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นิพนธ์ต้นฉบับ

ผลของสีที่ได้จากเชื้อรา *Monascus* ต่อฤทธิ์ก่อกลายพันธุ์ของสารที่เกิดจากปฏิกิริยาระหว่างอะมิโนไพรีนกับไนไตรท์ ซึ่งศึกษาด้วยวิธีเอมส์เทสต์

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บทคัดย่อ

ได้ทำการศึกษาฤทธิ์ต้านการก่อกลายพันธุ์ของสีธรรมชาติที่สร้างจากเชื้อรา *Monascus purpureus* สายพันธุ์ ATCC16365 และสายพันธุ์ NRRL2897 หรือสร้างจาก *Monascus* ที่ยังไม่มีการจำแนกสายพันธุ์ (KB13.1, KB10M16 และ KB20M10.2) ต่อผลิตภัณฑ์ที่ได้หลังจากการทำปฏิกิริยากันระหว่างโซเดียมไนไตรท์กับอะมิโนไพรีน โดยใช้วิธีเอมส์เทสต์ด้วยระบบที่ไม่มีเอนไซม์กระตุ้นความเป็นพิษทั้งแบบ frame-shift (ตรวจสอบด้วยสายพันธุ์ *Salmonella typhimurium* TA98) และ base-pair substitution (ตรวจสอบด้วยสายพันธุ์ *Salmonella typhimurium* TA100) ผลการศึกษาพบว่าในระดับความเข้มข้นสูงของแต่ละสีมีฤทธิ์ต้านการก่อกลายพันธุ์ในระดับสูง (มากกว่าร้อยละ 60) การศึกษาที่สองได้ทำการศึกษาผลของสีในการต้านการเกิดสารก่อกลายพันธุ์ โดยทำการเติมแต่ละสีลงในปฏิกิริยาระหว่างโซเดียมไนไตรท์และอะมิโนไพรีน ที่ปรับให้อยู่ในช่วง pH 3.0-3.5 คล้ายกับในกระเพาะอาหาร และบ่มที่อุณหภูมิ 37 องศาเซลเซียส นาน 4 ชั่วโมง จากการทดสอบดังกล่าวพบว่าสีที่ได้จาก *Monascus* ที่ยังไม่มีการจำแนกสายพันธุ์สามารถยับยั้งการเกิดสารก่อกลายพันธุ์ในปฏิกิริยาของไนไตรท์และอะมิโนไพรีน ในขณะที่การเติมสีที่ได้จาก *Monascus purpureus* ATCC 16365 และ NRRL 2897 ลงไปร่วมระหว่างการทำปฏิกิริยาของโซเดียมไนไตรท์และอะมิโนไพรีน ทำให้เกิดสารก่อกลายพันธุ์มากขึ้น อย่างไรก็ตามเมื่อนำแต่ละสีบ่มรวมกับสารตั้งต้นของสารก่อกลายพันธุ์ไนไตรท์ หรืออะมิโนไพรีนเป็นเวลา 1 ชั่วโมงก่อนที่จะเริ่มปฏิกิริยา พบว่าฤทธิ์การก่อกลายพันธุ์ที่เกิดขึ้นไม่ต่างกัน ซึ่งบ่งชี้ว่าสีไม่มีผลในการทำปฏิกิริยากับสารตั้งต้นของสารก่อกลายพันธุ์ สรุปแล้วกล่าวได้ว่าการยับยั้งการก่อกลายพันธุ์ที่เกิดขึ้นน่าจะเกิดเนื่องจากสีทำปฏิกิริยากับผลิตภัณฑ์สุดท้ายของปฏิกิริยา ซึ่งยังไม่ทราบถึงกลไกที่แน่ชัด และควรมีการศึกษา เช่น ปฏิกิริยาระหว่าง nitroreductase และสีที่สร้างจาก *Monascus* ว่ามีผลอย่างไรต่อการทำงานของเอนไซม์ในแบคทีเรีย

กุญแจคำ

สีจากเชื้อรา *Monascus*, ฤทธิ์ต้านการก่อกลายพันธุ์, อะมิโนไพรีน, โซเดียมไนไตรท์, เอมส์เทสต์

Original Article

Effects of *Monascus* Colorants on the Mutagenicity of Nitrite-Treated 1-Aminopyrene Using Ames Test

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Abstract

We studied the antimutagenicity of natural colorants synthesized by *Monascus* purpureus (ATCC 16365, NRRL 2897) and unidentified *Monascus* sp. (KB13.1, KB10M16 and KB20M10.2) against the reaction product (nitrite-treated 1-aminopyrene) produced from the reaction of sodium nitrite and 1-aminopyrene in Ames test on *Salmonella typhimurium* to detect frame-shift type mutation using TA98 and base-pair substitution type mutation using TA100 in the absence of metabolic activation. The results showed that high concentrations of the colorants had strong antimutagenicity (>60% inhibition) on the nitrite-treated 1-aminopyrene. The second study was designed to evaluate the effect of each colorant on the formation of mutagen by adding each colorant to the reaction mixture of sodium nitrite and 1-aminopyrene and started the incubation at 37°C for 4 h. The pH was adjusted to simulate the gastric digestion of pH 3.0-3.5. The colorants from unidentified *Monascus* sp. seemed to inhibit the formation of mutagens in the reaction of sodium nitrite and 1-aminopyrene. Interestingly, those from *M. purpureus* (ATCC 16365 and NRRL 2897) promoted the formation of mutagens. However, when each colorant was incubated with either mutagen precursor (nitrite or 1-aminopyrene) for 1 h before starting the reaction, there was no difference, indicating no interaction between each colorant and the precursors. Thus, it suggested that the inhibition of mutagenicity was due to the action of the colorant on the final reaction products of the reaction mixture. In conclusion, the mechanism of this effect is unknown and required further study e.g. on the interaction between nitroreductase and each colorant whether it has any effect on the bacterial enzyme activities.

Key words

Monascus colorants, Antimutagenicity, Aminopyrene, Sodium nitrite, Ames test

Introduction

Monascus pigments that are produced by various species of *Monascus* have been used as natural colorants and as traditional natural food additives in East Asia (1). They reduced serum triglyceride, LDL-cholesterol and total cholesterol (2). Red rice powder made from *M. anka* significantly inhibited acetaminophen-induced toxicity to the liver (hepatoprotective activity) of male rats (3). The mold *M. anka*, used for the fermentation of foods and beverages, expressed

strong antioxidant action (4). The *Monascus* sp. also has bacteriostatic effect on many micro-organism (5). *Monascus* colorants, both red and yellow, inhibited the mutagenicity of Trp-P-2(NHOH) (3-hydroxyamino-1-methyl-5H-pyrido [4,8-*b*] indole (6).

The colorant has been considered as a suitable substitute for conventional additives such as E-249 (nitrite salts) and E-252 potassium nitrate. Furthermore, the capability of the colorants to inhibit direct acting mutagens of nitro compounds is an interesting area of investigation. The objective of this study was to investigate the antimutagenic activity and anti-mutagen formation of the colorant extracts produced by *Monascus* sp., namely *M. purpureus* ATCC 16365, *M. purpureus* NRRL 2897, *Monascus* sp. KB13.1, *Monascus* sp. KB10M16 and *Monascus* sp. KB20M10.2 on the direct acting mutagen produced from nitrating reactions between sodium nitrite and 1-amino-pyrene. Antimutagenicity was determined using the pre-incubation method of Ames *Salmonella* assay without metabolic activation.

Materials and Methods

Chemicals

1-Aminopyrene was purchased from Aldrich (St. Louis, U.S.A.). D-Biotin, ammonium sulfamate were obtained from Sigma Chemical Company (St. Louis, Missouri, U.S.A.) L-Histidine monohydrochloride, dimethylsulfoxide and Bacto agar were supplied by E. Merck (Darmstadt, Germany). Oxoid nutrient broth No. 2 was bought from Oxoid Ltd., (Basingstoke, Hants, England). Sodium nitrite was purchased from BDH Chemicals Ltd., (Poole, England). Other chemicals were of laboratory grade.

Monascus *colorants*

The colorants synthesized from *Monascus* sp., namely *Monascus purpureus* ATCC 16365 (red color), *Monascus purpureus* NRRL 2897 (red color), *Monascus* sp. KB13.1 (red color), *Monascus* sp. KB10M16 (red color) and *Monascus* sp. KB20M10.2 (yellow color) were used throughout this study. Briefly, each *Monascus* sp. was inoculated on moistened rice in Erlenmeyer flasks for 15 days at 30-32°C (7). The fermented rice was extracted with 70% ethanol (20 g of dried sample with 100 ml ethanol) in a rotary shaker (300 rpm) for 9 h. The extract was centrifuged at high speed for 10 min (15°C) to obtain a clear supernatant and it was evaporated under vacuum (30°C). Finally, it was lyophilized. The dried material from each *Monascus* sp. was powdered. However, the colorants from *M. purpureus* NRRL 2897 and ATCC 16365 were sticky mass after lyophilization and used as they were. Various concentrations of each colorant from *Monascus* were prepared by dissolving them in dimethylsulfoxide before each study.

Preparation of standard mutagen

10 µl (tested on TA98) or 40 µl (tested on TA100) of 1-aminopyrene (0.0375 mg/ml) was mixed with 610-640 µl of 0.2N hydrochloric acid (sufficient to acidify the reaction mixture to pH 3.0-3.5). Then, 250 µl of 2M sodium nitrite was added. The final volume of the reaction mixture was 1,000 µl. Each reaction mixture was shaken at 37°C for 4 h and was placed in an ice bath to stop the reaction. Then, 250 µl of 2M ammonium sulfamate was added to the reaction mixture to remove excess sodium nitrite. The reaction product was shown to give direct acting mutagenicity in the Ames test (8).

Mutagenicity assay

The pre-incubation method of Ames test described by Yahagi *et al*. (9) was used to determine the mutagenicity of each sample throughout this study. Each colorant was mixed with 500 µl of 0.5M trisodium phosphate-potassium chloride buffer (pH 7.4) and 100 µl of each tester strain. The mixture was incubated at 37°C for 20 min. After incubation, molten top agar (2 ml) containing 5 mM L-histidine and 5 mM D-biotin (45°C) was added. The contents of the tube were mixed well and poured onto a minimal agar plate. The plate was evenly distributed by rotating and incubated at 37°C for 48 h before the numbers of histidine revertant colonies were determined. Each concentration was assayed in triplicate and done twice. Dimethylsulfoxide was used as the negative control. The non-toxic concentrations of colorants were those with no difference in the

number of spontaneous revertants, size of the colonies, and intensity of the background lawn compared with that of the negative control.

Effect of **Monascus** *colorants on mutagenicity of nitrite-treated 1-aminopyrene (direct-acting mutagens)*

Each tester strain (100 µl) was added to the test tube containing 500 µl of 0.5M trisodium phosphate-potassium chloride buffer (pH 7.4), 100 µl of each colorant that gave the appropriate concentrations depending on its solubility (as indicated in Table 2) and 100 µl of standard mutagen (products from 1-aminopyrene-nitrite reaction). The mixture was determined for its mutagenicity.

Effect of **Monascus** *colorants during formation of mutagen*

The effect of each colorant on the formation of mutagen occurring during the reaction of sodium nitrite and 1-aminopyrene was investigated. Each concentration of a colorant was added to the tube containing 10 µl (for TA98) or 40 µl (for TA100) of 1-aminopyrene (0.0375 mg/ml), 610-640 µl of 0.2N hydrochloric acid (sufficient to acidify the reaction mixture to pH 3.0-3.4), and 250 µl of 2M sodium nitrite. Each reaction tube was incubated at 37°C for 4 h in a shaking water bath. Then, 250 µl of 2M ammonium sulfamate was added to each reaction mixture. The tube was left in an ice bath for 10 min and the reaction mixture (100 µl) was determined for its mutagenicity.

The experiment further investigated the scavenging activity of each colorant on mutagen precursors during the formation of direct-acting mutagen in gastric-like pH. The colorants obtained from KB10M16 and KB20M10.2 that had high percent inhibitory effect on the formation of direct mutagens were selected to elucidate their role during mutagen formation. Three types of study were designed.

In study 1, 100 µl of KB10M16 that gave final 1,500 µg/plate or KB20M10.2 that gave final 2,000 µg/plate of the colorant was added to the tube containing 10 µl (with TA98), or 40 µl (with TA100) of 1-aminopyrene (0.0375 mg/ml), a volume of 0.2N hydrochloric acid sufficient to acidify the reaction mixture to pH 3.0-3.4, and 250 µl of 2M sodium nitrite. In study 2, 100 µl of colorant was pre-incubated with 1-aminopyrene (10 µl for testing on TA98 or 40 µl for testing on TA100) for 1 h in a shaking water bath; then, the mixture was added with 250 µl of 2M sodium nitrite and a volume of 0.2N hydrochloric acid sufficient to acidify the reaction mixture to pH 3.0-3.4. In study 3, colorant was pre-incubated with 2M sodium nitrite for 1 h in a shaking water bath. Then the reaction mixture was added with 1-aminopyrene (10 µl for testing on TA98 or 40 µl for testing on TA100) and a volume of 0.2N hydrochloric acid sufficient to acidify the reaction mixture to pH 3.0-3.4. The final volume of each reaction tube was 1,000 µl. The reaction tubes of all studies were incubated at 37°C for another 3 h in a shaking water bath. Then, they were immersed in an ice bath for 1 min. A 250 µl aliquot of 2M ammonium sulfamate was added into the reaction tube and the tube was placed in an ice bath for 10 min. Each reaction mixture (100 µl) was determined for its mutagenicity.

Data evaluation

Effects of each colorant on mutagenicity of standard direct mutagen and formation of standard direct mutagen were determined. Percentage of modulation of each colorant (either increase or decrease on mutagenicity of standard direct mutagen) with regular background lawn is calculated as follows:

$$\text{Percentage of modulation} = (A-B)/(A-C) \times 100$$

Where A is the number of histidine revertants induced by nitrite-treated 1-aminopyrene, B is the number of histidine revertants induced by nitrite-treated 1-aminopyrene in the presence of each colorant and C is the number of histidine spontaneous revertants induced in the presence of dimethylsulfoxide alone (negative control). The modulation is considered either strong, moderate or weak when its effect is higher than 60%, 40-60% or 20-40%, respectively, and no effect is affirmed when the change was less than 20% (10).

Results

Mutagenicity of Monascus *colorants*

The results show that almost all the *Monascus* colorants (unidentified *Monascus* sp. or *M. purpureus*) were not mutagenic on both *S. typhimurium* strains TA98 and TA100 (data not shown). Only the highest concentration (15,000 (g/plate) of M. purpureus NRRL 2897 and ATCC 16365 were mutagenic. It also shows that after interacting with excess nitrite in acid solution (pH 3.0-3.5), all colorants turned to be mutagenic (Table 1).

Table 1. Mutagenicity of nitrite-treated Monascus colorants on S. typhimurium strains TA98 and TA100

Source of colorants	Amount * ((g/plate)	Number of His^+ -revertants/plate [a]	
		TA98	**TA100**
Unidentified *Monascus*			
KB13.1	0 [b]	19 ± 1	92 ± 26
	100	20 ± 2	116 ± 18
	500	45 ± 6	288 ± 23
	1000	60 ± 5	295 ± 19
	1500	52 ± 7	343 ± 49
KB10M16	0 [b]	20 ± 2	109 ± 17
	100	21 ± 3	172 ± 16
	500	35 ± 13	250 ± 40
	1000	44 ± 11	351 ± 32
	1500	52 ± 8	367 ± 50
KB20M10.2	0 [b]	20 ± 2	109 ± 17
	500	30 ± 4	158 ± 18
	1000	42 ± 8	206 ± 20
	1500	48 ± 9	275 ± 36
	2000	68 ± 9	222 ± 11
Monascus purpureus			
NRRL 2897	0 [b]	13 ± 2	116 ± 13
	500	73 ± 11	132 ± 27
	1500	168 ± 31	376 ± 38
	2000	198 ± 14	455 ± 52
	4000	266 ± 20	524 ± 66
	6000	PK	567 ± 51
ATCC 16365	0 [b]	13 ± 2	116 ± 13
	500	35 ± 5	152 ± 14
	1500	55 ± 11	270 ± 28
	2000	92 ± 8	384 ± 29
	4000	147 ± 18	400 ± 82
	6000	PK	PK

* Amount/plate of *Monascus* colorants in DMSO.

[a] Value is mean ± S.D. of His^+-revertants/plate of two independent experiments (n=6).

[b] Used as a negative control to produce spontaneous revertants/plate

PK = Partial killing effect

Table 2. Inhibitory effect of *Monascus* colorants on the mutagenicity of nitrite-treated 1-aminopyrene on *S. typhimurium* strains TA98 and TA100

Source of colorants	Amount (µg/plate)	TA98 [a]		TA100 [a]	
		Revertants/ plate	Percent inhibition	Revertants/ plate	Percent inhibition
Unidentified *Monascus*					
KB13.1	0 [b]	1513 ± 65		1455 ± 59	
	1	1019 ± 62	33	948 ± 56	35
	10	540 ± 103	64	616 ± 27	58
	100	180 ± 24	88	359 ± 32	75
	1000	37 ± 10	98	36 ± 36	98
	5000	14 ± 11	99	2 ± 9	100
KB10M16	0 [b]	1136 ± 95		1455 ± 59	
	1	485 ± 41	57	758 ± 40	48
	10	414 ± 54	64	410 ± 7	72
	100	136 ± 19	88	289 ± 27	80
	1000	46 ± 2	96	37 ± 14	98
	2500	21 ± 8	98	0 ± 8	100
KB20M10.2	0 [b]	998 ± 75		1415 ± 59	
	1	492 ± 31	51	835 ± 61	41
	10	301 ± 43	70	536 ± 54	62
	100	179 ± 14	82	371 ± 33	74
	1000	140 ± 34	86	340 ± 31	76
	5000	76 ± 13	92	200 ± 62	86
	10000	102 ± 20	90	53 ± 10	96
M. purpureus					
NRRL 2897	0 [b]	929 ± 52		883 ± 64	
	200	781 ± 20	16	604 ± 77	32
	1000	546 ± 43	41	497 ± 56	44
	5000	443 ± 26	52	367 ± 31	58
	15000	268 ± 40	72	276 ± 24	69
ATCC 16365	0 [b]	929 ± 52		883 ± 64	
	200	846 ± 60	9	702 ± 78	21
	1000	769 ± 25	17	633 ± 40	28
	5000	410 ± 30	56	462 ± 65	48
	15000	335 ± 40	64	295 ± 44	67

[a] Mean ± S.D. of His^+-revertants/plate of two independent experiments conducted each in triplicate; spontaneous revertants have already been subtracted from the raw data

[b] Positive control (only revertants/plate induced nitrite treated 1-aminopyrene)

Effect of **Monascus** *colorants on the mutagenicity of nitrite-treated 1-aminopyrene*

All colorants, at the highest testing concentration, strongly inhibited (>60% inhibition) the mutagenicity of nitrite-treated 1-aminopyrene on both *S. typhimurium* strains (Table 2). The colorants from unidentified *Monascus* sp. (KB13.1, KB10M16, KB20M10.2) gave strong inhibition at the amount of 10 μg/plate or higher, while those from *M. purpureus* (ATCC 16365 and NRRL 2897) strongly inhibited the nitrite-treated 1-aminopyrene only at the amount equal to 15,000 μg/plate.

Role of **Monascus** *colorants during the formation of direct-acting mutagens (nitrite-treated 1-aminopyrene)*

The effect of colorants on the formation of direct-acting mutagenic product from the reaction between 1-aminopyrene and sodium nitrite is shown in Table 3. The result showed that colorants from unidentified *Monascus* sp. (KB13.1, KB10M16 and KB20M10.2) moderately or strongly inhibited the formation of frame-shift type mutagen at concentration of 1,500 μg/plate; whereas they expressed their moderate antimutagenic activity on the formation of base-pair substitution type mutagen at the same concentrations. On the other hand, the *M. purpureus* NRRL 2897 and *M. purpureus* ATCC 16365 colorants, which had sticky characteristic, promoted the formation of the direct mutagens.

The colorants from KB10M16 (1,500 μg/plate) and KB20M10.2 (2,000 μg/plate) that gave the highest antimutagenicity on the formation of mutagen were selected to explore the mechanism of inhibition of mutagen formation. As shown in Table 4, pre-incubation of each colorant with a mutagen precursor (1-aminopyrene) of the study 2 or nitrite of the study 3 showed similar inhibitory effect as that of the study 1. It suggests that the colorants did not have any inhibitory effect on the formation of direct mutagens during the nitrite treatment. The reduction of mutagenicity was rather the antimutagenicity against the product readily formed from the reaction. However, when the data in Tables 1 and 3 were constructed as Table 5 (effect of colorant from *Monascus* sp.), modulating effects (an antagonistic effect or a potentiating effect) can be seen.

Discussion

The colorants presently studied were separated into two groups, namely, from *Monascus* sp. (KB13.1, KB10M16 and KB20M10.2) and the others from *M. purpureus* (NRRL2897 and ATCC16365). Being lyophilized, the colorants of the first group were ground to fine powder, while those of the second group had a sticky mass characteristic. The distinct characteristic (powder or sticky mass) of colorants may be due to the difference in the characteristic of fungal amylase activity on hydrolyzing rice meal in the process of dye production.

Monascus colorants are safe, as they have been consumed for a long time as natural food colorants in oriental countries. The annual consumption of *Monascus* pigments in Japan alone was up to 600 tons (10). Kaio and co-workers (11) revealed that the *Monascus* colorants were nontoxic to animal. All the colorants from *Monascus* sp. showed no mutagenicity toward *Salmonella* tester strains without metabolic activation. It was noted that the highest concentration (15,000 μg/plate) of the colorant either from *M. purpureus* NRRL 2897 or ATCC 16365 slightly increased the number of revertants/plate above spontaneous reversion but it is still classified as non-mutagen. This confirmed the studies of Izawa *et al*. (6) and of Sabater-Vilar *et al*. (12) which found that they were not mutagenic in the majority of Ames tester strains with and without metabolic activation.

Demonstrating their direct mutagenicity after they were treated with sodium nitrite (500 mM) under acidic solution suggested that the colorants contained certain convertible compounds that can react with nitrite to produce mutagenic products causing frame-shift (TA98) and base-pair substitution (TA100) mutations. This finding may be common since most natural compounds generally reacted with nitrite and expressed their

Table 3. Modulating effects of *Monascus* colorants on the formation of direct mutagen between 1-aminopyrene and sodium nitrite

Source of colorants	Amount (µg/plate)	TA98 [a]		TA100 [a]	
		Revertants/ plate	Percent modulation [b]	Revertants/ plate	Percent modulation [b]
Unidentified *Monascus*					
KB13.1	0 [c]	402 ± 30		985 ± 91	
	100	196 ± 7	-51	1108 ± 119	+12
	500	264 ± 30	-34	863 ± 59	-12
	1000	164 ± 30	-59	642 ± 48	-35
	1500	134 ± 11	-67	526 ± 85	-47
KB10M16	0 [c]	363 ± 10		914 ± 45	
	100	170 ± 16	-53	816 ± 76	-11
	500	153 ± 22	-58	504 ± 43	-45
	1000	132 ± 17	-64	440 ± 57	-52
	1500	109 ± 14	-70	396 ± 75	-53
KB20M10.2	0 [c]	382 ± 38		917 ± 70	
	500	216 ± 32	-44	598 ± 53	-35
	1000	190 ± 5	-50	369 ± 42	-60
	1500	167 ± 13	-56	411 ± 15	-55
	2000	138 ± 15	-64	174 ± 27	-81
	5000	76 ± 13	-92	200 ± 62	-86
	10000	102 ± 20	-90	53 ± 10	-96
M. purpureus					
NRRL 2897	0 [c]	690 ± 52		1026 ± 42	
	500	1100 ± 43	+59.42	930 ± 52	-9.36
	1500	881 ± 43	+27.68	981 ± 81	-4.39
	2000	908 ± 62	+31.59	1083 ± 21	+5.55
	4000	875 ± 78	+26.81	902 ± 87	-2.09
	6000	PK		PK	
ATCC 16365	0 [c]	690 ± 52		1026 ± 42	
	500	919 ± 57	+33.18	1134 ± 78	+10.52
	1500	1076 ± 77	+55.94	1144 ± 81	+11.50
	2000	711 ± 63	+3.04	1086 ± 44	+5.84
	4000	621 ± 57	-10	1182 ± 56	+15.20
	6000	PK		PK	

PK = Partial killing effect

[a] Mean ± S.D. of His^+-revertants/plate of two independent experiments conducted each in triplicate; spontaneous revertants have already been subtracted from the raw data

[b] + or – indicates that the colorant increased or decreased the mutagenicity of the positive control, respectively.

[c] Positive control (only revertants/plate induced nitrite treated 1-aminopyrene)

Table 4. Determination of the role of *Monascus* KB10M16 (1,500 µg/plate) and KB20M10.2 colorant (2,000 µg/plate) as mutagen-precursor scavenger during the formation of direct-acting mutagen in gastric-like pH

Source of colorants	Study type	Calculated weight 1-AP (µg/plate)	*S. typhimurium*			
			TA98		TA100	
			Revertants/ Plate *	Percent inhibition	Revertants/ Plate *	Percent inhibition
KB10M16	-	0.03	481 ± 54		ND	
	1[a]	0.03	172 ± 14	67	ND	ND
	2[b]	0.03	174 ± 8	66	ND	ND
	3[c]	0.03	177 ± 12	66	ND	ND
	-	0.12	ND		1172 ± 48	
	1[a]	0.12	ND	ND	330 ± 18	83
	2[b]	0.12	ND	ND	327 ± 37	83
	3[c]	0.12	ND	ND	360 ± 50	80
KB20M10.2	-	0.03	455 ± 36		ND	
	1[a]	0.03	156 ± 16	68	ND	ND
	2[b]	0.03	148 ± 16	70	ND	ND
	3[c]	0.03	227 ± 21	52	ND	ND
	-	0.12	ND		1112 ± 58	
	1[a]	0.12	ND	ND	320 ± 22	81
	2[b]	0.12	ND	ND	313 ± 52	82
	3[c]	0.12	ND	ND	360 ± 39	77

ND = not determined
Spontaneous revertants were 18 ± 2 and 154 ± 9 for TA98 and TA100, respectively.
* Mean ± S.D. of six plates from two independent experiments.
[a] Colorant was added into the reaction mixture at time 0.
[b] Pre-incubating colorant with 1-aminopyrene for 1 h before adding sodium nitrite.
[c] Pre-incubating colorant with nitrite for 1 h before adding 1-aminopyrene.
1-AP = 1-aminopyrene

mutagenicity. For instance, the extracts of raw and pickled vegetables and fruits, e.g. garlic, cabbage, shallot, mushroom, cucumber, ginger, Chinese mustard, bamboo shoot and mango when treated with nitrite in the absence of metabolic activation exhibited direct-acting mutagenicity in *Salmonella* assay (13).

The role of *Monascus* colorants as antimutagen seems to be more important than their effect on the formation of mutagens. This might be expected, since the colorants themselves also interacted with nitrite to form new mutagens as described previously. Therefore, incubating each colorant along with each precursor gave lesser inhibitory effect.

Pre-incubation of the colorant from *Monascus* sp. KB10M16 or KB20M10.2 with 1-aminopyrene (study 2 of antimutagen formation study) or nitrite (study 3 of antimutagen formation study) demonstrated no difference in the inhibitory effect, indicating that the colorants did not scavenge either 1-aminopyrene or nitrite. Thus, *Monascus* colorants might have no role in antimutagen formation during digestion in the stomach.

The results present possible advantage of *Monascus* colorants as inhibitors of nitrite-treated

Table 5. Modulating effect* occurred during the incubation of 1-aminopyrene (1-AP) at 0.03 or 0.12 µg/plate (when tested on TA98 or TA100, respectively), 0.5M sodium nitrite and each colorant

Nitrite-treated compound	Amount[a] (µg/plate)	*S. typhimurium* (revertants/plate)					
		TA98			TA100		
		A[b]	B[c]	C[d]	A[b]	B[c]	C[d]
1-AP[e]	-	-	402	-	-	985	-
KB13.1[f]	100	-	20	-	-	116	-
	500	-	45	-	-	288	-
	1000	-	60	-	-	295	-
	1500	-	52	-	-	343	-
1-AP + KB13.1	100	422	196	226	1101	1108	
	500	447	264	183	1273	863	-7
	1000	462	164	298	1280	642	410
	1500	454	134	320	1328	526	638
1-AP[e]	-	-	363	-	-	914	802
KB10M16[f]	100	-	21	-	-	172 ± 16	-
	500	-	35	-	-	250 ± 40	-
	1000	-	44	-	-	351 ± 32	-
	1500	-	52	-	-	367 ± 50	-
1-AP + KB10M16	100	384	170	214	1086	816	270
	500	398	153	245	1164	504	660
	1000	407	132	275	1265	440	825
	1500	415	109	306	1281	396	885
1-AP[e]		-	382	-	-	917	
KB20M10.2[f]	500	-	30	-	-	158 ± 18	-
	1000	-	42	-	-	206 ± 20	-
	1500	-	48	-	-	275 ± 36	-
	2000	-	68	-	-	222 ± 11	-
1-AP + KB20M10.2	500	412	216	196	1075	598	477
	1000	424	190	234	1123	369	754
	1500	430	167	263	1192	411	781
	2000	450	138	312	1139	174	965
1-AP[e]	-	-	690	-	-	1026	-
NRRL 2897[f]	500	-	73	-	-	132 ± 27	-
	1500	-	168	-	-	376 ± 38	-
	2000	-	198	-	-	455 ± 52	-
	4000	-	266	-	-	524 ± 66	-
1-AP + NRRL 2897	500	763	1100	-337	1158	930	228
	1500	858	881	-23	1402	981	421
	2000	888	908	-20	1481	1083	398
	4000	956	875	81	1550	902	648

Table 5. (Continued)

Nitrite-treated compound	Amount[a] (µg/plate)	*S. typhimurium* (revertants/plate)					
		TA98			TA100		
		A[b]	B[c]	C[d]	A[b]	B[c]	C[d]
1-AP[e]	-	-	690	-	-	1026	-
ATCC 16365[f]	500	-	35	-	-	152 ± 1[*]	-
	1500	-	55	-	-	270 ± 2	-
	2000	-	92	-	-	384 ± 29	-
	4000	-	147	-	-	400 ± 82	-
1-AP + ATCC 16365	500	725	919	-194	1178	1134	44
	1500	745	1076	-331	1296	1144	152
	2000	782	711	71	1410	1086	324
	4000	984	621	363	1426	1182	244

Spontaneous revertants/plate of TA98 and TA100 were 20 ± 2 and 109 ± 17, respectively.
* indicated by the number of actual revertants/plate which is lower than arithmetic summation of revertants/plate induced by nitrite-treated 1-aminopyrene and of revertants/plate induced by nitrite-treated colorant from *Monascus*
[a] amount/plate of *Monascus* colorants dissolved in dimethylsulfoxide
[b] summation of revertants/plate induced by nitrite-treated 1-aminopyrene (from Table 3) and revertants/plate induced by nitrite-treated colorant (from Table 1)
[c] actual experimental data from incubation of 1-AP, nitrite and each colorant
[d] difference between arithmetic summation of revertants/plate (A) and actual experimental data (B)
[e] nitrite-treated 1-aminopyrene (from Table 3)
[f] nitrite-treated colorant (from Table 1)

products that can induce mutations. The colorants inhibited the mutagenicity of the standard mutagen on both strains of *S. typhimurium*, suggesting that scavenging of the toxic compounds by the colorants might be the possible mechanism of inhibition. The crude colorants extracted from fermented rice may consist of some pigments, which could form extra-cellular complexes with standard direct-acting mutagens; therefore, they could limit the bioavailability of direct-acting mutagens to the bacterial DNA. Another proposed mechanism could be that the colorants activated the degradation of the toxic compounds. Izawa *et al.* (6) demonstrated that the antimuta-genicity of either pure *Monascus* red or *Monascus* yellow compound suppressed the mutagenicity of the synthesized Trp-P-2(NHOH), simulating the bacterial metabolic activated intermediate of Trp-P-2, and several activated heterocyclic amines in the *Salmonella* mutagenicity test. They reported that incubation of *Monascus* red or yellow pigments with the food mutagen Trp-P-2(NHOH) accelerated its spontaneous degradation as measured by HPLC and UV/visible spectrophotometer. In that experiment, *Monascus* red pigment was more than twice as active as *Monascus* yellow in accelerating the degradation of the carcinogen. The concentrations of the pigments remained unchanged during the course of the incubation (6). Watanabe *et al*. (14) reported that *Monascus* pigments catalytically acted for the degradation of Trp-P-2(NHOH) and IQ(NHOH). However, there has been no study on the effect of such colorants on the degradation of nitrite-treated 1-aminopyrene or other related compounds. Thus, intensive study on chemical structure elucidation, as well as optimi-zation for specific target compound containing fermented rice, should be carried out in the future.

Inhibition on bacterial enzymes for metabolic activation of nitro compounds may be another mechanism of inhibition. Nitro-polycyclic aromatic hydrocarbons that are the possible

products of nitration of 1-aminopyrene required bacterial intracellular metabolic conversion to express their mutagenesis toward bacterial DNA (15-16). Natural food colorants from *Monascus* might reduce mutagenicity of direct-acting mutagens by inhibition of bacterial activating enzymes, such as nitro-reductase and/or *O*-acetyltransferase. It was proposed that nitro compounds required reduction of the nitro group to a hydroxylamine intermediate and esterification of the OH function by several transferases, especially the *O*-acetyltransferase, to metabolites that spontaneously decomposed to an arylnitrenium ion, which initiated mutagenesis (17-18). Therefore, the inhibition of activating enzymes in the presence of *Monascus* colorants may also be the cause of the decrease in the number of histidine revertant colonies per plate induced by nitrite-treated 1-aminopyrene. Further experiment to prove this hypothesis is required.

The results present the possible benefit of colorants as inhibitors of mutagenicity induced by direct mutagen that occurs within gastric-like solution. In addition, the results of investigation on the interaction between 1-aminopyrene and nitrite in the presence of each colorant exhibited antagonistic or potentiating characteristics. This was a surprisingly unexpected effect because both 1-aminopyrene and each colorant could interact with nitrite and expressed their mutagenicity toward both *S. typhimurium* tester strains. The mechanism of this effect was unknown and required further study e.g. on the interaction between nitroreductase and 1-aminopyrene or nitroreductase and each colorant, whether it competitively inhibits or potentiates the bacterial enzyme activities.

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