>205; positive ion mode, *m/z* 224>178, 224>182.

Chemical Reactivity of CSE with Tyr To determine optimal reaction time, the fresh CSE solution was gently mixed with 2 mM Tyr solution for 0.017 (1 min), 1, 3, 6, 12 and 24 h at 37°C. After the reaction, the solutions were analyzed in the SRM mode. The peak area in the SRM chromatogram of reaction products at each reaction time was compared with that at 24 h.

Synthesis of *N*-(3-Oxobutyl)-Tyr *N*-(3-Oxobutyl)-Tyr, whose IUPAC name is 3-(4′-hydroxyphenyl)-2-(3″-oxobutylamino)propanoic acid, was synthesized by mixing 5.5 eq of Tyr, methyl vinyl ketone and $NaHCO_3$ in 100 mL water at 80°C for 4 h. The reaction mixture was condensed and then the title compound was purified and collected by HPLC, under the following conditions: mobile phase, 5% CH_3CN and 95% water containing 0.1% trifluoroacetic acid; flow rate, 1.4 mL/min; Cosmosil 5C_{18}-AR-II packed column (3.0 mm×150 mm). ^{1}H-, ^{13}C-, hetero-nuclear multiple quantum coherence (HMQC) and hetero-nuclear multiple-bond connectivity (HMBC) NMR spectra were obtained on an ECP-500 (JEOL) at 500 MHz in CD_3OD solution. High-resolution FAB mass spectra were obtained by a JMS-700 (JEOL Ltd., Tokyo, Japan).

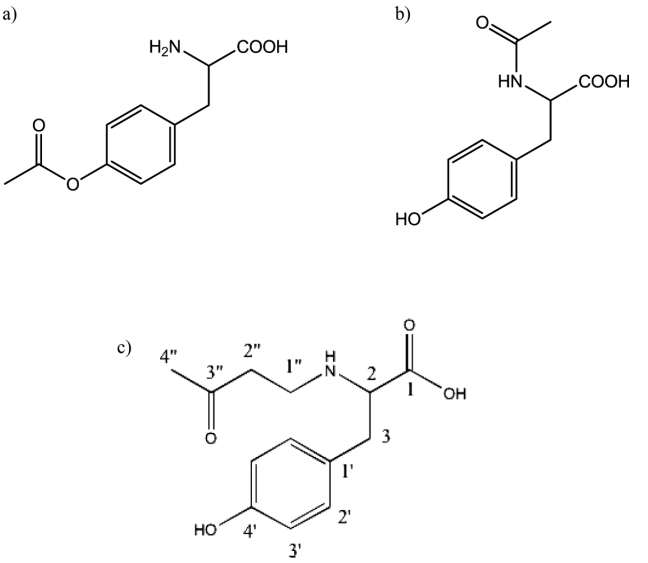

Fig. 2. Structures of *O*-Acetyl Tyrosine (a), *N*-Acetyl Tyrosine (b) and *N*-(3-Oxobutyl)-tyrosine [3-(4′-Hydroxyphenyl)-2-(3″-oxobutylamino)-propanoic Acid] (c)

Results and Discussion

Analysis of Compounds Produced by Reaction of Tyr with CSE To analyze the compounds produced by reaction of Tyr with CSE, LC/MS analysis, the MS/MS method (product ion scan, neutral loss scan) and highly sensitive SRM were used. First, we optimized the MS parameters and obtained the protonated and deprotonated molecular ions of Tyr, $[Tyr+H]^+$, *m/z* 182 and $[Tyr-H]^-$, *m/z* 180 under positive and negative ESI conditions. Next, we compared differences in the mass spectra of the CSE solutions before and after reaction with the Tyr solution (Fig. 1). As a result, three compounds displaying a constant neutral loss scan (*m/z* 46 u) on mass spectra were detected. The loss of *m/z* 46 u, that is, the decrement from $[Tyr+H]^+$ *m/z* 182 to *m/z* 136, is characteristic of Tyr in MS/MS spectra. We next tried to determine their structural formulas. The molecular weight (M_r) of the three peaks was presumed to be 223 for retention times (t_R) of 15.1 and 16.8 min, and 251 for t_R 11.2 min from the mass value of the positive and the negative ion mode mass spectra. The compounds of M_r 223 (Tyr+42) were estimated to be acetylated forms of Tyr, because M_r 223 has *m/z* 42 u higher mass values than Tyr (M_r 181). *N*-Acetyl-Tyr and *O*-acetyl-Tyr were identified by using their respective authentic standards on the basis of the retention time and product ion spectral pattern of their LC/MS chromatograms (Table 1). Ultimately, the compound of t_R 15.2 min was identified as *O*-acetyl-Tyr and that of t_R 16.8 min as *N*-acetyl-Tyr (Figs. 2a, b).

Subsequently, the elemental composition of M_r 251 (Tyr+70) was determined using a high-resolution mass spectrometer. The accurately measured mass was 252.1233±0.00048. The difference between the accurately measured mass and the calculated exact mass of $C_{13}H_{18}NO_4$ was −0.00017 u, and the mass error was 0.67 ppm. This indicates that the elementary composition (molecular formula) of the compound Tyr+70 is $C_{13}H_{18}NO_4$.

Time Courses of the Amounts of Compounds Produced by Reaction of Tyr with CSE The time courses of the product yields of three Tyr derivatives, *N*-acetyl-Tyr, *O*-acetyl-Tyr and Tyr+70, which were produced by reaction of Tyr with CSE, were examined to determine their respective optimal reaction times by using LC/MS and SRM methods. The mass transition pattern of each product was monitored by the product ion mass spectra (Table 2). The peak area in the SRM chromatogram of each compound was determined at 0.017 (1 min), 1, 3, 6, 12 and 24 h after reaction of Tyr with CSE. The yield of each compound was expressed as the ratio against the value at 24 h. The peak area of *O*-acetyl-Tyr of t_R

Table 2. Mass Transition Patterns of Three Tyr Derivatives Obtained by Reaction of Tyr with CSE

t_R (min)	Estimated M_r	Mass transition pattern from $[M+H]^+$ and $[M-H]^-$
11.2	*N*-(3-Oxobutyl)-Tyr	*m/z* 252>194, 148
	251	*m/z* 250>180
15.2	*O*-Acetyl-Tyr	*m/z* 224>178, 180
	223	*m/z* 222>205
16.8	*N*-Acetyl-Tyr	*m/z* 224>182, 178
	223	*m/z* 222>180

HPLC conditions: flow rate, 0.4 mL/min; column oven temperature, 27°C; linear gradient analysis system.

15.2 min (M_r 223) reached maximum at around 1 min after the reaction and thereafter the value was sustained for at least 24 h. The production rate of *O*-acetyl-Tyr of t_R 15.2 min (M_r 223) was the fastest among the three compounds and that of *N*-acetyl-Tyr of t_R 16.7 min (M_r 223) was second. Its yield was about 50% after 1 h and almost 100% after 24 h. Tyr+70 (t_R 11.2 min, M_r 251) showed the slowest production rate and its yield was about 50% after 3 h and almost 100% after 24 h. The peaks of those compounds were not detected in the CSE solution itself.

GC/MS Analysis of Active Ingredients in CSE To identify the active ingredients in CSE that can produce the acetylated compounds Tyr+42 and the compound Tyr+70 by reacting with Tyr, GC/MS analysis was used. With regard to compounds Tyr+42, it was confirmed that the acetic acid contained in CSE does not react with Tyr at all, but the acetic anhydride easily reacts with Tyr in PBS solution to produce the acetylated Tyr derivatives, *N*- and *O*-acetyl-Tyr. This result indicates that acetic anhydride is present in the fresh CSE solution and gradually hydrolyzes to acetic acid with time. Thus, analytical data for the authentic standard of acetic acid were compared with those of acetic anhydride by GC/MS. Peaks of acetic acid and acetic anhydride were observed at t_R 23 min on the TIC chromatograms and also appeared with the same base peak of *m/z* 43 in both mass spectra. From these results, we can surmise that acetic anhydride is easily decomposed to acetic acid by hydrolysis during passage through the GC column.

Next, we tried to identify the active ingredients in CSE that can produce compound Tyr+70. Two compounds were detected at t_R 6.3 min and 9.5 min from the mass chromatogram of *m/z* 70. From library search of their mass spectra, we identified the peak at t_R 9.5 min as crotonaldehyde and the peak at t_R 6.3 min as methyl vinyl ketone. These retention times were the same as those of their respective authentic samples. From the results of quantification in the SIM mode, the concentrations in the CSE solution of crotonaldehyde and methyl vinyl ketone were approximately 450 μM and 550 μM, respectively.

Identification of Compound Tyr+70 To clarify the structure of compound Tyr+70, crotonaldehyde and methyl vinyl ketone, components assumed to be present in CSE, were reacted with Tyr at 37°C. The peak of compound Tyr+70 appeared on the SRM spectra of the mixed solution of Tyr and methyl vinyl ketone, but was hardly detected on that of the mixed solution of Tyr and crotonaldehyde. This shows that methyl vinyl ketone reacts with Tyr to produce compound Tyr+70. Analysis data for compound Tyr+70 synthesized by reaction of methyl vinyl ketone with Tyr agreed with those of compound Tyr+70 obtained by reaction of CSE with Tyr. The structure of compound Tyr+70 was identified as *N*-(3-oxobutyl)-Tyr[3-(4′-hydroxyphenyl)-2-(3″-oxobutylamino)-propanoic acid] (Fig. 2c) by ^{1}H-, ^{13}C- and 2D- (HMQC, HMBC) NMR spectra. This compound was newly formed in the mixture. The analysis data of ^{1}H-, ^{13}C-NMR and high-resolution FAB-MS were as follows: ^{1}H-NMR (CD_3OD) δ: 2.17 (3H, s), 2.91 (2H, dd, J=6.4 Hz), 3.19 (2H, d, J=6.4 Hz), 3.26 (2H, d, J=6.4 Hz), 4.18 (H, dd, J=6.4 Hz), 6.77 (2H, d, J=8.7 Hz), 7.11 (2H, d, J=8.7 Hz). ^{13}C-NMR (CD_3OD) δ: 29.6, 35.9, 39.6, 43.1, 63.0, 116.9, 125.6, 131.6, 158.5, 170.9, and 207.6. HR-FAB-MS *m/z* ($[M+H]^+$); Calcd for $C_{13}H_{18}NO_4$, 252.1235. Measured: 252.1238.

These results show that the synthesized *N*-(3-oxobutyl)-Tyr is obtained by the Michael reaction, not the Schiff base reaction of carbonyl compounds with primary amines. Recently, a similar result has been reported in the literature; α,β-unsaturated carbonyl compounds, methyl vinyl ketone and acrolein, react with lysine residues in proteins *via* their unsaturated bond to form Michael addition adducts, and this reaction is more efficient than Schiff base adduction *via* their carbonyl group.[12] On the other hand, it has been shown fluorometrically that crotonaldehyde and acrolein in CSE can form carbonyl adducts at lysine, cysteine, and histidine residues in human serum albumin.[13] The future challenge is to evaluate whether CSE reacts with such amino acids to produce carbonyl adducts.

Conclusion

The present study demonstrated that CSE can easily react with Tyr at 37°C to produce *N*-acetyl-Tyr, *O*-acetyl-Tyr and *N*-(3-oxobutyl)-Tyr. It is noteworthy that the *O*-acetylation reaction of Tyr proceeded extremely quickly. We also elucidated the presence of active ingredients in CSE, *i.e.*, methyl vinyl ketone and acetic anhydride, although the latter compound could not be definitely determined because the spectral data of acetic acid and acetic anhydride are very similar. It is certain, however, that acetic anhydride is present in CSE. We also clarified that Michael addition adducts are formed by the reaction of Tyr with methyl vinyl ketone in CSE. Our findings, although preliminary, offer a clue to further elucidating the health risks of smoking. However, it is not certain whether CSE can react with Tyr residues in peptides and proteins as with Tyr itself. Further studies are needed to assess what reaction products of CSE with peptides containing Tyr are produced.

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