Detection of 2-Amino-1-methyl-6-(4-hydroxyphenyl)imidazo[4,5-*b*]pyridine in Broiled Beef

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2-Amino-1-methyl-6-(4-hydroxyphenyl)imidazo[4,5-*b*]pyridine (4'-OH-PhIP) showed mutagenicity in *Salmonella typhimurium* TA98 in the presence of S9 mix, inducing 180 revertants per 100 μ g. Since creatinine, tyrosine and glucose, which are present in meat, are expected to be involved in the formation of 4'-OH-PhIP, its presence in broiled beef was examined. 4'-OH-PhIP was detected in broiled beef by high-performance liquid chromatography and UV-spectrometry after extraction with 0.1 N HCl and purification by blue cotton treatment and ion exchange column chromatography. Its level was estimated to be 21.0 ng per g of broiled beef, which is comparable to that of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP).

Key words: 4'-OH-PhIP — PhIP — Mutagen — Broiled beef — Heterocyclic amine

A series of mutagenic and carcinogenic heterocyclic amines have been found in cooked meat and fish, beef extract, cigarette smoke condensate and airborne particles.¹⁻⁹⁾ Among them, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), which is the most abundant heterocyclic amine in cooked meat and fish, was recently shown to be carcinogenic in mice and rats.¹⁰⁻¹²⁾ Studies on the formation of PhIP in a model system suggested that its aminoimidazole moiety is derived from creatinine or creatine and the remaining part of the molecule is derived from Maillard reaction products of phenylalanine and glucose or of phenylalanine itself.¹³⁻¹⁵⁾

When PhIP was incubated with isolated rat hepatocytes, 4-(2-amino-1-methylimidazo[4,5-*b*]pyrid-6-yl)-phenyl sulfate (4'-PhIP sulfate) was identified as a major metabolite and 2-amino-1-methyl-6-(4-hydroxyphenyl)imidazo[4,5-*b*]pyridine (4'-OH-PhIP), an unconjugated derivative of the sulfate, as one of the others.¹⁶⁾ 4'-OH-PhIP was also shown to be formed together with the *N*-hydroxy derivative of PhIP on incubation of PhIP with liver microsomes from rats, mice and rabbits *in vitro*.¹⁷⁻¹⁹⁾ Moreover, the 4'-hydroxy derivative of PhIP was detected in feces of rats given PhIP.²⁰⁾

The exocyclic primary amino group of this PhIP derivative is suspected to be responsible for the mutagenicity of this compound, as in other mutagenic and carcinogenic heterocyclic amines,^{1,19,21)} although 4'-OH-PhIP was reported to be non-mutagenic to *Salmonella*

typhimurium TA98, both with and without a metabolic activation system.¹⁸⁾ Moreover, by analogy with the formation of PhIP, 4'-OH-PhIP is probably formed from creatinine, tyrosine and glucose and from creatinine and tyrosine. Therefore, we tested the mutagenicity of 4'-OH-PhIP and also attempted to isolate this compound from broiled beef and identify it.

4'-OH-PhIP, supplied by the Nard Institute, Osaka, was synthesized from 2-chloro-1-methyl-6-phenylimidazo[4,5-*b*]pyridine as follows. Briefly, the 2-chloro compound was nitrated with nitric acid in sulfuric acid to give the 4'-nitro derivative. This nitro compound was reduced to the 4'-amino compound with Fe/HCl. Treatment of the 4'-amino compound with ammonia displaced the chlorine, yielding 4'-amino-PhIP. Finally, 4'-amino-PhIP was treated with an equimolar amount of sodium nitrite at 20°C for 15 min under acidic conditions and the reaction mixture was heated at 150°C for 1 h, then the resulting compound was crystallized from dimethylformamide. In this way, 4'-OH-PhIP was obtained in an overall yield of 12%. The electron ionization mass spectrum of the synthetic 4'-OH-PhIP showed a molecular ion at *m/z* 240, and fragment ion peaks at *m/z* 239 and 212. Its UV-absorption spectrum in methanol exhibited maxima at 239, 275 and 321 nm. Its ¹H-NMR spectrum in dimethyl sulfoxide-*d*₆ showed the presence of the following protons; δ (ppm) 3.54 (s, 3H), 6.84 (d, 2H, *J* 8.4 Hz), 6.87 (s, 2H), 7.48 (d, 2H, *J* 8.4 Hz), 7.62 (d, 1H, 2.0 Hz), 8.18 (d, 1H, 2.0 Hz) and 9.44 (s, 1H). These physical properties were well consistent with those of 4'-

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OH-PhIP isolated as one of the metabolites of PhIP in previous experiments.^{16,18)} Furthermore, on high-performance liquid chromatography (HPLC), the retention time of 4'-OH-PhIP synthesized chemically was the same as that of the arylsulfatase hydrolysis product of 4'-PhIP sulfate, which was isolated from the urine of rats fed PhIP, as reported previously.¹⁶⁾

The mutagenicity of this chemically synthesized 4'-OH-PhIP was tested in *S. typhimurium* TA98 and TA-100. 4'-OH-PhIP induced revertants of TA98 with S9 mix in a dose-dependent manner; 50, 100 and 200 μg of this compound per plate gave 90, 180 and 330 net revertant colonies, respectively. No mutagenicity was observed in *S. typhimurium* TA98 without S9 mix or in TA100 with or without S9 mix at doses of up to 500 μg per plate.

Broiled beef was tested for the presence of 4'-OH-PhIP. Sliced lean beef (60 mm $\times$ 40 mm $\times$ 4 mm per piece) was broiled for 2.5 min on each side over a naked gas flame. Then, a sample of 25 g was minced, homogenized with 250 ml of 0.1 N HCl for 1.5 min in a Polytron homogenizer, shaken for 20 min, and centrifuged at 5,000 rpm for 10 min. The precipitate was extracted twice more, and the pooled supernatant was mixed with trichloroacetic acid at a final concentration of 5% and allowed to stand overnight. The mixture was centrifuged at 8,000 rpm for 20 min, and the supernatant was adjusted to about pH 7.0 with 6 N NaOH. This solution was treated three times with 1 g portions of blue cotton (Funakoshi Co. Ltd., Tokyo) and adsorbed materials were extracted with methanol-ammonia water as previously reported.²²⁾ The extract was evaporated and the residue (23 mg) was dissolved in 20 ml of methanol and applied to a cation exchange TIN-100 H05E fiber column (1.5 $\times$ 7 cm, Toray Ind. Inc., Tokyo). The column was washed with 100 ml of methanol and adsorbed materials were eluted with 350 ml of methanol-ammonia water (10:1, v/v). This eluate was evaporated and the residue (1.6 mg) was further purified by HPLC in a CCPM apparatus equipped with a UV-8000 spectrophotometer (Tosoh Corp., Tokyo) at the ambient temperature with monitoring of the eluted material for absorption at 315 nm.

A sample of the evaporated residue was dissolved in 150 μl of 50% methanol and injected into a semi-preparative TSKgel ODS-120A column (10 μm particle size, 7.8 $\times$ 300 mm, Tosoh Corp.). Material was eluted with 16% acetonitrile in 25 mM phosphate buffer (pH 2.0) at a flow rate of 2 ml/min. The fraction with the same retention time (12–14 min) as the authentic compound was collected and evaporated. The residue was dissolved in 200 μl of 50% methanol and an aliquot was separated on a cation exchange, SP-2SW column (5 μm particle size, 4.6 $\times$ 250 mm, Tosoh Corp.) with a mobile

Fig. 1. Detection of 4'-OH-PhIP in broiled beef. The peak fraction with the same retention time as authentic 4'-OH-PhIP on an SP-2SW column, indicated by an arrow (A), was collected and purified further on an ODS column (B). 4'-OH-PhIP was detected as a single peak, shown by an arrow, in the same position as authentic 4'-OH-PhIP. The eluates from both columns were monitored for absorption at 315 nm.

phase of 35% acetonitrile in 100 mM phosphate buffer (pH 3.0) at a flow rate of 0.9 ml/min. As shown in Fig. 1A, a peak of material was detected at a retention time of 14.5 min, which corresponds to that of authentic 4'-OH-PhIP. This peak fraction was collected and further purified on a YMC A303 ODS column (5 μm particle size, 4.6 $\times$ 250 mm, Yamamura Chemical Laboratories, Kyoto). The column was developed with 15% acetonitrile and 10% methanol in water with 0.1% diethylamine adjusted to pH 7.0 with acetic acid at a flow rate

Fig. 2. UV-absorbance spectra of samples of broiled beef and authentic 4'-OH-PhIP recorded with a photodiode array detector on an ODS column. Samples of the fraction containing ~120 ng of 4'-OH-PhIP prepared from broiled beef (A) and 200 ng of authentic 4'-OH-PhIP (B) were analyzed. The chemical structure of 4'-OH-PhIP is inset in (B).

of 0.7 ml/min. A single peak of material was detected at the same elution position as that of authentic 4'-OH-PhIP (Fig. 1B). The amount of 4'-OH-PhIP detected on the ODS column was estimated to be equivalent to 10.5 ng/g of broiled beef from a standard curve for the authentic compound. A similar value was also estimated from the size of the peak on the SP-2SW column.

To confirm the structure of the compound in the peak fraction with a retention time of 14 min on the analytical

ODS column, we collected the peak fractions obtained by HPLC chromatography on the SP-2SW column in repeated purifications. A sample of approximately 120 ng of this compound was examined with an SPD-M6A photodiode array detector (Shimadzu Corporation, Kyoto) on a YMC A303 ODS column under the same conditions as described above. As shown in Fig. 2, its UV spectrum was identical with that of authentic 4'-OH-PhIP. The fluorescence emission spectrum of the compound recorded with an FS-8011 fluorometric detector (Tosoh Corp.) with excitation at 315 nm on the ODS column was also the same as that of authentic 4'-OH-PhIP. Thus, the compound isolated from broiled beef was concluded to be 4'-OH-PhIP.

The overall recovery of 4'-OH-PhIP during the purification process was found to be 50% by spiking with an amount of authentic 4'-OH-PhIP equivalent to that detected by HPLC. By correcting the amount of 4'-OH-PhIP detected by HPLC for this recovery, the original level of this compound was estimated to be 21 ng per g of broiled beef. As 1 g of broiled beef induced 2000 revertants of TA98 with S9 mix, 4'-OH-PhIP accounted for less than 0.1% of the total mutagenicity of broiled beef.

PhIP, which is reported to be the most abundant heterocyclic amine in cooked food, has been detected in various cooked foods at levels of 0.56–69.2 ng/g, and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), the second most abundant compound, has been found at levels of 0.64–6.44 ng/g.^{2,5)} Broiled beef contains PhIP and MeIQx at levels of 15.7 and 2.11 ng/g, respectively.^{2,5)} Thus, the content of 4'-OH-PhIP is comparable to that of PhIP. The mutagenicity of 4'-OH-PhIP in *S. typhimurium* TA98 is 10^3 - and 8×10^4 -fold less than those of PhIP and MeIQx, respectively.^{1,5,21)} However, mutagenicities of heterocyclic amines in *Salmonella* are not quantitatively correlated with the carcinogenicities in rodents.²¹⁾ Humans are expected to be exposed to 4'-OH-PhIP derived from cooked food and also through metabolism of PhIP.²⁰⁾ Therefore, a long-term 4'-OH-PhIP feeding experiment should be carried out in animals for carcinogenicity evaluation.

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