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Characterization of Genotoxicity of Kojic Acid by Mutagenicity in Salmonella and Micronucleus Induction in Rodent Liver

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Three lots of kojic acid (KA) which were produced for use as a reagent, food additive and in cosmetics were shown to be mutagenic in *S. typhimurium* TA100 with or without S9 mix, with a specific activity of around 100 revertants per mg of KA. Since there are contradictory reports on genotoxicity of KA, we examined, using HPLC, whether the mutagenicity to *S. typhimurium* is due to KA itself, or due to contaminants present in the KA samples. Although two UV absorbing fractions were separated by HPLC, mutagenicity was detected only in the major fraction and the specific mutagenic activity of KA did not change before and after HPLC separation. The material in the major peak fractions on HPLC was confirmed to be KA by NMR. Thus it was demonstrated that KA itself is mutagenic and no mutagenic contaminants were detected in the three lots of samples. Since KA is known to produce liver tumors in mice, we further examined the genotoxicity of KA in the liver of rodents. KA induced micronuclei (MN) in the regenerating liver of adult mice by its gastric intubation at 1 g per kg body weight. However, no MN were induced in young mice (3 weeks old) without partial hepatectomy. Since it was recently found that KA had no tumor-initiating activity in the liver of mice in a two-step carcinogenicity study, there is no evidence that the genotoxicity detected in the mouse liver is involved in liver carcinogenesis.

Key words: mutagenicity, liver micronuclei, genotoxicity, *S. typhimurium*, kojic acid.

Introduction

Kojic acid (KA) [5-hydroxy-2-hydroxymethyl]-4*H*-pyran-4-one; CAS No. 501-30-4; (Fig. 1)] is a natural substance produced by various fungi, *Penicillium* spp., *Aspergillus orizae, flavus and lamarii*, and also by certain bacteria (1). KA which had been used as a food additive for the prevention of enzymatic browning of shell fish, raw crabs and shrimp, owing to its inhibitory activity on tyrosinase, was found to be genotoxic *in vitro*, inducing *his*$^+$ reverse mutations in *S. typhimurium* (2–5). It was also found to be genotoxic *in vivo*, inducing micronuclei (MN) in peripheral blood of rats (6).

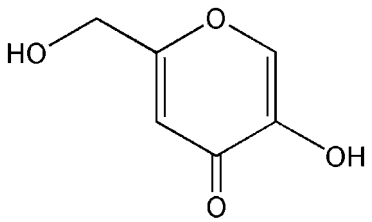

Fig. 1. Structure of kojic acid (KA).

KA had been reported to induce hepatomas and thyroid adenomas in mice (7). Thyroid adenomas or hyperplasia production in the mouse or rat was suggested to be due to promoting activity of KA (7–9). In contrast, the possibility of involvement of genotoxicity of KA in mouse hepatoma development could not be excluded (10). In 2003, the Ministry of Health, Labour and Welfare, Japan noticed that KA was no longer used for prevention of browning of shell fish in market due to the development of modern technology, and KA was withdrawn from the list of existing food additives in the same year. Since KA is a fungal product, soy sauce, miso and sake were suspected to contain KA. Out of 32 samples of fermented foods examined for the presence of KA, three samples were found to be positive, but the levels were not so high, being at the maximum, 1 ppm (personal communication by Dr. Tamio Maitani, National Health Sciences, Tokyo, Japan, Dec. 7, 2005). Further, KA is still used in cosmetics as a skin lightening agent (quasi-drug) at concentrations of up to 1.5% due

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to its inhibiting action on human melanocyte tyrosinase (11). Thus, the safety of KA needs to be confirmed from various points.

Recently, results of an extensive study on genotoxicity of KA have been reported, in which KA showed only weak mutagenicity without a dose-dependent response in *S. typhimurium* (12). Many contradictory or inconsistent results had been reported for the genotoxicity of KA. KA induced MN in peripheral blood, but not in the liver of young rats by a single application through gastric intubation (6). However, it did not induce MN in bone marrow of mice (12,13) by a single application through intraperitoneal injection. Furthermore, MN were not induced in human keratinocytes SVK14 nor in human hepatocellular carcinoma cells, HepG2, *in vitro* (12) but were induced in human lymphoblastoid cells, TK6 and WTK-1 (personal communication by Dr. Masamitsu Honma, National Institute of Health Sciences, Tokyo Japan, December 12, 2005). KA did not induce $HPRT^-$ mutations in Chinese hamster V79 cells or mouse L5178Y cells (12), but induced TK^- mutation in TK6 ($TK^{+/-}$) and WTK-1 ($TK^{+/-}$, $P53^{-/-}$) cells (unpublished observations, Yu F Sasaki). As for chromosome aberrations and sister chromatid exchanges *in vitro*, positive results were reported in Chinese hamster cells (5).

Since there had been no standards for composition of KA in food hygiene law, while the manufacturers' specifications for cosmetic use was at least 97% pure, there was a possibility that the discordance reported for genotoxicity was due to differences in composition of KA samples used. Thus, it is important to clarify whether the mutagenicities of some KA samples were due to KA itself or contaminant included. In the present study, samples of various lots of KA produced by different companies that are used as a food additive, reagent and cosmetics ingredient were purified by HPLC. The resulting fractions were tested in order to determine whether the reported mutagenicity in *S. typhimurium* was due to KA itself or to another component present in the KA samples. This testing showed that the mutagenicity is due to KA itself and not to contaminants. As KA has been reported to be hepatocarcinogenic in mice (7), we further examined whether KA shows *in vivo* genotoxicity in rodent liver, by MN assay.

Materials and Methods

Chemicals: Kojic acid, reagent grade, lot no. 052K2516 was purchased from Sigma (St. Louis), 5312 used for food additive (content is 100.6%) was supplied from Alps Pharmachemical Industry (Gifu, Japan) and 2Y181 [at least 97% pure, but more typically (by HPLC) was >99% pure] used for cosmetics was supplied from Sansho Seiyaku Co., Ltd (Fukuoka, Japan). For *in vivo* genotoxicity studies, KA for food additive provided by Alps Pharmachemical Industry was used. Cyclophospamide, diethylnitrosamine (DEN), dimethyl sulfoxide (DMSO), 4-nitroquinoline 1-oxide and trifluoroacetic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan), and 1,2-dimethylhydrazine (1,2-DMH) and benzo[*a*]pyrene were from Tokyo Kasei Kogyo (Tokyo, Japan). Solutions of KA were prepared immediately before use.

HPLC separation: Analytical HPLC was performed under three systems. System 1; an LC-20A series from Shimadzu Co. (Kyoto, Japan) equipped with Shimadzu SPD-M20A photodiode detector. A Mightysil RP-18 GP column (5 μm particle, 3 mm i.d. $\times$50 mm; Kanto Chemical Co., Tokyo, Japan) was used at 40°C, with an isocratic eluent of methanol-0.05 % v/v trifluoroacetic acid (3:97) and a flow rate of 0.15 mL/min: System 2; a Shimadzu LC-10A series was used with a Mightysil RP-18 GP column (5 μm particle, 4.6 mm i.d. $\times$250 mm), an eluent of methanol-0.05 % v/v trifluoroacetic acid (3:97) and a flow rate of 0.7 mL/min: System 3; the same equipment and column with system 2, but the eluent used was 0.1% propionic acid-0.05N perchloric acid and a flow rate of 0.7 mL/min. Preparative HPLC was performed on an LC-8A series from Shimadzu Co., using a large volumetric flow cell in the UV detector. A Mightysil RP-18 GP column (5 μm particle, 10 mm i.d. $\times$250 mm; Kanto Chemical Co., Tokyo, Japan) and an eluent of methanol-0.05 % v/v trifluoroacetic acid (3:97) were used with a flow rate of 4.0 mL/min. After separation, each fraction was lyophilized and weighed. All fractions were subjected to mutagenicity testing.

NMR analysis: ^1H and ^{13}C spectra were recorded on an ECP-600 spectrometer (JEOL Ltd., Akishima, Tokyo) using DMSO-d_6 as a solvent.

Mutagenicity assay: The mutation assays were performed using *S. typhimurium* TA100 with and without S9 mix (14). The S9 purchased from Oriental Yeast Co. Ltd (Tokyo, Japan) was prepared from Crj:CD (SD), male rat treated with phenobarbital and 6-naphthoflavone. KA was dissolved in 0.1 mL of distilled water and the assay was performed by the method of preincubation (15). For the mutagenicity test, after separation of samples by HPLC, 1 mg of dried residues was applied to a plate for each of fractions 6, 8 and 9. For fraction 7, a dose-dependent response with 0.5, 1.0 and 1.5 mg/plate was examined and the specific activity was calculated based on the linear regression of the least square method. For other fractions, the residues were dissolved in 500 μL water, and 10 or 100 μL were applied to each plates. Statistic analysis was performed by multiple regression analysis.

Animal and KA administration: Male ddY mice of 3 and 8 weeks old, and male Fischer 344 rats of 8 weeks

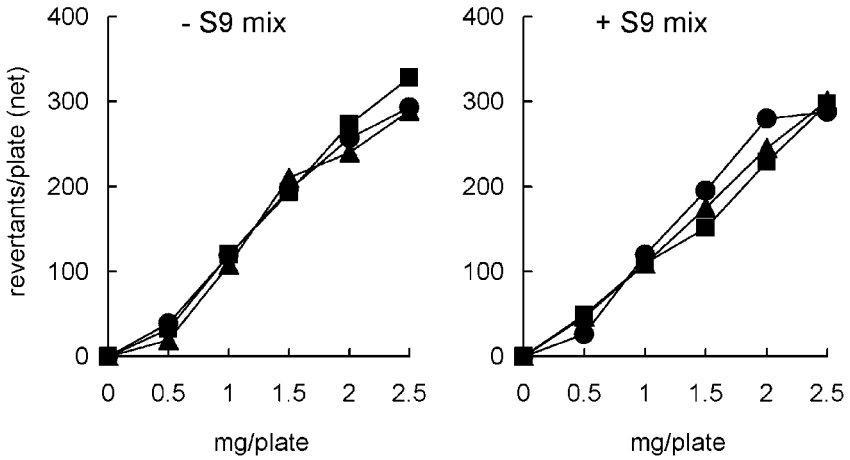

Fig. 2. Mutagenicities of kojic acid (KA) in *S. typhimurium* TA 100 in the absence (left) or presence (right) of S9 mix. The values were averages of two plates, subtracted with numbers of spontaneous revertants of 128 (−S9 mix) or 167 (+S9 mix). Positive control, 0.03 μg of 4-nitroquinoline 1-oxide induced 400 revertants (−S9 mix) and 5 μg of benzo[*a*]pyrene induced 1545 revertants (+S9 mix) over spontaneous one. No statistic differences were observed among these three samples. ●; lot.052K2516, ■; lot.5312, ▲; lot.2Y181.

old were purchased from SLC Japan (Shizuoka, Japan). Eight week old animals were acclimatized for one week at 24±2°C with a relative humidity of 55±5%, with basal diet of MF pellets (Oriental Yeast Industries, Tokyo, Japan) and tap water ad libitum, and were used at age 9 weeks. Three week old mice were used immediately. For gastric intubation, KA was suspended at 0, 50 and 100 mg/mL in 0.5% sodium carboxymethyl-cellulose and immediately administered to animals at a dose of 10 mL/kg, corresponding to KA doses of 0, 500 and 1000 mg/kg. The doses administered to the animals were based on the approximate maximum tolerated dose for each species determined by simple acute toxicity experiments: oral gavage with 2000 mg/kg KA resulted in death of 4/4 mice and 4/4 rats within 3h.

Micronucleus assay: Four mice and rats were used for each KA-dose group and three for positive control groups, following the reported method (16,17). Twenty-four hours after administration of KA, partial hepatectomy (two-thirds) was performed on 9 week old animals by removing three major lobes of the liver, the left lateral, left medial and the right lateral lobes under ether-anesthetized conditions. After four days, the animals were anesthetized with ether, and their livers were perfused *in situ* for 5 min with Liver Perfusion Medium [Hanks' balanced salt solution (Ca^{2+}/Mg^{2+} free) containing 0.5 mM EGTA and 10 mM HEPES (pH 7.2–7.3)] (GIBCO-Invitrogen, Carlsbad, CA), followed by 5 min with Liver Digest Medium (Hanks' balanced solution (Ca^{2+}/Mg^{2+} free) containing 0.05% collagenase type IV, 50 μg/mL trypsin inhibitor, 10 mg/mL bovine serum albumin, 10 mM HEPES, and 560 μg/mL $CaCl_2$) (GIBCO-Invitrogen). Both solutions were maintained at 37°C and delivered at a flow rate of 14 mL/min. The perfused livers were minced in a Petri dish containing Liver Digest Medium; the minced tissue was then passed through gauze, centrifuged at 200 g for 1 min, and the cell pellet obtained was fixed with 1 mL of 10% neutral formaldehyde. Ten μL of the cell suspension were mixed with 10 μL of 500 μg/mL acridine orange, the mixture placed on a glass slide and covered with a coverslip. The number of micronucleated hepatocytes (MNHEPs) among 1000 hepatocytes was recorded for each animal under a fluorescence microscope (×400 or greater), with a blue excitation filter and a yellow barrier filter. MNHEP was defined as a hepatocyte with two nuclei, one being less than 1/4 in diameter of the other nucleus. For 3 week old mice, MN assays were performed by the same procedure without partial hepatectomy, and livers were removed at 72, 96 and 120 h. The differences between the frequencies of MN in KA treated and KA 0 control animals were analyzed by one-way ANOVA followed by pairwise comparisons using the Dunnett test. A P-value of less than 0.05 was considered statistically significant.

All animal experiments were carried out following the guidelines set out by Hachinohe National College of Technology in the Guide for the Care and Use of Laboratory Animals.

Results

Mutagenicity in *S. typhimurium* of KA for reagent, food additive and cosmetics: Three lots of KA, 052K2516 (reagent), 5312 (food additive) and 2Y181 (cosmetics) showed similar mutagenic activities in TA 100, under conditions without or with S9 mix (Fig. 2). All samples showed linear dose-dependent response between 0.5 and 1.5 mg/plate of KA. The specific mutagenic activities calculated by linear regression by

Table 1. Weight and mutagenicity in *S. typhimurium* TA100 of each fraction obtained by HPLC of KA samples

Fr. No.	Lot. 052K2516			Lot. 5312			Lot. 2Y181		
	Weight (mg/Fr.)	Revertants/Fr.		Weight (mg/Fr.)	Revertants/Fr.		Weight (mg/Fr.)	Revertants/Fr.	
		−S9 mix	+S9 mix		−S9 mix	+S9 mix		−S9 mix	+S9 mix
1	0.2	0	0	0.6	0	20	0.1	0	0
2	0.2	0	0	0.4	0	0	0.1	0	0
3	0.2	0	0	0.3	0	0	0.0	0	0
4	0.2	0	0	0.4	0	0	0.1	0	0
5	0.0	0	0	0.0	0	30	0.1	0	0
6	11.3	1402	1232	14.2	2321	1734	10.2	1129	946
7	22.8	2686	3051	27.0	4260	3504	33.4	3176	3377
8	15.1	1595	1700	17.4	2153	2205	26.6	2258	2471
9	7.4	749	853	9.0	945	1359	11.6	1170	961
10	2.9	292	213	2.3	259	94	4.1	410	250
11	2.0	0	0	0.3	25	0	1.2	0	0
12	0.8	0	0	0.3	85	0	0.2	0	0
13	0.4	0	0	0.4	85	0	0.2	0	0
14	0.4	0	0	0.1	40	0	0.2	0	0
Total weight	63.9			72.5			88.1		

the least square method for 052K2516, 5312 and 2Y181 were 116, 115 and 106 revertants/mg, respectively, without S9 mix and 113, 106 and 111, respectively, with S9 mix. No statistic differences were detected among these different sources of samples at significance of ≤ 0.05.

Separation of mutagenic substance in KA samples by HPLC: To clarify whether the mutagenicity was derived from KA itself or some contaminants in the samples, HPLC separations were performed. A KA sample solution in distilled water of 0.5 μg/0.5 μL was eluted by analytical HPLC under the conditions described in Materials and Methods (systems 1–3). The chromatograms under system 1 of three lots of KA monitored by absorption at 270 nm were similar, and revealed a single peak at 3.8 min (Fig. 3a). Only when a very large amount of KA was applied, a small shoulder peak was detected at the foot of the major peak (data not shown). Using two other different HPLC systems 2 and 3, similar chromatograms were obtained with three samples of KA (data not shown), with their small and very large amounts. These results indicated only a minor UV absorbing material(s) are contaminated in all three samples at similar levels.

Preparative HPLC was then applied to each lot of KA to facilitate the determination of the mutagenicity of the various constituents of the KA samples. A 500 μL aliquot of 25 mg/mL KA solution in distilled water was applied to a column, under the conditions described in Materials and Methods. Typical 270 nm chromatograms of the three samples are shown in Fig 3b. From the chromatograms, it can be seen that all of the samples include an impurity detected as a small peak shoulder, which was eluted after the major peak (arrows in Fig 3b). Circa 100 mg of each KA sample was separated by repeating the HPLC eight times, then all corresponding fractions of the eight runs were pooled and lyophilized to dryness. The contaminant distributed in fractions 10 and 11 in all three samples. The residual weight of each fraction (Table 1) correlated well with its absorption at 270 nm (Fig 3b).

When each fraction was examined for mutagenicity in *S. typhimurium* TA100, fractions 6–10 of all three samples were mutagenic with and without S9 mix, while no significant mutagenicity was detected in the other fractions, under either condition. The total mutagenicity of each fraction of the three samples (Table 1) correlated well with its absorption at 270 nm (Figs 3). All three lots of KA supplied were slightly colored, but after preparative HPLC, the purified KA (fractions 6–9) was almost colorless, and fractions 10 and 11 were strongly colored according to the original color shade.

Table 2 shows the specific activities (revertants/mg) of fractions 6–10. Since fraction 10 contained significant amounts of contaminant, the average specific activities of fractions 6–9 of each sample are also indicated, together with those of the original KA samples, calculated from the data presented in Fig 2, by linear regression by least square method. The specific activities of the three samples were almost the same before and after separation by HPLC, with and without S9 mix.

Structure confirmation by NMR: The dried residues of fractions 6–9 of 052K165, 5312 and 2Y181 were subjected to ^{1}H and ^{13}C-NMR analysis. All of the protons in the KA structure (Fig. 1) were detected in the ^{1}H-NMR spectra, with the following chemical shifts; 4.29 (doublet, CH_2), 5.65 (triplet, CH_2-OH, exchanges with D_2O), 6.33 (singlet, 5-H), 8.02 (singlet, 2-H), and

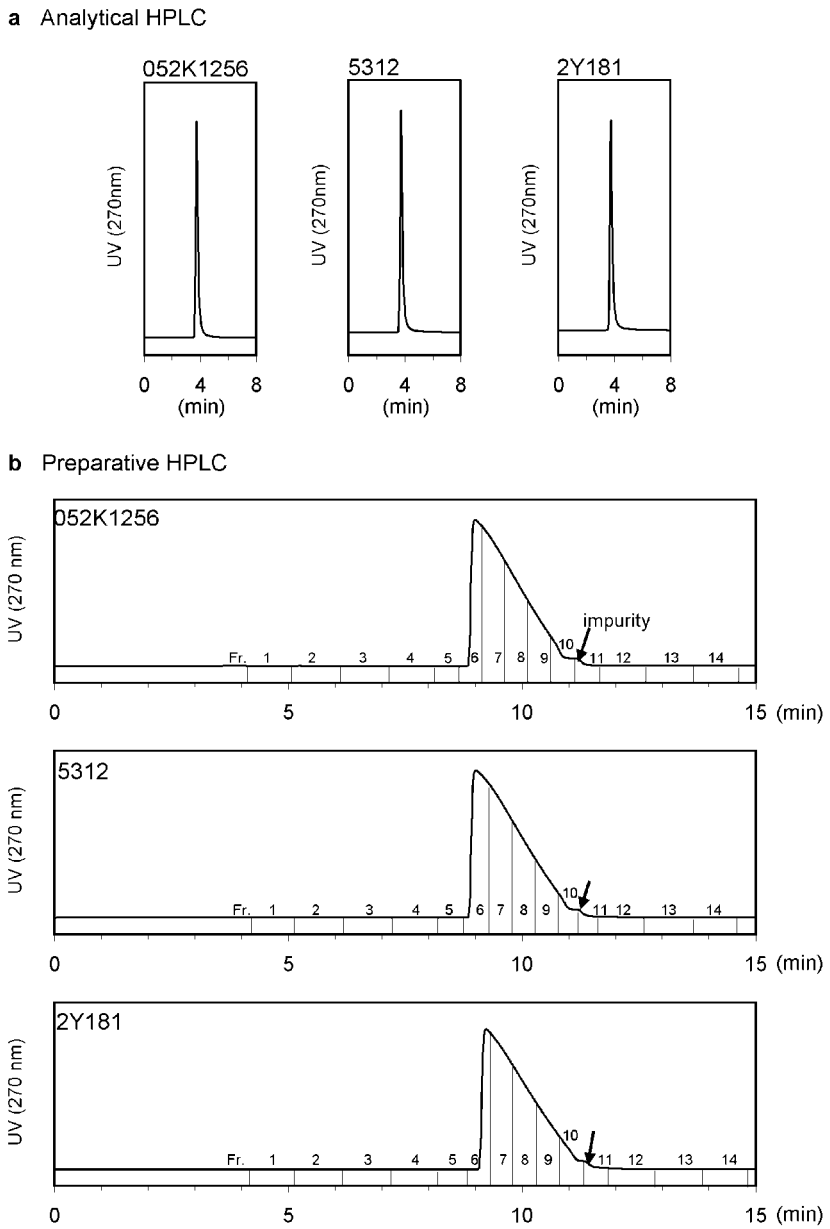

Fig. 3. Analytical (a) and preparative HPLC chromatograms (b) of three samples of KA (lot. 052K1256, 5312, 2Y181) (a): A 0.5 μL aliquot of KA solution (1 mg/mL) in distilled water was loaded on an analytical column (Mightysil RP-18 GP, 3 × 50 mm), and separated by an eluent of methanol − 0.05 v/v% trifluoroacetic acid (3:97) with a flow rate of 0.15 mL/min and absorption at 270 nm was recorded. (b): A 500 μL aliquot of KA solution (25 mg/mL) was applied to a preparative column, Mightysil RP-18 GP (5 μm, 10 × 250 mm), and separated by the same eluent as that used for analytical HPLC with a flow rate of 4 mL/min. In addition to the major peak of KA, a small shoulder peak of impurity was detected as indicated by arrow.

9.05 (singlet, C = C-OH, exchanges with D_2O) ppm. Further, the KA carbon skeleton was detected by ^{13}C-NMR at 60.01 (exocyclic carbon ($\underline{C}H_2$)), 110.37 (C3), 139.79 (C6), 146.25 (C5), 168.61 (C2), and 176.09 (C4(C = O)) ppm. No impurity peaks were detected in any of the samples analyzed. Thus, the major 270 nm absorbing substances in these three samples were confirmed to be KA (data not shown).

***In vivo* genotoxicity of KA:** *In vivo* genotoxicity of KA was examined by MN assay in regenerating livers of mice and rats. Four days after partial hepatectomy (120 h after KA administration), mean values of MNHPCs in mice increased dose dependently and with 1000 mg/kg of KA, the value was significantly increased as compared with 0 dose of KA (Table 3). In rats, however, no increase was observed. Furthermore, KA was found to have no MN inducing ability in infant mice without partial hepatectomy (3 weeks old) (Table 4).

Table 2. Specific mutagenicity in *S. typhimurium* TA100 of KA samples after HPLC separation

Fraction No.	Revertants/mg					
	Lot. 052K1652		Lot. 5312		Lot. 2Y181	
	−S9 mix	+S9 mix	−S9 mix	+S9 mix	−S9 mix	+S9 mix
6	124	109	164	120	111	93
7	118	134	158	130	95	101
8	106	113	124	127	85	93
9	101	115	105	151	101	83
10	95	71	113	41	128	76
Average ± SEM*	112 ± 5.3	118 ± 5.6	138 ± 14	132 ± 6.7	98 ± 5.4	93 ± 3.7
Before HPLC**	116	113	115	106	106	111

Fractions 6, 8 and 9 were analyzed with 1 mg/plate of dried substance. Fraction 7 was tested with 0.5, 1.0 and 1.5 mg/plate of the dried substance, specific mutagenicity was calculated from the linear regression by the least square method. Other fractions were tested with 2 and 20% of dried substances, and results of tests with 20% of total residue of each fraction are indicated. With 2% weight of each fraction, no more than 20% of spontaneous mutagenicity was detected. For fraction 10, the mg amount applied to a plate is; lot.052K1652, 0.58 mg; lot. 5312, 0.46 mg; lot. 2Y181, 0.64 mg. Means of duplicate assays are indicated.

*Average of specific activities of fractions 6 ~ 9 ± standard error of means (SEM).
**Values were calculated from Fig. 2, based on the linear regression by the least square method.

Table 3. MN induced in regenerating liver of mice and rats treated with single gavages of KA

Species	Chemical	Dose (mg/kg)	MNHPCs/1000 HPCs (mean ± SEM)
Mouse	KA	0	2.33 ± 0.33
		500	5.00 ± 1.00
		1000	10.3 ± 1.45*
	DEN	160	15.7 ± 1.20*
Rat	KA	0	1.67 ± 0.33
		500	2.00 ± 0.58
		1000	1.33 ± 0.33
	DEN	160	17.3 ± 2.01*

KA, kojic acid; DEN, diethylnitrosamine
MNHPCs, micronucleated hepatocytes; HPCs, hepatocytes.
*Significantly different from control: $p < 0.05$.

Table 4. Micronuclei induced by KA in the liver of young mice

Chemical	Dose (mg/kg)	MNHPCs/1000HPCs (Mean ± SEM)		
		72 h	96 h	120 h
KA	0	0.75 ± 0.48	1.75 ± 0.75	1.5 ± 0.65
	500	0.75 ± 0.25	2.00 ± 0.82	1.75 ± 0.75
	1000	2.00 ± 0.41	2.00 ± 0.41	2.75 ± 0.75
Cyclophosphamide	20		8.75 ± 1.38*	

MNHPCs, micronucleated hepatocytes; HPCs, hepatocytes.
*Significantly different from KA 0 control: $p < 0.05$ by student *t* test.

Discussion

In the present study, KA samples used as a reagent, food additive and cosmetics ingredient showed mutagenicity in *S. typhimurium* TA100. The mutagenic activities of the samples were almost the same with or without S9 mix. The specific activity was weak, being around 100 revertants per mg (Table 2). The major, 270 nm absorbing component of each sample was isolated by preparative HPLC at a retention time of 8.5–10.5 min (Fig. 3b) and identified as KA by NMR analysis. Each KA sample supplied contained some UV absorbing materials which eluted later than KA (indicated by arrow in Fig. 3b). However, the impurity fraction, fraction 11, did not show any mutagenicity. When the mutagenic activities per unit weight of the three lots of KA supplied are compared with those of the major peak fractions 6–9 obtained by HPLC separation, there are no appreciable differences (Table 2). Although preparation methods of these three KA samples supplied are not available, features of these three samples are different and it is suspected that their preparation methods are different. Although a possibility of contamination of mutagenic non-UV absorbing material(s) which behave in the same way as KA on HPLC could not be completely negated, this possibility can be expected as negligible. Thus, it is indicated that all three lots did not contain mutagenic contaminants, and the mutagenic activity of the KA samples is solely derived from KA.

KA, at a dose of 1 g/kg, induced MN in the regenerating liver of mice. Thus, it seems that KA is genotoxic to liver. However this genotoxicity of KA was not detected in the liver of infant mice, on which partial hepatectomies were not performed. The reason for not detecting genotoxicity of KA in infant mice might have been because, while the mitotic index is expected to rise to a very high level after partial hepatectomy, it would be much lower in infant mice (3 weeks old). Further, differences in the metabolisms of infant mice and adult mice having received partial hepatectomies may play

some roles in this difference in genotoxicity (18). It is also noteworthy that KA did not induce MN in bone marrow of mice (12,13).

In contrast, KA was not genotoxic in regenerating rat's liver (Table 3). KA was also recently reported by Suzuki *et al*. (6). not to induce MN in young rats (4 weeks old). However, KA induced MN in peripheral blood of young rats (6). At present, neither the molecular mechanism of the genotoxicity of KA, nor the difference in metabolisms between rats and mice is known.

During preparation of this manuscript it has been reported in a two step carcinogenesis study, in which mice were fed a diet containing 3% KA at initiation step and phenobarbital was used as a tumor promoter, that there is no liver tumor initiating activity (19). At present, it is not clear whether the partial hepatectomy which was performed two weeks after the beginning of phenobarbital administration was at an appropriate time to detect initiating activity of KA or not. It is possible that the difference in the timing of the partial hepatectomy after cessation of KA administration might be one of the reasons for the discrepancy between KA being positive in MN induction and negative in initiating activity.

Although in the present study, genotoxicity of KA was detected in the mouse liver, it was not proved that this genotoxicity is involved in hepatic tumor development in mice.

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