Formation of Tyramine and Histamine during Soybean Paste (Miso) Fermentation

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The concentrations of seven non-volatile amines, tyramine (Tym), histamine (Him), phenethylamine (Phm), putrescine (Put), cadaverine (Cad), spermidine (Spd) and spermine (Spm), during miso fermentation were studied. Tym of these amines which is generally known to be present in miso was detected during fermentation by the conventional production method. Him and Phm were not detected, and concentrations of Put, Cad, Spd and Spm, which were present in the raw starting material for miso, did not change significantly during fermentation. However, Tym and Him were produced when the miso starting materials, *i.e.*, cooked soybeans, rice koji and mashed miso, were added to liquid medium and allowed to stand. Tym- and Him- producing bacterial strains were isolated from the liquid culture media containing the miso starting materials. The Tym-producing strain was a gram-positive coccus, and the Him-producing strain was a gram-positive rod. Thus, the results show that the Tym and Him observed in culture were produced by the two different species.

During miso fermentation, Tym and Him are apparently produced within a short period of time when the respective optimum conditions for their growth are present. With the exception of Phm which did not exist in the miso starting materials, other amines including Put, Cad, Spd and Spm were not produced and microorganisms producing them are not believed to be present during miso fermentation.

Keywords — amine formation; tyramine; histamine; miso; non-volatile amine; gram-positive coccus; gram-positive rod; fermentation

Introduction

Fermented and brewed foods such as cheese, beer, wine, miso (a fermented soybean paste) and soy sauce contain various amounts of amines including tyramine (Tym), histamine (Him), phenethylamine (Phm), putrescine (Put), cadaverine (Cad), spermidine (Spd), spermine (Spm) and others.¹⁻³⁾ Although the origins of some these amines are still unknown, it is generally supposed that microorganisms are partly related to their production. Edwards and Sandine²⁾ reported that some bacteria isolated from cheese show

tyrosine (Tyr) and histidine (His) decarboxylase activities, while Umezumi *et al.*⁴⁾ reported that some yeasts, nitrate-reducing bacteria and *Lactobacilli* used in the brewing of sake show amine production. Further, Chander *et al.*⁵⁾ showed that *L. bulgaricus* has Tym producing activity. Thus, it has been suggested that these microorganisms might be related to the amines contained in foods. Regarding miso, on the other hand, Takagi and Okamoto⁶⁾ showed that tryptamine was formed, and Chin and Koehler⁷⁾ reported that Tym and Him were formed during the aging process, though the relationship between

these amines and any microbial strains producing them has not yet been clarified.

We previously examined the contents of amines in marketed foods including wine,³⁾ miso and soy sauce,⁸⁾ and suggested that the amines contained in foods, particularly miso and soy sauce, can be viewed as either those already contained in the raw ingredients or those formed during the production process itself, for example, Tym, Him and Phm. Accordingly, in the present study we prepared miso samples by usual production methods in order to determine the origins of the amines contained therein and to examine the mechanisms of their formation. We also monitored the relationship between the concentrations of these amines and simultaneous detection of microorganisms.

Materials and Methods

Mashed Miso — The ingredients used in the production of miso, rice koji, cooked soybeans and mashed miso supplied by a miso manufacturing company (M Co.). Table I shows the composition of original mashed miso.

The Miso Starting Materials — cooked soybeans, rice koji and mashed miso.

Basic Liquid Medium — Peptone (Nihon Seiyaku Co., Ltd.) 5 g, yeast extract (Difco, Detroit) 3 g, and glucose 1 g was added to 1000 ml distilled water (pH7) and autoclaved.

Liquid Medium A — Cycloheximide (Wako Pure Chemical Industries, Ltd.) 10 mg was added to 1000 ml of basic liquid medium to suppress growth of fungi.

Liquid Medium B — Sodium azide (Wako Pure Chemical Industries, Ltd.) 10 mg, to suppress growth of aerobic bacteria was added to 1000 ml

of liquid medium A.

BCP Agar Plate — Plate count agar with BCP (bromocresol purple) was purchased from Nissui Pharmaceutical Co., Ltd.

Determination of Amines — The procedure described in a previous paper⁸⁾ was followed. Briefly, 2 ml of medium was diluted with 0.1 N HCl to a total volume of 20 ml for use as the test solution.

Colony Counts — The culture medium of mashed miso was diluted in the conventional manner using pour-plate technique with a BCP agar plate. After incubation at room temperature (23 to 25°C) for 3 d, colonies were counted.

Determination of Amino Acids — Each sample was diluted 100-fold with 0.02N HCl and analyzed with an amino acid analyzer (835 type Hitachi, Ltd., Japan)

Thin Layer Chromatography (TLC) — Kieselgel 60 HPTLC plate (Merck Co., Ltd.) and acetone–ammonia water (9: 1, V/V) as a solvent were used.

Determination of Amines during Miso Fermentation — The concentration of amines and bacterial counts in miso samples mashed by M Co. on June 17, 1989 were analyzed. A total of 4 miso batches were used prepared with two types of cooked soybeans, *i.e.*, conventional ground cooked soybeans (① and ②) and whole ones (③ and ④). Samples of 2.2 kg were packed in pickling containers provided with springs which were sealed, following which they were allowed to stand in the shade outdoors which is similar to the conditions of general miso fermentation. Twenty g portions of each miso sample were collected to determine the amine concentration. Bacterial colony counts of samples ① and ③ and pH values of samples ① to ④ were also determined.

Production of Amines in the Miso Starting Materials — The miso starting materials were added to the respective liquid media, cultured, and examined for the production of amines. Approximately 0.5 g of cooked soybeans, rice koji and mashed miso from whole or ground soybeans (①–④) which had been frozen was added to 2 ml of liquid media A and B at salt concentrations

TABLE I. Composition of Mashed Miso

Cooked soybeans	6.9kg
Rice koji	1.8kg
NaCl	1.3kg
10%NaCl solution	300ml

ranging from 0 to 12% and allowed to stand at room temperature (23 to 25°C) for 2 d. Then, each culture was subjected to TLC to determine the production of amines.

Amine-Production Patterns in Liquid Medium — Ten g of mashed miso from whole soybeans ③ which had been frozen was added to 50 ml of liquid medium B and allowed to stand at room temperature (23 to 25°C) for 90 d. Liquid medium B contained mashed miso had been autoclaved was used as a control. During this period, the culture medium was sampled periodically, and amine content, amino acid content, pH and total bacterial count were monitored.

Results and Discussion

1. Determination of Amines and Bacterial Counts during Miso Fermentation

Amine concentration was determined in each miso sample on the days shown in Table II. Neither Phm nor Him was detected in any sample at any time during the analysis period, while, Put, Cad, Spd, and Spm were present from the first day of fermentation in all samples. Although Tym was not detected in any samples up to day 42, miso sample ③ contained Tym on day 90. It was thus assumed that Tym was produced sometime between days 42 and 90, paralleling the hot

weather (August to September). Tym concentration showed no significant increase thereafter, and began to decrease on day 143. No difference was observed in Tym content in various parts of the container. In contrast, the concentrations of Put, Cad, and Spm changed little from those at the start of fermentation, although the content of Spd decreased somewhat with the lapse of time. Thus, these amines were not believed to have been produced during the aging period. Although Phm and Him were not detected in this study, these amines have been found to contain in some marketed miso products.⁸⁾ In this work it was considered that neither bacterial species producing these amines was present nor, if present, the conditions were those allowing the amine production.

Bacterial counts and amine-producing bacteria in samples ① and ③ were examined simultaneously. Both the samples contained only bacteria until day 14, and only one species of yeast colonies was observed on the plates from day 26 to 42. The cultures yielded few colonies of either bacteria or yeasts after day 90.

Approximately 20 colonies grown on each BCP agar plate were isolated and inoculated into a basic liquid medium. After cultivation

TABLE II. Amine Concentrations during Miso Fermentation

($\mu\text{g/g}$)

Day	Phm				Put				Cad				Him				Tym				Spd				Spm			
	①	②	③	④	①	②	③	④	①	②	③	④	①	②	③	④	①	②	③	④	①	②	③	④	①	②	③	④
1	—	—	—	—	42.0	27.1	33.8	20.9	3.6	3.7	3.8	3.4	—	—	—	—	—	—	—	—	5.6	18.6	17.0	23.0	1.3	4.4	3.7	5.5
3	—	—	—	—	36.2	19.8	20.2	23.3	3.2	3.4	3.3	2.6	—	—	—	—	—	—	—	—	8.9	29.5	15.4	15.5	1.2	6.7	3.4	3.4
7	—	—	—	—	42.5	38.9	33.6	33.4	4.2	4.1	4.3	4.3	—	—	—	—	—	—	—	—	4.5	12.0	12.0	16.6	5.1	4.4	4.4	6.1
14	—	—	—	—	39.8	39.8	34.9	30.9	4.6	4.7	4.5	3.5	—	—	—	—	—	—	—	—	8.3	9.0	15.9	20.9	1.3	4.1	3.2	5.7
26	—	—	—	—	35.9	34.4	30.8	29.4	3.5	4.2	4.0	4.0	—	—	—	—	—	—	—	—	2.8	8.8	15.5	13.8	1.2	4.1	3.0	3.1
42	—	—	—	—	43.7	45.2	29.4	35.0	4.2	4.2	3.6	3.5	—	—	—	—	—	—	—	—	11.4	5.1	15.0	12.8	1.5	0.8	2.7	0.3
90	—	—	—	—	35.4	30.7	29.0	27.3	3.1	2.6	2.6	2.8	—	—	—	—	26.5	—	—	—	7.0	10.0	15.5	16.8	2.2	1.7	3.1	3.3
124	—	—	—	—	29.3	30.7	31.6	28.7	2.7	2.7	3.2	2.9	—	—	—	—	26.9	—	—	—	3.0	4.9	6.9	9.4	0.9	1.3	2.3	3.0
143	—	—	—	—	32.2	31.0	29.9	28.6	2.6	2.6	2.7	2.8	—	—	—	—	17.2	—	—	—	3.9	5.0	7.7	9.3	1.5	1.2	1.8	2.6
203	—	—	—	—	30.0	37.1	30.0	30.8	2.5	3.0	2.5	3.6	—	—	—	—	11.0	—	—	—	4.5	3.8	7.1	10.2	1.3	1.1	1.2	3.6
451	—	—	—	—	31.1	26.1	28.8	27.8	2.1	2.1	2.1	2.2	—	—	—	—	—	—	—	—	0.9	1.3	1.3	1.0	—	—	0.5	0.4

① ② : mashed miso from ground cooked soybeans, ③ ④ : mashed miso from whole cooked soybeans,

Phm : Phenethylamine, Put : Putrescine, Cad : Cadaverine, Him : Histamine, Tym : Tyramine, Spd : Spermidine, Spm : Spermine,
— : not detected.

at room temperature for 2 days, 10 μ l of the culture was subjected to TLC to determine the production of Him, Tym and Phm. In the cultures of samples ① and ③, no bacterial species which could have produced Tym or other amines was detected, regardless of whether or not Tym was detected in sample ③. This may be due to the fact that no sampling was performed during the period of Tym production.

The pH values of all samples were around 6.1 at the mashing point, following which they decreased over time, being 5.4 to 5.7 on day 90. The pH then showed a slow change, ranging from 5.2 to 5.4 on day 203 and from 5.1 to 5.2 on the final day 451. These findings indicate that microorganisms producing Tym, such as lactic acid bacteria, might be related to the Tym production observed during the period of pH decrease, even though no amine-producing bacteria was detected and no relation between bacterial count and production of Tym was established. Tym was not detected in ground cooked soybeans but was found in whole cooked soybeans in sample ③. Salt does not easily permeate whole cooked soybeans, so that the production of Tym may be affected by salt concentration.

In fermenting miso, Tym was detected in only one of 4 samples, while Him was not detected in any sample. Various factors may account for this, including salt concentration, the influence of coexisting microorganisms, or aerobic/anaerobic conditions.

2. Production of Amines in the Miso Starting Materials

Although no amine-producing bacterial species was detected during the fermentation of miso samples, Tym was found in one of these samples. Thus, it was considered that the amine-producing bacteria was already present in the miso starting materials or that the materials became contaminated with these bacteria during the production process.

Table III shows the results of amine-assays of the cultures of miso starting mate-

TABLE III. Production of Tyramine and Histamine in the Miso Starting Materials Cultured in Liquid Medium

Sample	Medium	Salt (%)	Results	
			Tym	Him
S	A	0-12	(-)	(-)
	B	0	(+)	(+)
	B	4	(+)	(-)
K	B	8,12	(-)	(-)
	A	0-12	(-)	(-)
	B	0	(+)	(-)
	B	4-12	(-)	(-)
W	A	0	(+)	(-)
	A	4	(+)	(-)
	A	8,12	(-)	(-)
	B	0	(+)	(+)
	B	4	(+)	(-)
G	B	8,12	(-)	(-)
	A	0	(+)	(-)
	A	4-12	(-)	(-)
	B	0	(+)	(-)
	B	8	(+)	(-)
	B	4,12	(-)	(-)

S: cooked soybeans, K: rice koji, W: mashed miso from whole soybeans, G: mashed miso from ground soybeans.

rials. Tym was detected in all the starting materials, while Him was detected in cooked soybeans and mashed miso from whole soybeans. Phm was not found in any starting material and no sample contained Him alone. Bacterial growth was observed within a salt concentration range of 0 to 8%, but no bacterial growth was seen in any sample at the salt concentration of 12%. Tym was detected within the salt concentration range of from 0 to 8%, while Him was detected exclusively at the concentration of 0%. These findings indicate that the Him-producing bacteria was contained only in the cooked soybeans. However, since these soybeans had been cooked, it seems that these Tym- and Him-producing bacteria may always be present in the miso starting materials, and that salt, other starting materials and equipment in the factory may be continuously exposed to them.

3. Detection of Amine-Producing Bacteria

Culture media in which Tym or Him were detected, *i.e.*, liquid medium B containing

Fig. 1. Concentrations of Tyramine, Histamine, Tyrosine and Histidine and Total Bacterial Counts in Mashed Miso Cultured in Liquid Medium during the First 90 d of Fermentation

—●—, tyramine; —■—, histamine; —▲—, bacterial counts; —○—, tyrosine; —□—, histidine; —×—, pH.

cooked soybeans at the salt concentration of 0% (S-B-0 as shown in Table III) and those containing mashed miso at the salt concentrations of 0 and 4% (G-B-0 and W-B-4, respectively) were diluted with sterile saline for pour culture in BCP agar plate medium containing 2% salt, 10 ppm cycloheximide, and 10 ppm sodium azide. Colonies which changed the color of the medium to yellow after standing at room temperature (10 colonies from S-B-0, 21 from W-B-4 and 39 from G-B-0) were isolated and cultured in basic liquid medium for 3 d and observed for production of amines and amine-producing bacteria.

Of 10 S-B-0 colonies which showed the same uniform colony type formation on the BCP agar plate, 9 produced Him, while only 1 of 21 W-B-4 colonies produced Tym. None of 39 M-B-0 colonies produced any amines. This unexpected finding of only one Tym-producing bacteria may be because the population of amine-producing bacteria had declined or been replaced by other amine-nonproducing bacteria at the time of culture.

The Tym-producing bacteria (referred to

as T-strain) produced only Tym, and the Him-producing bacteria (H-strain) produced only Him. Microscopic observations of gram-stained specimens revealed that T-strain was a gram-positive coccus and that H-strain was a gram-positive rod, thus the strains producing Tym and Him were different species.

4. Tym- and Him-Production Patterns in Liquid Medium

Since it was determined that the Tym and Him in the miso starting materials were produced by different bacterial species, production of these two amines during cultivation in the liquid medium as described below was monitored with the lapse of time. The simultaneous production of amines by the two species, as well as its dependence on pH, bacterial count, salt concentration, and the concentrations of His and Tyr, which are the precursor amino acids of the respective amines, was studied in Fig. 1.

Bacterial counts showed rapid increase to 1.2×10^8 /ml from 50 h to 5 d after the start of culture. On day 11, the total bacteria count was 1.2×10^8 /ml, following which it decreased to 3.3×10^7 /ml on day 15 and then increased

again, peaking at 7.3×10^7 /ml on day 26, after which it decreased.

The first increase in total bacterial count was accompanied by the initiation of Tym production. Tym concentration was almost constant ($145 \mu\text{g/ml}$) on day 11 when the bacterial count began to decrease, and on day 21 it also began to decrease.

The production of Him began 5 d after Tym production began. The concentration of Him showed a rapid increase during the 2 days before day 15, when the bacterial count began to increase again. It then decreased as the bacterial count decreased, though a moderate time-lag was observed; that is, products of amines were almost in agreement with the growth of respective bacterial counts.

We attempted to detect the amine-producing bacterial species in the same samples by isolating approximately 10 colonies from the BCP agar plate. For instance, on day 3, when the production of Tym was observed, 60 colonies were isolated and their amine production was examined; no bacterial species producing Tym was detected in any colony, however. In contrast, 2 of 10 colonies obtained on day 15 and 2 of 15 obtained on day 26 showed Him production.

Thus, total bacterial count apparently did not always agree with the counts of Tym- and Him-producing species, indicating that there were various competing amine-nonproducing species in addition to the Tym- and Him-producing species in the culture. It was further considered, that the species producing Tym and Him were present only in very small populations, even though they produced considerable quantities of Tym and Him.

While it was found in this study that the T-strain appeared first and was followed by the H-strain, either the T- and H-strain were inoculated at the same time into single culture, or the strain inoculated into the other strain existing culture, could grow and produced the corresponding amine. Thus, it is believed that the growth of the H-strain start-

ed after the growth of the T-strain by chance and that these strains did not compete with each other.

In the control sample, neither bacterial growth nor production of Tym or Him was observed.

The pH value decreased in inverse proportion to the bacterial increase, being 6.1 on day 0, decreasing to 4.2 on day 5 and 3.8 on day 8, and remaining almost constant thereafter. Thus it was assumed that the first bacterial growth represented lactic acid bacteria in the miso starting materials.

The concentration of either amino acids His or Tyr decreased with increase in Him or Tym concentration, respectively. The His concentration was $144 \mu\text{g/ml}$ on day 6 and decreased to $53 \mu\text{g/ml}$ on day 26. The Tyr concentration was $191 \mu\text{g/ml}$ on day 2 and decreased to $114 \mu\text{g/ml}$ on day 5. The initiation of production of each amine corresponded to the beginning of decrease in the concentration of the corresponding amino acid. When the amine concentration became constant, the concentration of the amino acid increased again. Each amino acid was thus decarboxylated to the corresponding amine. The finding that the amino acid concentrations increased after amine concentrations stabilized suggests that the amino acids were continuously supplied by the mashed miso. The concentration of the amino acid Arginine (Arg) which was determined simultaneously, was $212 \mu\text{g/ml}$ at day 0 and $393 \mu\text{g/ml}$ on day 3. However, no Arg was detected on day 6, indicating that it was completely consumed during the course of bacterial growth; it was detected again on day 90, when the growth of the bacteria had apparently completely ceased. As the H-strain was present during the period when no Arg was present and when pH was decreased, it was assumed that this strain was able to grow after the growth of other bacteria which needed Arg had ceased.

The salt concentration of the culture medium was 2.1% at day 0 and remained

constant thereafter. Salt concentration, in particular, may have affected amine production, as it was higher in fermenting miso than in mashed miso cultured in liquid medium. But both the T- and H-strains grew in the liquid medium simultaneously, so it can be assumed that these strains might also grow in miso fermentation.

When the miso sample of day 90 in which Tym had been detected (whole soybean miso sample ③) was added to medium B and allowed to stand, neither Tym nor Him was produced. This supports the assumption that exchange or competition of amine-nonproducing bacterial species occurs during the aging process and that strains producing amines are immediately replaced by other amine-nonproducing bacteria after the production of amines.

Only slight variation in Put, Cad, Spd and

Spm concentrations was observed during miso fermentation, and microorganisms producing them apparently did not exist during the fermentation in this study. That the production of Phm was not observed might be because these particular samples contained no bacterial species which produce Phm. Thus, we consider it likely that the microbial species producing Tym, Him and Phm in miso are different.

Identification of T-strain and H-strain is now under investigation and will be reported elsewhere.

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