MUTAGENICITY ASSAY OF SOME SPICES USING BACTERIAL MUTANTS

AHMAD DAMHOERI, AKIYOSHI HOSONO, TAKATOSHI ITOH AND AKIRA MATSUYAMA

AP4 Project, Faculty of Agricultural Engineering and Technology, Bogor Agricultural University, Bogor, Indonesia

ABSTRACT

Mutagenicity of eleoresins prepared from red pepper, bird pepper, shallot and nutmeg fruit was assayed with *Salmonella typhimurium* TA98 and TA100, their streptomycin-dependent derivatives, SD7823 and SD1018, respectively, and *E. coli* WP2 try$^-$hcr$^-$. All oleoresins from these spice materials except dried and stored nutmeg kernel exhibited the dose-response mutagenicity effects with both SD1018 and SD7823 in plate tests. The oleoresin from shallot was of the lowest activity and that from mace displayed the highest activity. Extracts from young leaves of the "kemang" tree were found to have an antimutagenic activity against the mutagenicity of capsicum peppers.

INTRODUCTION

Spices are economical plant products which are widely used over the world for culinary, curing and pharmaceutical purposes. Their constituents exhibit various biological and pharmacological activities including antimicrobial and cytotoxicological effects. These activities may prompt us to investigate the safety aspects of spices and their processed products, although spices have been generally recognized as safe because of their long historical and widespread use. So far, carcinogenic and mutagenic effects of some spices have been reported[1~7]. Since a close correlation between carcinogenicity and mutagenicity has been well known[8~10] and a mutagenesis process may be also involved in aging[11], determinations of mutagenic activities in spices would be worthwhile to make clear their safety aspects. In respect of food mutagenicity assays *in vitro,* Kada *et al.*[12] recently isolated streptomycin-dependent (SM^d) strains derived from Ames strains TA98 and TA100 and demonstrated the feasibility of the new system for the SM^d $\rightarrow$ SM^{ind} mutation in mutagenicity screening tests of food samples containing histidine.

In this study, attempts were also made to isolate SM^d strains from TA98 and TA100 and, employing these strains, examine oleoresins of some spices harvested in Indonesia for *in vitro* mutagenicity.

MATERIALS AND METHODS

Bacterial Tester Strains.

In spot tests, *Salmonella typhimurium* TA98 and TA100 which were kindly provided by Prof. B.N. Ames of California University, U.S.A., *Escherichia coli* WP2 $try^{-}hcr^{-}$ supplied by Dr. T. Kada, National Institute of Genetics, Japan, and two SM^{d} mutants SD1018 and SD7823 which were derived from TA100 and TA98, respectively, in this laboratory, were used. In the plate tests, the latter two SM^{d} mutants were employed.

Isolation of Streptomycin-Dependent Derivatives from *S. typhimurium.*

The procedures employed in this study were the same as the method of Kada *et al.*[12] In brief, the log-phase cells of TA98 and TA100 were treated with 50 μg/ml N-methyl-N'-nitrosoguanidine (MNNG) for 60 min at 37°C. After the post-treatment incubation, decimal dilution series of the culture were plated and colonies of SM^{d} derivatives were picked up comparing the growth on B2 agar plates with and without 20 μg/ml streptomycin (SM). The isolates were subjected to the test of sensitivity to a diagnostic mutagen, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF2). During maintenance of tester SM^{d} strains, their SM (20 μg/ml) dependence, AF2 and spontaneous mutagenesis and ampicillin (25 μg/ml) resistance were monitored and, if necessary, the reisolation of effective tester strains was tried using AF2 as a diagnostic mutagen.

Test Samples.

Samples for mutagenicity tests such as red pepper (*Capsicum annuum* Linn.), bird pepper (*Capsicum frutescens* Linn.), shallot (*Allium ascalonicum* Linn.) and nutmeg fruits (*Myristica fragrans* Houtt.) were commercially purchased at local markets and their oleoresins were prepared using ethanol. With nutmeg fruits, oleoresins were prepared from their flesh, red-colored mace and kernel. Two different kernels were tested, one from young seeds with light brownish or semi-redish mace and the other from seeds dried and stored after harvest. Care was taken to use the fresh sample materials in order to avoid any mycotoxin problem. Sample materials were washed with water, drained, slightly dried and cut into small pieces. Then they were macerated with 1.6–2.8 parts of ethanol (v/w), homogenized and kept at room temperature overnight. After filtration, ethanol was evaporated at about 50°C using a rotary evaporator to obtain concentrates or oleoresins. Kernels of nutmeg seeds were similarly treated after removing their shell. Test samples of oleoresins were made sterile by autoclaving or filtration through a 0.45– μm Millipore filter. For the antimutagen tests, the young leaves of "kemang" trees (*Mangifera caesia* Jack ex Wall.) were milled together with small amounts of

water, homogenized, filtered through gauze, filter paper and finally a membrane filter for eliminating microbial contaminants. Dry matter contents of the test samples were determined by heating at 105°C to represent the concentration of the sample incorporated into the plate agar bed or top agar in the dry matter content per unit volume.

Procedures for Spot Tests and Plate Test.

Spot tests with TA98, TA100 and *E. coli* WP2 $try^{-}hcr^{-}$ were conducted in principle according to the method of Maron and Ames[13]. Overnight cultures of tester strains grown in B2 broth (10 g Kyokuto beef extract, 10 g Daigo-Eiyo polypeptone and 5 g NaCl per liter, pH 7.0) at 37°C with shaking were used as the inoculum. The soft agar seeded with 0.1 ml overnight tester strain culture grown in B2 broth was overlayed on the minimal agar plate semi-enriched with B2 broth (20 ml B2 per liter of minimal agar) and, after solidifying the top agar, each 20-μl aliquot of the test sample solution was applied to a sterile 8 mm filter paper disk which had been placed on the agar plate, followed by incubation at 37°C for 2 to 3 days. With SD1018 and SD7823, each 0.1-ml aliquot of the 20-times diluent of the tester-strain overnight culture grown in B2 broth containing 20 μg/ml SM was spread directly on the surface of a B2-broth agar plate and then a paper disk was placed to apply the sample.

Plate tests were mostly performed by a method using agar plates incorporated the sample (SA method) and in a few experiments by a slight modification of the plate incorporation method with a preincubation step described by Maron and Ames[13] (PI method). In SA method, 9-ml molten B2 broth agar was mixed well with 1-ml sample solution and promptly plated. Each 0.1-ml aliquot of an overnight culture of the tester strain, which had been diluted 20 times with 0.067M phosphate buffer, was spread on a surface of the hardened B2 agar plate and then incubated at 37°C for 1 to 3 days and revertant colonies developed were scored. In the PI method, the mixture of both 0.1-ml aliquots of the tester-strain culture and the sample solution prepared in a test tube was pre-incubated at 37°C for 20 min with gentle shaking. After the pre-incubation, 2-ml molten top agar maintained at 45°C were added, promptly mixed well and applied on the B2 agar plate. Numbers of revertant colonies per plate were counted at an appropriate time during 1—3 days of incubation at 37°C. Plate tests were conducted in duplicate runs and included positive and negative control groups. As a positive control or diagnostic mutagen, the aqueous solutions of AF2 were used throughout this study. In both SA and PI methods, sample concentration-survivor relations were also determined using B2 agar plates containing 20 μg/ml SM.

Reagents.

AF2 and SM were kind gifts of Dr. T. Kada of National Institute of Genetics, Japan, and Indonesia Meiji Co., Ltd., respectively. MNNG used was GR grade of Takeda Chemical Co., Ltd.

RESULTS

Isolation of Streptomycin-Dependent Mutants Derived from *S. typhimurium* TA98 and TA100.

Two SM^d strains, SD1018 and SD7823, were isolated in this laboratory from TA100 and TA98, respectively, according to the method of Kada *et al.*[12]. The dose-response curves of the two isolates obtained for the diagnostic mutagen (AF2) are shown in Fig. 1. In the PI method, the number of revertants per plate revealed a linear dose-response relation for the concentrations of AF2 in the top agar lower than about 1.5×10^{-3} μg/ml. In the SA method, the fairly good linear standard curve for AF2 concentrations up to about 1.2×10^{-3} μg/ml in the agar plate was obtained with both strains after incubation of 3 days, although dose-response curves after one-day incubation showed some upward curvature at the lower concentrations. In both methods, the linear relation between revertants per plate and AF2 concentrations was up to the AF2 concentration which gave 60—80% survivors. The SD7823 strain indicated a little more sensitive response to AF2 mutagenesis than the SD1018 strain. For the same amount of AF2 per plate, scores of revertant colonies per plate obtained in the PI method were bigger about 6 times than those in the SA method with both SM^d strains. The numbers of spontaneous revertants per plate after 1-day incubation were 20—81 for SD1018 and 36—93 for SD7823 and those after 3-day incubation 44—174 for SD1018 and 61—283 for SD7823, respectively.

Spot Tests for Mutagenicity with Some Spices.

Spice oleoresins prepared from red pepper, bird pepper, shallot and component parts of the nutmeg fruit were tested for mutagenicity using *S. typhimurium* TA100 and TA98, *E. coli* WP2 try^-hcr^- and two SM^d mutants, SD1018 and SD7823. The results are indicated in Table 1. Capsicum peppers such as red pepper and bird pepper were positive with some killing zone around a paper disk charged with the sample for *E. coli,* SD1018 and SD7823. Shallot was negative for TA98 and slightly positive for TA100 and *E. coli,* while it was weakly positive for two SM^d strains. Nutmeg fruit flesh and kernel were negative for TA98, TA100 and *E. coli.* In contrast, samples of nutmeg fruits such as fruit flesh, red mace and kernel from raw young seed were positive for SD1018 and SD7823. However, the nutmeg oleoresin prepared from dried and stored seed displayed no mutagenic effect for all strains.

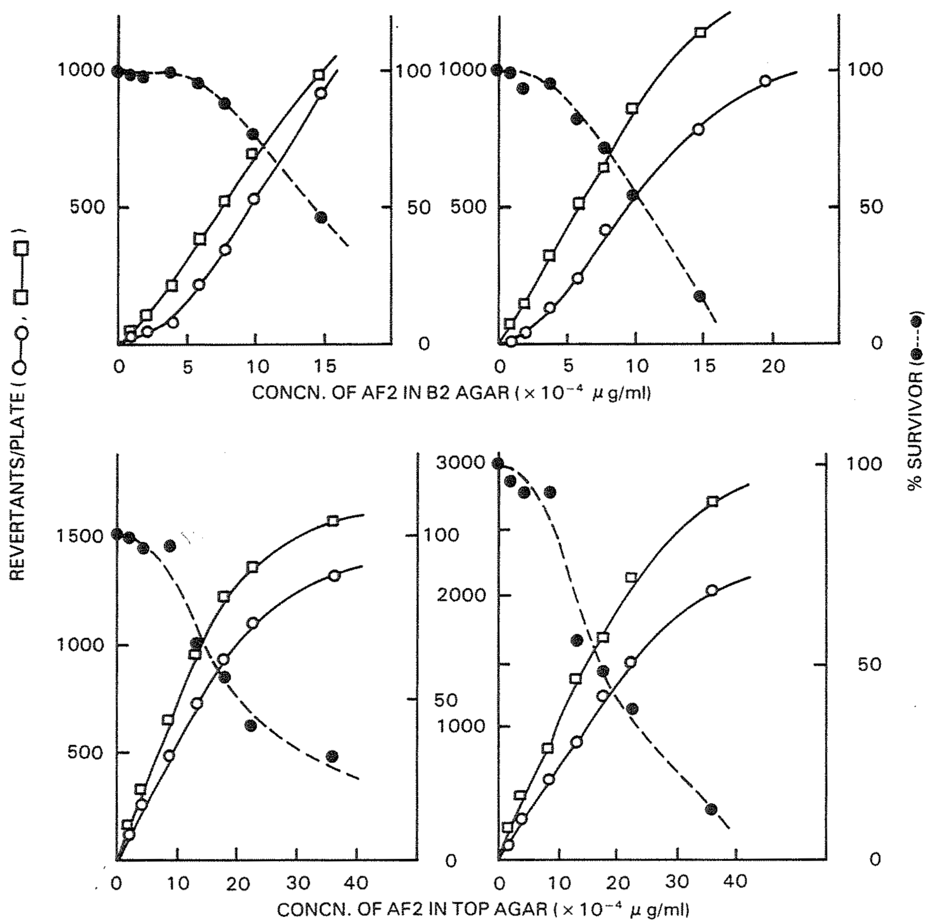

Fig. 1. Dose-Response Curve of *S. typhimurium* SM^d Strains, SD1018 and SD7823 for AF2.
A: SD1018, SA method; B: SD7823, SA method; C: SD1018, PI method; D: SD7823, PI method; Open circle, after 1-day incubation; Open square, after 3-day incubation; Spontaneous revertants have been substracted.

Table 1. Spot tests for mutagenicity of spice oleoresins and related materials.

Sample	*S. typhimurium*		*E. coli* WP2 try⁻hcr⁻	*S. typhimurium* SM[d]	
	TA98	TA100		SD1018	SD7823
Capsicum pepper					
Red pepper	±	+	+	+	+
Bird pepper	—	±	±	+	+
Shallot	—	—	—	±	±
Nutmeg fruit					
Fruit flesh	—	—	—	+	+
Mace	*	*	*	+	+
Kernel	—	—	—	+	+
Myristicin	*	*	*	+	+
Capsaicin	*	*	*	±	±
AF2[a]	+	+	+	+	+

* Not tested.

a Diagnostic mutagen.

Plate Tests for Mutagenicity with Some Spices.

The dose-response effects for the mutagenicity and cell killing of the oleoresins prepared as above mentioned were determined with SD1018 and SD7823. With all samples except the oleoresin from dried and stored nutmeg, their obvious dose-response effects for the mutagenicity were found more or less for both SM[d] strains (Figs. 2 ~ 7). Revertants per gram of raw samples, from which the oleoresins were prepared, calculated from the linear part of the dose-response curve are listed in Table II. Under the experimental condition in this study, values of revertants per gram raw sample (RPG) were found to vary in a wide range. In the SA method, RPG values were in the order of 10 for shallot and around 10^2 for capsicum peppers. In contrast, the RPG values for nutmeg fruit oleoresins were higher, in the order of 10^3 to 10^4, and the value for mace was highest. In the PI method, RPG values for red pepper were in the order of 10^3, higher than those in the SA method. It is also noted that the oleoresin of nutmeg kernel prepared from raw seeds with light brownish or semi-red mace indicated the dose-response effect, while that from dried and stored seeds did not, although both of them revealed the appreciable bactericidal effect (Fig. 7). This difference was confirmed also in a simultaneous determination with both kinds of nutmeg oleoresins (data not shown). The mutagenic effects of red pepper and shallot were unaffected by different sterilization methods, autoclaving at 121 °C and filtration through a membrane filter (Millipore type HA) (Fig. 2). This suggests the mutagenic factor in these spices is thermostable. On the extraction of red pepper oleoresin with hexane,

Table 2. Revertants per plate per gram sample[a].

Sample	Test method	Tester strain	
		SD1018	SD7823
Red pepper[b]	SA	1.4×10^2	–
" [b]	SA	7.3×10	–
" [c]	SA	8.1×10	–
" [b]	PI	5.9×10^3	7.9×10^3
Bird pepper[b]	SA	1.0×10^2	–
" [c]	SA	3.5×10	6.3×10
Shallot[b]	SA	2.6×10	–
" [c]	SA	2.5×10	–
Nutmeg fruit			
Fruit flesh[b]	SA	1.3×10^3	1.1×10^3
Mace[b]	SA	2.9×10^4	4.6×10^4
Raw kernel[b]	SA	7.9×10^2	1.1×10^3
" [b]	SA	–	2.1×10^3
AF2[d]	SA	7.0×10^{10}	8.5×10^{10}
"	PI	3.4×10^{11}	4.8×10^{11}

[a] Raw material, from which the oleoresin was prepared.
[b] Autoclaved.
[c] Filtered through a membrane filter.
[d] Diagnostic mutagen.

its major mutagenic as well as bactericidal factors were found to remain in the residues (Fig. 3). With bird pepper oleoresin, the number of revertants per plate decreased in the lower concentrations as compared with the negative control (Fig. 4), although this decrease observed with the membrane-filtered oleoresin was not found with the autoclaved oleoresin (Fig. 8).

Antimutagenic Factors Against Capsicum Pepper Mutagenesis.

The "kemang" tree are native to the west Java area and their young leaves are locally used to prepare the traditional vegetable salad. The same amount of the kemang leaf extracts (13.1 mg dry matter/ml) was added to different concentrations of capsicum pepper oleoresins for examining the antimutagenic effect of the leaf extracts. In the test using SD1018, the water extracts of kemang young leaves were found to display their antimutagenic effect on the mutagenesis of capsicum peppers (Fig. 8).

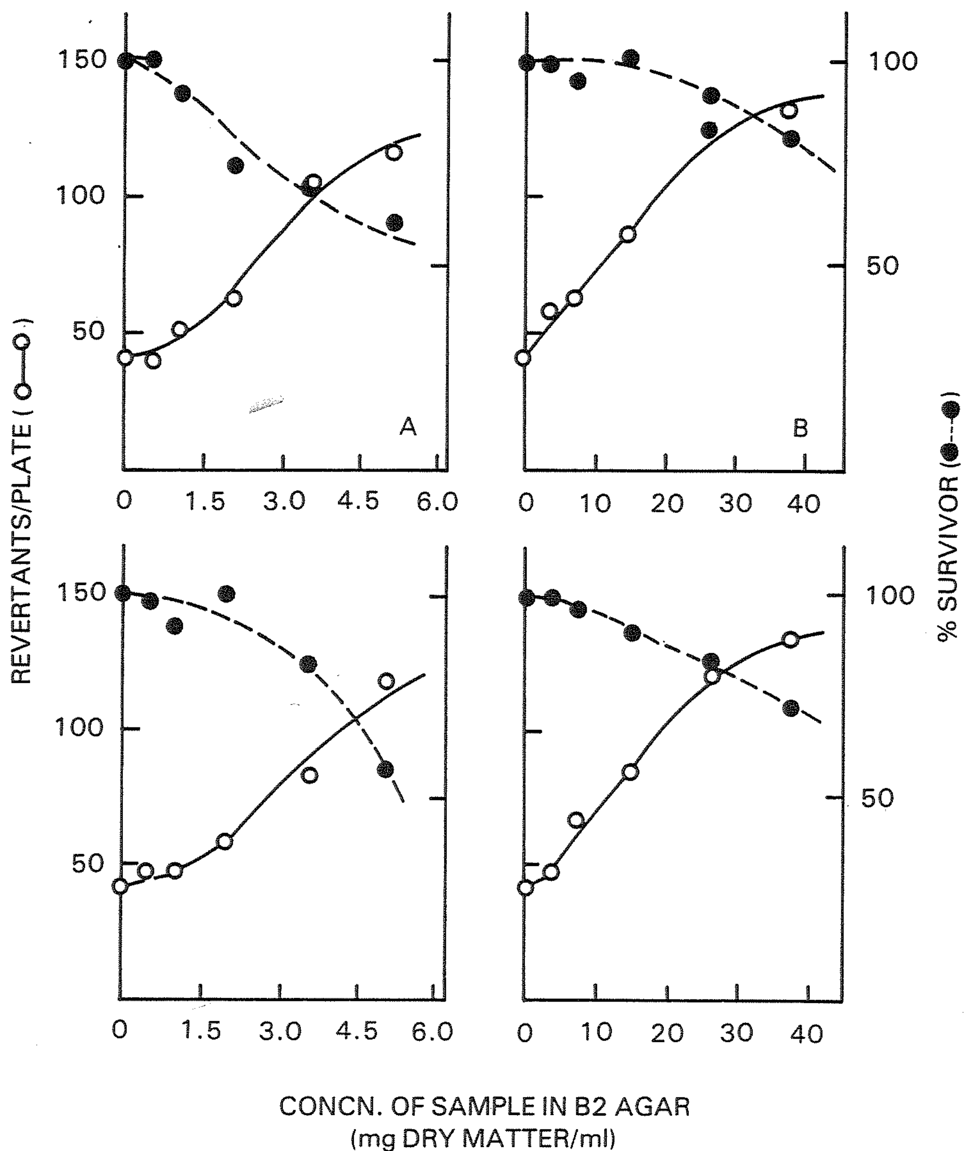

Fig. 2. Dose-Response Effects of Red Pepper and Shallot with SD1018. Sample: A & C, red pepper; B & D, shallot; Sterilization of sample solution: A & B, filtered through a membrane filter; C & D, autoclaved; Test method: SA.

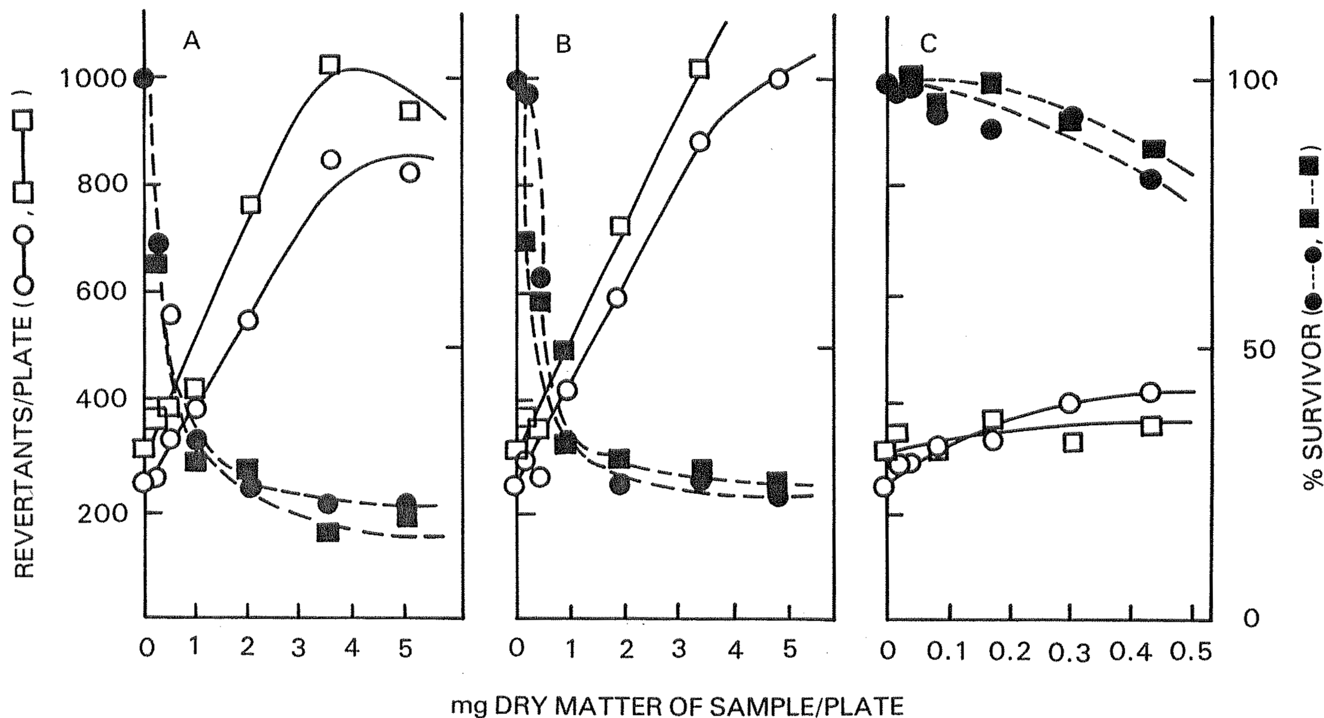

Fig. 3. Dose-Response Effects of the Whole and Fractionated Samples of Red Pepper with SD1018 and SD7823.

A: whole; B: residue of hexane extraction; C: hexane extracts; After hexane extraction of the whole red pepper oleoresin sample (51.5 mg dry matter/ml), the volumes of residues and extracts were adjusted to the same volume as the whole sample and their dry matter contents were found to be 48.7 mg/ml and 4.4 mg/ml, respectively. Each 0.1-ml aliquot was used per plate in the PI method.

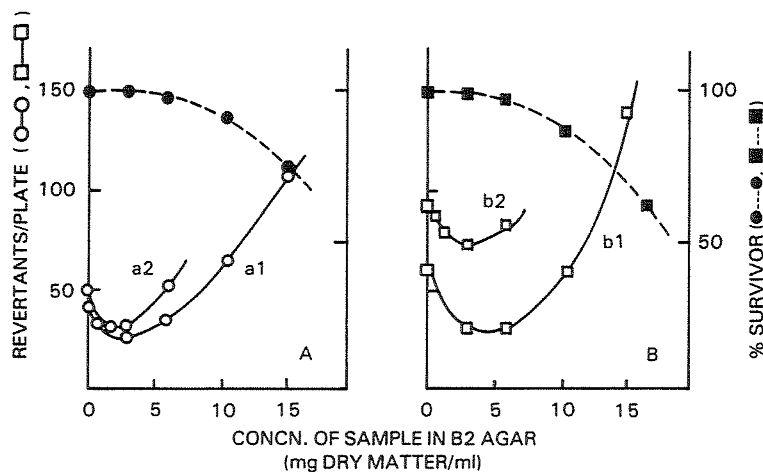

Fig. 4. Dose-Response Effects of Bird Pepper with SD 1018 and SD 7823. A: SD 1018; B: SD 7823; a2 and b2 represent the reproduced data for a1 and b1, respectively, obtained in a second determination; Test method: SA.

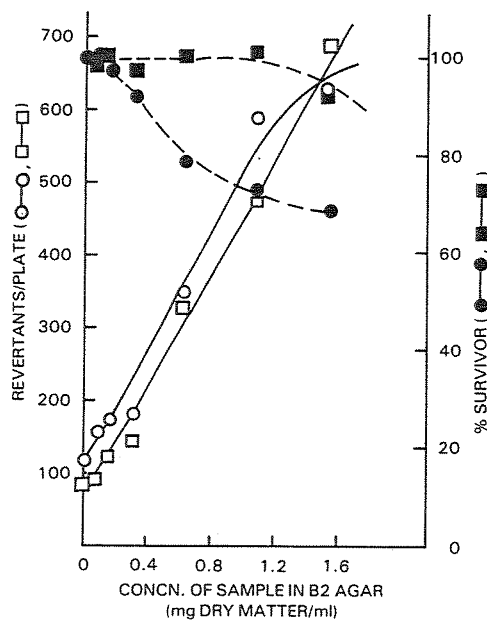

Fig. 5. Dose-Response Effects of Nutmeg Fruit Flesh Oleoresin with SD1018 (circle) and SD7823 (square). Test method: SA.

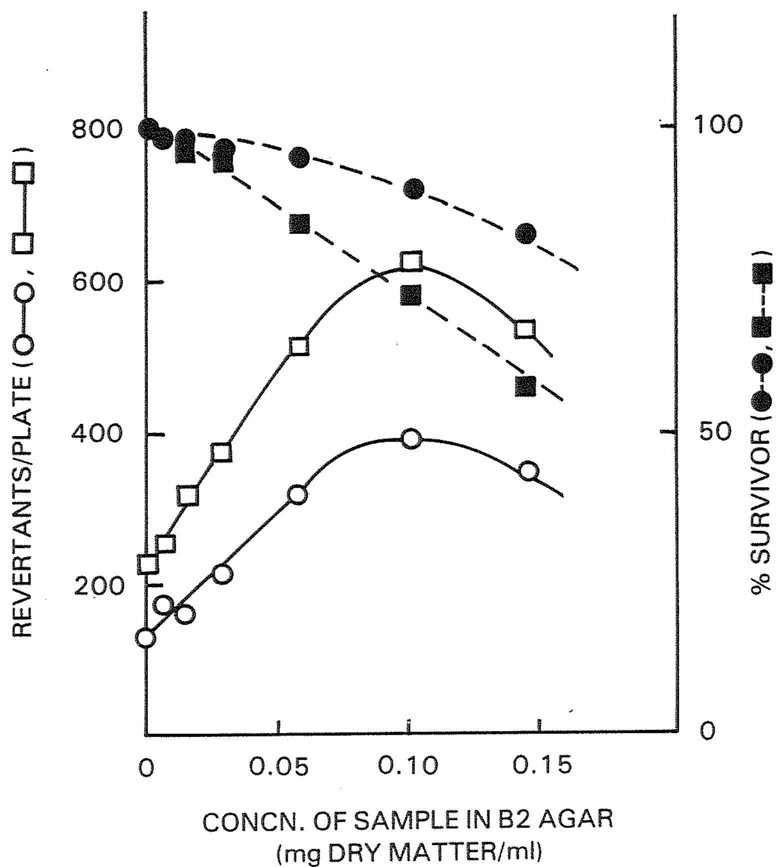

Fig. 6. Dose-Response Effects of Mace Oleoresin with SD1018 (circle) and SD7823 (square).

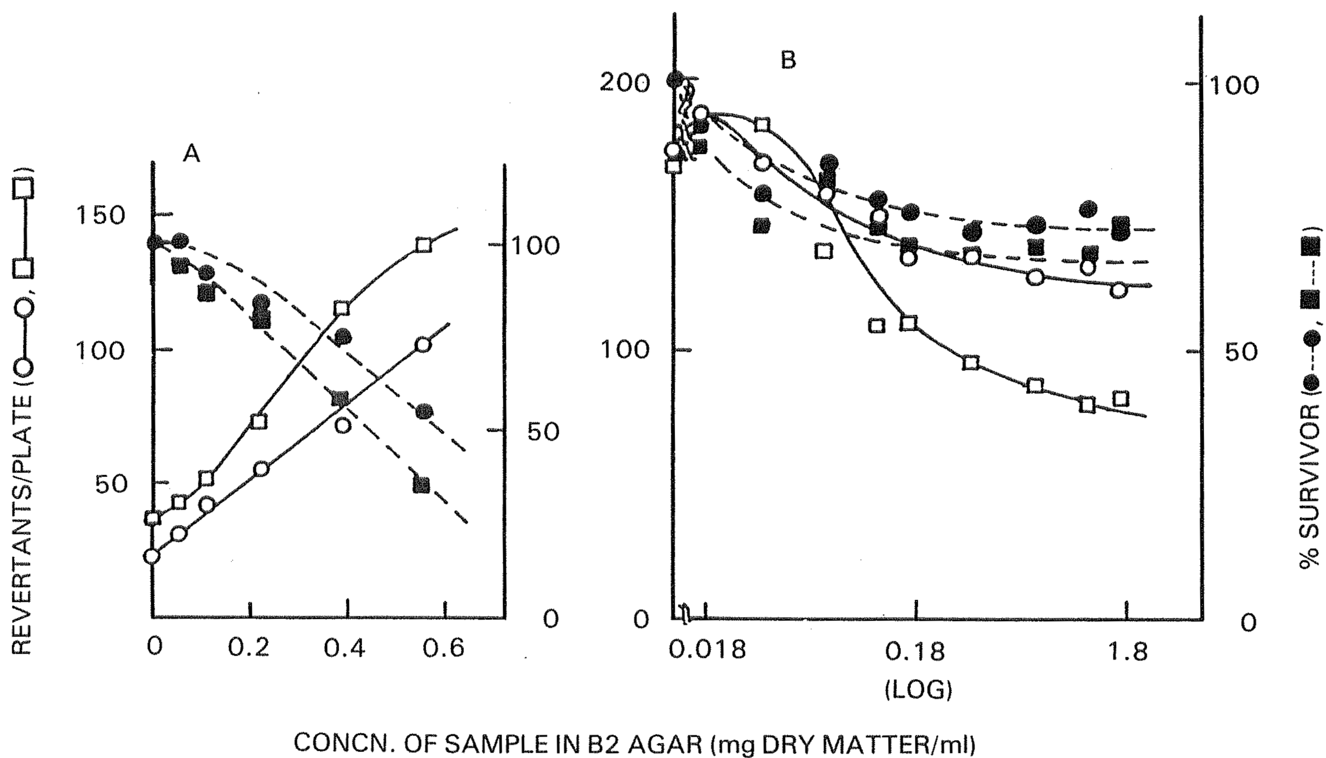

Fig. 7. Dose-Response Effects of Nutmeg Kernel Oleoresin with SD1018 (circle) and SD7823 (square).

A: assayed with kernel from raw fruit seeds with orange-colored mace; B: assayed with kernel from dried and stored seed (commercially obtained); Test method: SA.

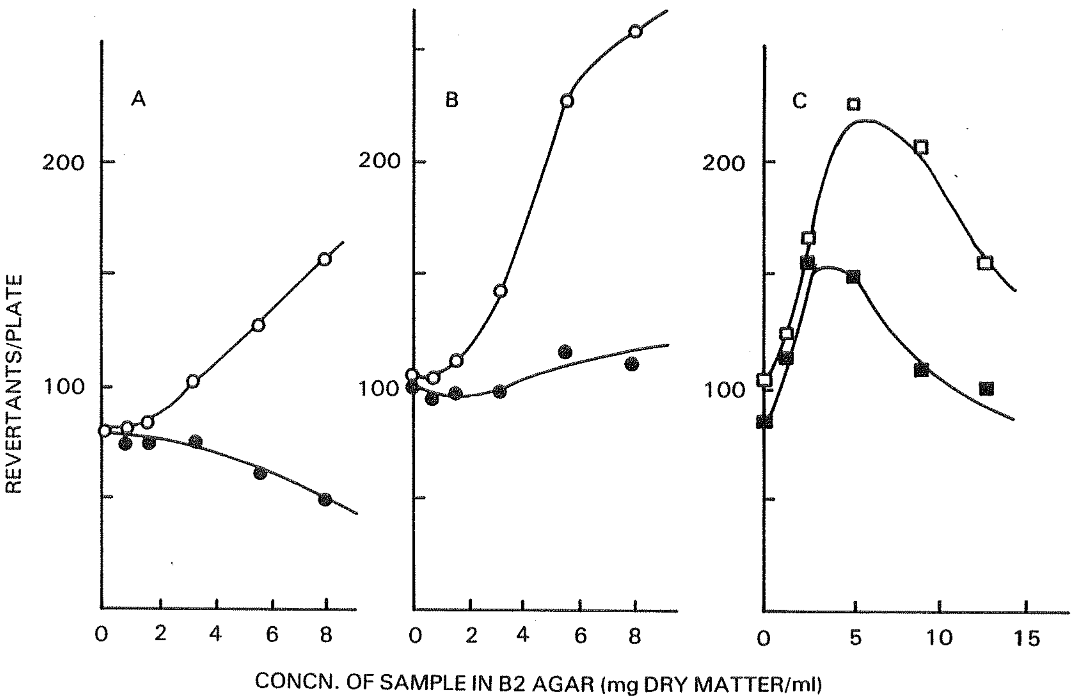

Fig. 8. Effects of Kemang Young-Leaf Extracts on the Mutagenicity of Capsicum Pepper Oleoresin with SD1018.

A: red pepper, after 1-day incubation; B; red pepper, after 2-day incubation; C: bird pepper, after 2-day incubation; Open circle: without kemang leaf extracts; Closed circle: with equal amount of kemang extracts (13.1 mg dry matter/ml B2 agar); Test method: SA.

DISCUSSION

In the mutagenicity tests, the feasibility of SM^{d} tester strains with samples containing histidine such as foods was demonstrated by Kada *et al.*[12]. Employing SM^{d} strains derived from TA98 and TA100 in this laboratory, a mutagenicity screening of some tropical spice matterials was conducted without metabolic activation of samples. Oleoresins from capsicum peppers and shallot were found to display very weak but yet obvious mutagenic activities and those from nutmeg fruits appreciable activities of the mutagenicity in the $SM^{d} \rightarrow SM^{ind}$ system. The former spices are widely used among inhabitants in tropical areas. The latter nutmeg components are of the appreciable mutagenic activities and involve various pharmacological substances, although their usual intake is not so much. It would be useful to elucidate the possible chronic effects of the long-term intake of these spices commonly used in tropical areas. Some of them might be linking with their mutagenicity, e.g. cancer induction and aging.

Nutmeg is one of the most popular spices, especially for cooking and processing meats. Nutmeg fruit flesh is locally used to make a candied fruits called "manisan pala" in Indonesia. In this study, oleoresins were prepared from fruit flesh, mace and kernel of nutmeg fruits and all of them were found to display appreciable mutagenic activities except the oleoresin from dried and stored nutmeg seeds. Nutmeg and mace are known to contain myristicin, myristicol, eugenol and so on. In respect of the mutagenic activity of nutmeg, Rockwell and Raw[14] described that nutmeg extracts are mutagenic, while Buchanan *et al.*[15] found that neither nutmeg oleoresin nor myristicin preparation was mutagenic in *Salmonella*/mammalian microsome mutagenicity assay employing his^{-} tester strains. However, Buchanan *et al.*[15] also mentioned that the interpretation of their negative data for myristicin is difficult, considering the similarlity of the chemical structure and toxicological characteristics of myristicin to those of a hepatocarcinogen, safrole[3,17].

Capsicum pepper contains capsaicin which is its major pungent principle. Buchanan *et al.*[15] also reported none of chili pepper oleoresin and capsaicin revealed significant *in vitro* mutagenicity in *Salmonella*/mammalian microsome mutagenicity assay. However, it should be noted that nonmutagenicity *in vitro* in some certain assay system does mean neither nonmutagenicity in other assay systems nor noncarcinogenicity. In addition, spice oleoresins generally contain various biologically active principles and this chemical characteristics lead us to the difficulty in drawing the definite conclusion on their mutagenic and carcinogenic potentialities. As for the mutagenicity and carcinogenicity of red pepper, the production of liver tumors in rats fed capsicum peppers was first reported by Hoch-Ligetti[1,2]. Later, the findings of Hoch-Ligetti was interpreted by other authors that the test diet used might be contaminated by aflatoxins, a highly potential hepatocarcinogen group and capsicum peppers

enhanced the carcinogenic effect of aflatoxins and thus that capsicum peppers behaved as a co-carcinogen[4,16]. In this study, the tests using SM^d strains indicated the dose-response mutagenic effects with nutmeg fruit and capsicum pepper oleoresins. Therefore, further studies on mutagenic and antimutagenic factors involved in these spices and their interaction would be required. It should be also worthwhile to mention that a leaf extracts prepared from kemang leaves obviously reduced the mutagenic effects of capsicum peppers and the present result may suggest the possibility to find out more anti-mutagenic food materials.

Acknowledgements.

The authors thank Dr. T. Kada of the National Institute of Genetics, Japan, for his helpful advices and kind supply of strains and reagents. They are indebted to Prof. B.N. Ames and his associates of California University, U.S.A., for the strains and references and also to Dr. K. Honma of Hokkaido National Agricultural Experiment Station, Japan, for his discussion on terminology of spice samples. The work was supported by a grant from Japan International Cooperation Agency.

REFERENCES

1. C. Hoch-Ligetti (1951). Acta Unio Intern. Contra Le Cancrum, *7,* 606.
2. C. Hoch-Ligetti (1952). Texas Rept. Siol. Med., *19,* 996.
3. I.K. Adamuya (1971). Acad. Nank. Gruz., S.S.R., Soobseheida, *65,* 237.
4. T. Osawa, H. Ishibashi, M. Yamanaka, M. Namiki, M. Yamanaka and K. Namiki (1981). Mutat. Res., *91,* 291.
5. J.M. Concon, T.W. Swerczek and D.S. Newburg (1981). In "Antinutrients and Natural Toxicants in Foods", ed. by R.L. Ory, Food & Nutrition Press, Inc., Westport, Connect., U.S.A., p. 359.
6. M. Ungsurungsie, O. Suthienkul and C. Paovalo (1982). Fd. Chem. Toxic., *20,* 527.
7. H. Nakamura and T. Yamamoto (1982). Mutat. Res., *103,* 119.
8. B.N. Ames, W.E. Durston, E. Yamasaki and F.D. Lee (1973). Proc. Natl. Acad. Sci. (U.S.A.), *70,* 2281.
9. J. McCann, E. Choi, E. Yamasaki and B.N. Ames (1975). *ibid., 72,* 5135.
10. T. Kawachi, T. Yahagi, T. Kada, Y. Tajima, M. Ishidate, M. Sasaki and T. Sugiyama (1980). "Molecular and Cellular Aspects of Carcinogen Screening Tests" by R. Montesano, H. Bartsch and L. Tomatis, IARC Scientific Publications No. *27,* Lyon, p. 323.
11. T. Sugimura (1981). Abstracts of 3rd Intern. Conf. on Environmental Mutagens, p. 7.
12. T. Kada, K. Aoki and T. Sugimura (1983). Environmental Mutagenesis, *5,* 9.
13. D.M. Maron and B.N. Ames (1983). Mutat. Res., *113,* 173.
14. P. Rockwell and I. Raw (1979). Nutr. Cancer, *1,* 10.
15. R.L. Buchanan, S. Goldstein and J.D. Budroe (1981). J. Food Sci., *47,* 330.
16. J.D. Hendricks, R.O. Sinnhuber, P.M. Loveland, N.E. Pawlowski and J.E. Nixon (1980). Science, *208,* 309.
17. V.L. Singleton and F.H. Kratzer (1969). J. Agric. Food Chem., *17,* 497.