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Traditional Japanese Fermented Foods Free from Mycotoxin Contamination

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Abstract

Miso (soybean paste), shoyu (soy sauce), sake (rice wine) and katsuo-bushi (dried bonito), which are traditional Japanese fermented foods, have been consumed for a long period of time and are considered to be the safest foods. However, fermented foods may be contaminated with mycotoxins. Fungi used for the fermentation of miso, shoyu, and sake consist of *Aspergillus oryzae* and *A. sojae*. Although *A. oryzae* and *A. sojae* belong to the *A. flavus* group, which is known to produce aflatoxins, none of the strains examined actually produced aflatoxins. Aflatoxin contamination of rice, miso, shoyu, sake and katsuo-bushi could not be detected. Aflatoxigenic fungi do not occur in areas with a mean temperature below 16ºC. Since the mean temperature in most areas of Japan is lower than 16ºC, it appears that food contamination with aflatoxigenic strains does not occur in most parts of Japan. Some koji molds are known to produce kojic acid (KA) and cyclopiazonic acid (CA). Production of KA and CA was examined and the producing strains were eliminated from the commercial fermented foods. The fate of KA and CA in shoyu fermentation was also examined and it became clear that the contents of KA and CA decreased during shoyu fermentation. Contamination of katsuo-bushi with aflatoxins, sterigmatocystin and ochratoxin A could not be detected. As mentioned previously, mycotoxin contamination, which is presently very low, should be completely eliminated in future.

Discipline: Food / Postharvest technology
Additional key words: aflatoxins, kojic acid, cyclopiazonic acid, sterigmatocystin, ochratoxin A

Aflatoxins

Miso (soybean paste), shoyu (soy sauce) and sake (rice wine) which are traditional Japanese fermented foods have been consumed for a long period of time, and are considered to be the safest foods. However, the discovery of aflatoxins became a cause for concern for the producers of traditional Japanese fermented foods. *Aspergillus flavus* and *A. parasiticus,* which are known to produce aflatoxins, belong to the *A. flavus* group. Koji mold is considered to be composed of *A. oryzae* and *A. sojae*. Koji mold involved in the first step of fermentation is also classified into this group. Aflatoxins exhibit a strong acute toxicity and the strongest carcinogenicity found in the natural world to animals. Koji mold first and then the other microorganisms are involved in the fermentation process. In order to examine the production of aflatoxins by koji mold strains practically used in the Japanese fermentation companies, we collected commercially used koji mold. A total of 212 strains of koji mold were tested for their aflatoxin production. Though substances similar to aflatoxins were observed on the TLC (thin layer chromatography) plate under UV light, it was found that none of them produced aflatoxins, as shown in Table 1 based on the analysis of the UV spectra.

Rice is a staple food in Japan and also a raw material of traditional Japanese fermented foods. The possibility

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Table 1. Production of aflatoxin-like substances by koji molds used for various purposes

Strains	Production of aflatoxins	Production of aflatoxin-like substances						Number of strains
		+		±		–		
For miso	0	6	(18%)	4	(12%)	23	(70%)	33
For shoyu	0	14	(29%)	7	(15%)	27	(56%)	48
For sake	0	4	(22%)	0	(0%)	14	(78%)	18
For tamari shoyu	0	3	(50%)	0	(0%)	3	(50%)	6
For shochu	0	1	(50%)	0	(0%)	1	(50%)	2
For hon-koji	0	0	(0%)	0	(0%)	1	(100%)	1
Others	0	21	(20%)	11	(11%)	72	(69%)	104
Total	0	49		22		141		212
%	0	23		10		67		100

Aflatoxins were analyzed by TLC first. Then aflatoxin-like substances that were observed under UV light could not be identified as aflatoxins based on UV spectra.

of contamination with aflatoxins was examined. Ninety-eight rice samples produced in Japan in 1966 and 1967 were analyzed at a rate of 2 samples from each prefecture. Two rice samples from Burma (Myanmar), 1 rice sample from Thailand, 3 samples from USA, 3 rice samples from the People's Republic of China, 1 rice sample from Taiwan, and 1 rice sample from Spain imported between 1966 and 1967 were also analyzed. Aflatoxins were not detected from any of these samples.

One hundred and eight commercial miso samples, 33 home-made miso samples and 28 koji (molded rice inoculated with koji mold) samples were also analyzed. Aflatoxins were not detected from any of these samples. Therefore, it appears that miso is free from aflatoxin contamination. The decomposition of aflatoxins in miso was studied by spiking aflatoxins before fermentation. Although aflatoxins B_1 and G_1 were decomposed at the primary stage of fermentation, the contents of most of aflatoxins B_2 and G_2 did not decrease. These results indicate that, in order to prevent aflatoxin contamination of miso, it is necessary to use aflatoxin-free raw materials and to prevent the koji mold from becoming contaminated with aflatoxin-producing fungi.

Aflatoxin contamination of shoyu was also examined[4]. Thirty-nine shoyu samples were collected from all parts of the country and analyzed. No aflatoxins were detected from these samples. Therefore, it was concluded that shoyu samples were not contaminated with aflatoxins.

The geographical distribution of the fungi that produce aflatoxins in soil in Southeast Asia and Japan was also studied[2,3]. It appeared that the frequency of isolation of aflatoxin-producing fungi in soil increased from the subtropical zone to the tropical zone as shown in Fig. 1. No aflatoxigenic strains were isolated from areas where the mean temperature was lower than 16ºC. Therefore, it was concluded that in most parts of the Japanese islands *A. flavus* and *A. parasiticus* do not occur.

Kojic acid

Kojic acid is known to be produced by *A. oryzae*. Kojic acid does not display a strong toxicity, but shows a weak mutagenicity in the Rec assay and Ames test. Production of kojic acid by *A. oryzae* strains was examined[6, 7] and is shown in Fig. 2. Most of the strains used for the production of sake and shoyu did not produce detectable amounts of kojic acid. Twelve out of 17 strains used for miso fermentation produced kojic acid. These strains were supplied by the Japan Association of Koji-starter. Based on these results, we informed the Japan Association of Koji-starter that the strains with a high production of kojic acid should not be used for koji-starter. As Sakaguchi's medium is considered to be suitable for the production of kojic acid, Sakaguchi's medium was used for time course studies of kojic acid production by koji molds at 30ºC. The production of kojic acid which was negligible after 2 or 3 days of incubation, increased rapidly during the 3 to 14-day incubation period. After 2 weeks of incubation, the amount of kojic acid in the culture medium decreased. As rice is used for koji (molded rice) production, rice was used for the time course studies of kojic acid production by koji molds at 30ºC. The results were almost similar to those obtained in Sakaguchi's medium. However, the amount of kojic acid in the rice culture medium did not decrease after 2 weeks. In

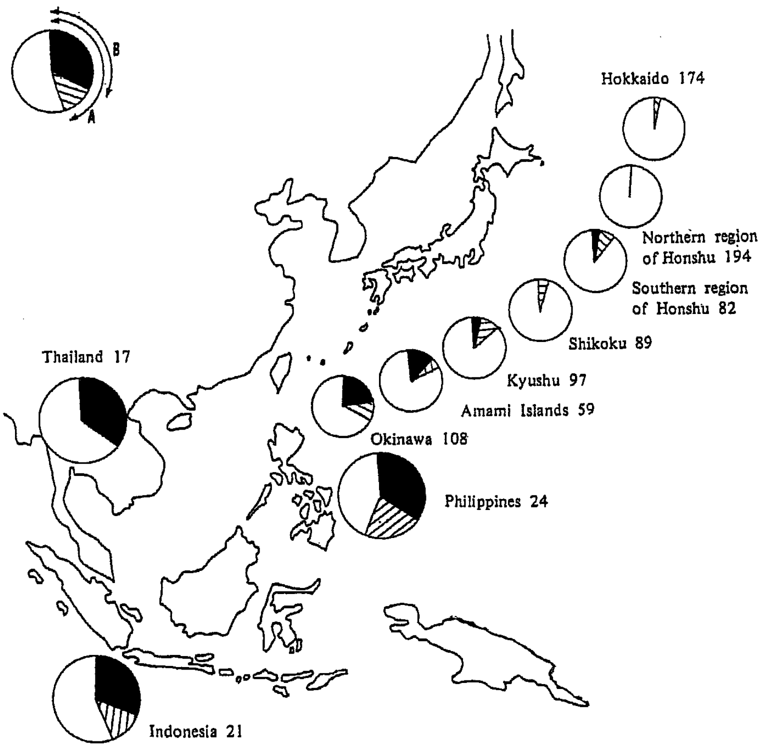

Fig. 1. Detection ratio of aflatoxin-producing fungi in soil samples collected from several countries of Southeast Asia and Japan
A: Ratio of samples where *A. flavus* and *A. parasiticus* were detected.
B: Ratio of samples where aflatoxin-producing fungi were detected.
Number: number of examined samples.

the Japanese fermented food industries, the incubation period required for making koji to produce sake, shoyu and miso is usually within 2 days. It was observed that kojic acid in koji after 2 days of incubation could not be detected or that the amount was very low, even when strains with a high production of kojic acid were used. We expect that kojic acid-producing strains will be eliminated from the koji molds used in the Japanese fermented food industries in the near future. The decomposition of kojic acid during shoyu fermentation was examined. When kojic acid was spiked during shoyu fermentation, kojic acid could not be detected after 150 days. Presently, the usual shoyu fermentation period is about 180 days. Therefore, even if kojic acid were to be produced during the koji-making period, it might be decomposed during shoyu fermentation and the probability that kojic acid remains in the final shoyu product would be very low.

Cyclopiazonic acid

It was reported that several strains of *A. oryzae* produce cyclopiazonic acid (CA). Since CA is also a kind of mycotoxin and shows a comparatively strong acute toxicity, the production of this toxin by several *Aspergillus* strains was examined[1]. The results are shown in Table 2. Thirty-six strains of *A. oryzae*, which were used in traditional Japanese fermented foods, were examined. As a

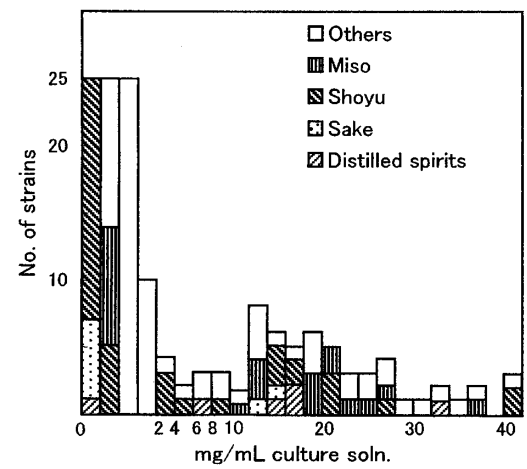

Fig. 2. Production ability of kojic acid by strains of koji molds

Table 2. Production of cyclopiazonic acid by several *Aspergillus* strains

Aspergillus species	Strains No.	Cyclopiazonic acid	Character-istics
Aspergillus oryzae	NFRMCA01	N.D.	
	NFRMCA02	N.D.	
	NFRMCA03	N.D.	
	NFRMCA04	N.D.	
	NFRMCA05	N.D.	
	NFRMCA06	N.D.	
	NFRMCA07	N.D.	
	NFRMCA08	N.D.	
	NFRMCA09	N.D.	
	NFRMCA10	N.D.	
	NFRMCA11	N.D.	
	NFRMCA12	N.D.	
	NFRMCA13	19.6 µg/g	
	NFRMCA14	N.D.	
	NFRMCA15	N.D.	for shoyu
	NFRMCA16	N.D.	for shoyu
	NFRMCA17	N.D.	for shoyu
	NFRMCA18	N.D.	for shoyu
	NFRMCA19	N.D.	
	NFRMCA20	N.D.	
	NFRMCA21	N.D.	
	NFRMCA22	N.D.	
	NFRMCA23	N.D.	
	NFRMCA24	N.D.	
	NFRMCA25	24.4 µg/g	
	NFRMCA26	N.D.	
	NFRMCA27	5.5 µg/g	
	NFRMCA28	N.D.	
	NFRMCA29	N.D.	
	NFRMCA30	N.D.	
	NFRMCA31	N.D.	
	NFRMCA32	N.D.	
	NFRMCA33	N.D.	
	NFRMCA34	N.D.	
	NFRMCA35	N.D.	
	IFO4278	1.9 µg/g	
Aspergillus sojae	MFR-S-52	N.D.	for shoyu
	NFR-NO9	N.D.	for shoyu
Aspergillus flavus	TH-1	16.0 µg/g	aflatoxigenic
	US-F-2	5.2 µg/g	aflatoxigenic
Aspergillus parasiticus	NRRL2999	N.D.	aflatoxigenic
	NRRL3145	N.D.	aflatoxigenic
	ATCC15517	N.D.	aflatoxigenic
Aspergillus niger		N.D.	

N.D.: Not detected.

result, the 4 strains were found to produce CA. When CA production by *A. flavus* and *A. parasiticus* was compared, all of the *A. flavus* strains produced CA while none of the *A. parasiticus* strains produced CA. Four strains of *A. oryzae* used for shoyu fermentation and 2 strains of *A. sojae* also used for shoyu fermentation did not produce CA. Three strains of *A. oryzae*, which showed comparatively high levels of production of CA, were used in order to determine the time course of production of CA using liquid media. These experiments showed that none of the strains produced detectable amounts of CA until 40 h after inoculation. Koji mold, which is used for the production of traditional fermented foods, only proliferates for about 40 h at most after inoculation. CA was not produced in detectable amounts until 40 h, even when strains with a high CA production ability were inoculated to the media. Most of the *A. oryzae* strains did not produce any detectable amount of CA. These results suggested that the possibility of CA contamination of Japanese fermented foods was extremely low, but that it is preferable not to use CA-producing strains for the production of foods. Therefore, these CA-producing strains were eliminated from commercial koji mold. Decomposition of CA was also examined[8]. When CA was spiked in shoyu before fermentation, about 70% of the amount of CA decreased in the first 40-day period. This decrease was induced mainly by yeast, especially *Zygosaccharomyces rouxii*, by decomposition or assimilation.

Katsuo-bushi

Katsuo-bushi (dried bonito) is a kind of traditional fermented food used for the seasoning of soup base. For the production of dried bonito, fungi belonging to the *A. glaucus* group have been used. *Aspergillus* strains are inoculated to boiled, smoked and half-dried bonito. The inoculation had been naturally performed in a special room where *Aspergillus* strains had been grown over a long period of time. Twenty-six samples of katsuo-bushi were collected from all of the producing areas of Japan. As most of the mold growing on katsuo-bushi belonged to *Aspergillus*, the possibility of contamination with aflatoxins, ochratoxin A and sterigmatocystin was examined[5]. The results showing the biological effects of the extracts from dried bonito are presented in Table 3. Toxins were not detected from any of the samples examined. Most of the isolated strains from katsuo-bushi belonged to the *A. glaucus* group, *A. ochraceus* group, and *A. versicolor* group. Twelve representative strains were selected and the production ability of mycotoxins such as aflatoxins, ochratoxin A and sterigmatocystin was examined. Eleven strains did not produce any of the mycotoxins listed above. Only one strain of *A. flavus*, which seemed to be attached to the surface of katsuo-bushi, and was isolated from the washing solution of katsuo-bushi, produced aflatoxin B_1 at the level of 1.1 ppm on rice medium. Inoculation was also performed using 3 mycotoxin-producing strains. These 3 strains grew well on the katsuo-bushi at 28ºC. From a sample of katsuo-bushi inoculated with *A. flavus* and cultured, 10 ppm of aflatoxin B_1 was detected, and from one sample inoculated with *A. ochraceus* and cultured, 5 ppm of ochratoxin A was detected. Sterigmatocystin was not detected from samples of katsuo-bushi inoculated and cultured with *A. versicolor*. However, strains with a stronger toxicity may produce sterigmatocystin, if they infect katsuo-bushi. Also, if the starter of katsuo-bushi becomes infected with mycotoxin-producing strains, mycotoxin contamination of katsuo-bushi may occur. Therefore, strains that do not produce mycotoxins were selected and have been used for katsuo-bushi fermentation.

As mentioned above, in the present study, several Japanese fermented foods were found to be free from contamination with mycotoxins such as aflatoxins, sterigmatocystin, kojic acid and cyclopiazonic acid.

Further studies should be carried out to ensure that other traditional Japanese fermented foods are also safe.

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Table 3. Chemical analysis of mycotoxins in katsuo-bushi (dried bonito) and biological test for the extracts from dried bonito

Sample	Source	Aflatoxins	Ochratoxin A	Sterigmatocystin	Chick embryo test[a]		Brine shrimp test[a]	
No.					24 h	48 h	24 h	48 h
1	Chikura, Chiba Prefect.	–	–	–	2/15	2/15	1/49	3/49
2		–	–	–	0/15	0/15	2/35	3/25
3		–	–	–	2/15	3/15	1/45	8/45
4	Nishiizu, Shizuoka Prefect.	–	–	–	0/14	2/14	1/48	5/48
5	Gozensaki, Shizuoka Prefect.	–	–	–	0/15	2/15	1/71	4/71
6	Yaidzu, Shizuoka Prefect.	–	–	–	1/15	3/15	0/55	3/55
7		–	–	–	0/15	0/15	2/38	4/38
8		–	–	–	0/15	0/15	0/61	2/61
9		–	–	–	2/15	3/15	1/42	2/42
10		–	–	–	2/15	2/15	0/37	2/37
11	Daioh, Mie Prefect.	–	–	–	2/13	2/13	0/39	4/39
12		–	–	–	0/15	1/15	1/48	3/48
13	Tosashimidzu, Kochi Prefect.	–	–	–	3/15	3/15	1/50	3/50
14		–	–	–	0/15	0/15	1/82	2/82
15		–	–	–	0/15	0/15	3/56	4/56
16		–	–	–	0/15	0/15	1/41	2/41
17		–	–	–	2/15	4/15	1/79	4/79
18	Yamakawa, Kagoshima Prefect.	–	–	–	0/15	0/15	2/39	3/39
19		–	–	–	2/15	3/15	2/29	5/29
20		–	–	–	2/14	2/14	1/36	5/36
21	Makurazaki, Kagoshima Prefect.	–	–	–	0/15	0/15	1/99	14/99
22		–	–	–	0/15	0/15	0/68	4/68
23		–	–	–	0/15	0/15	4/61	10/61
24		–	–	–	0/14	0/14	0/110	5/110
25		–	–	–	0/15	0/15	0/116	6/116
26	Miyako Island, Okinawa Prefect.	–	–	–	2/15	0/15	0/70	5/70

a): Denominator denotes the numbers of tested samples and numerator denotes the numbers of dead individuals.
– : No production of corresponding mycotoxin.

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