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NOTE

Novel antimutagenic pigment produced by *Bacillus licheniformis* SSA3

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We discovered that the *Bacillus licheniformis* SSA3, fermenting traditional Korean soy sauce and soybean paste, involved in the synthesis of a dark-brown pigment. This pigment produced in the minimal medium supplemented with tyrosine only as precursor. We showed that this pigment is novel, and differed from melanoidin and melanin, and an antimutagenic substance.

Studies on the pigments produced by microorganisms have been done for long time. Gessard (6), Muschel (14) and Dawid (4) reported that certain species of *Bacillus* produced melanin. Clark and Smith (3) reported that *Bacillus niger* synthesized a black pigment from tyrosine and Barnett et al. (1, 2) identified the kinds and quality of a brown pigment produced non-enzymatically from the precursor secreted by *Bacillus subtilis*.

So far studies on the pigments of traditional Korean soy sauce have been performed non-systematically as follows: "standardization of Korean soy sauce" by Lee and Koh (10), "a study on the amino-carbonyl reaction" by Shin and Yang (18), "pigment-forming bacteria in the presence of L-tyrosine and their possible role in the browning of fermented soybean products" by Park and Kyung (15).

Kim (8), Park and Kim (9) identified that *Bacillus licheniformis* SSA3 ferments traditional Korean soy sauce and soy bean paste. The color of fermented traditional Korean soy sauce and soybean paste is either black or brown. In this study we report that *Bacillus licheniformis* SSA3 produces a dark-brown pigment from tyrosine. This pigment was identified as a novel antimutagenic substance.

We investigated whether *Bacillus licheniformis* SSA3 can produce a dark-brown pigment. This bacterium was inoculated into the minimal broth supplemented with each of 22 amino acids, respectively, and cultured by agitating at 150~200 rpm for 15 days at 30°C. The results showed that *Bacillus licheniformis* SSA3 could synthesize a dark-brown pigment in the minimal medium (5) when supplemented with tyrosine only as shown in Table 1.

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So far it has been known that the most important factors in the non-enzymic browning reaction (Maillard reaction) are the carbonyl and amino groups. (16) However, the dark-brown pigment in this study was produced specifically by tyrosine only.

Another interesting previous result (9) showed that the mutant strains of *Bacillus licheniformis* SSA3, *Bacillus licheniformis* SSA3-2M1 could not produce the dark-brown pigment even in the presence of tyrosine in the minimal medium.

From these results it is understood that the synthesizing mechanism for this pigment is enzymatic, which is a very different process from Maillard reaction for melanoidin. Therefore we conclude that this pigment is not melanoidin.

To characterize this pigment we purified it using the following methods. From the minimal broth supplemented with 0.1% (w/v) tyrosine, *Bacillus licheniformis* SSA3 was inoculated and cultured by agitating at 150~200 rpm for 15 days at 30°C for pigmentation. The pigmented culture was centrifuged for 15 min at 15,000 rpm to get rid of any cell debris and then the supematant was dried by freeze-drying techniques (7).

From the minimal agar plates supplemented with 0.1% tyrosine, *Bacillus licheniformis* SSA3 was spread on the plates and incubated for 5 days at 30°C for pigmentation. The pigment was extracted from the incubated plates by NH_4OH and then dried by freeze-drying.

The dried pigment from the liquid and solid media was redissolved in distilled water, then passed through a Sephadex G-150 column (5 × 30 cm) by dropping with secondary distilled water. The pigment fraction was collected by measuring the absorbance at 430nm according to Barnett et al. (1) and dried again by freeze-drying.

The obtained pigment was used to test the solubility and the result was compared with those of melanin (11) as shown in Table 2. The comparison shows that this pigment was highly soluble in H_2O, but that melanin wasn't soluble at all. Therefore we concluded that this pigment is novel, and not melanin.

We further conducted an antimutagenicity test (12, 13, 17) for this novel pigment substance as shown in Table 3. The result showed that 10 μg of this pigment reduced the mutagenecity by up to 41% against *Salmonella typhimurium* TA100 in the presence of 1μg of aflatoxin B1 and brought us to the conclusion that this pigment is a novel substance of antimutagenicity.

Table 1. Pigmentation test of *Bacillus licheniformis* SSA3 in the minimal broth supplemented with one of the following amino acids

Amino acids	Pigmentation	Amino acids	Pigmentation
L-Alanine	-	L-Isoleucine	-
L-Arginine Hydrochloride	-	L-Leucine	-
L-Asparagine	-	L-Lysine Hydrochloride	-
L-Aspartic acid	-	L-Methionine	-
L-Cysteine, Free Base	-	L-Phenylalanine	-
L-Cystine	-	L-Proline	-
L-Glutamic acid	-	L-Serine	-
L-Glutamine	-	L-Threonine	-
Glycine	-	L-Tryptophan	-
L-Histidine Hydrochloride	-	L-Tyrosine	+
Trans-4-Hydroxy-L-proline	-	L-Valine	-

To conduct pigmentation test, each 0.1% (w/v) of 22 amino acids (21 L-amino acids and glycine) were added separately in the minimal broth (10 g dextrose, 7.0 g dipotassium phosphate, 2.0 g monopotassium phosphate, 0.5 g sodium citrate, 0.1 g magnesium sulfate, 1.0 g ammonium sulfate in 1 liter of distilled water, pH7.0). Pigmentation was determined from the three replicate experiments.

Table 2. Comparison of the solubility between the pigment produced by *Bacillus licheniformis* SSA3 and melanin (11)

Solvent	The pigment produced by *Bacillus licheniformis* SSA3	Melanin (11)
H_2O	soluble	insoluble
Ethanol	insoluble	insoluble
Acetone	insoluble	insoluble
Chloroform	insoluble	insoluble
NH_4OH	soluble	-

The pigment produced by *Bacillus licheniformis* SSA3 was highly soluble in H_2O and NH_4OH, and slightly soluble in methyl alcohol (melanin was not), but not soluble at all in ethanol, acetone and chloroform. The solubility of melanin was referenced from Lingappa, et al. (11). −: not determined.

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Table 3. Antimutagenicity test for the pigment produced by *Bacillus licheniformis* SSA3

Treatment	revertanats/plate			
	S. typhimurium TA98	% inhibition	*S. typhimurium* TA100	% inhibition
AFB(1μg)(control)	1788 ± 194	0	2777 ± 190	0
AFB1 + 1μg	1759 ± 36	2	2490 ± 150	10
AFB1 + 5μg	1624 ± 126	9	2259 ± 256	19
AFB1 + 10μg	1581 ± 70	12	1650 ± 732	41
AFB1 + 20μg	1336 ± 188	25	1507 ± 110	46

The mutagenecity was induced by aflatoxin (AFB) B1 (AFB1, 1 μg/plate) in *Salmonella typhimurium* TA98 and TA100.
Spontaneous: TA98-32, 25, 30 (29± 4)
TA100-170, 164, 199 (178± 19).
% inhibition was determined from the three replicate experiments.

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