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Pigment-forming bacteria in the presence of L-typrosine and their possible role in the browning of fermented soybean products

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Abstract

A hypothesis that Korean home-made fermented soybean products are brown-pigmented in large part by contaminated bacteria is proposed. Twenty six strains of bacteria forming brown pigments in the presence of L-tyrosine were isolated from home-made soybean paste. They were characterized and all were identified as strains of *Bacillus subtilis*. The isolates produced dark brown to brownish black pigmentation on yeast extract-peptone-glucose agar (YPGA) supplemented with 0.1% L-tyrosine in 72 hours but not on YPGA. They also caused different degress of lighter pigmentation on potato dextrose agar and nutrient agar. When an arbitrarily chosen pigmenting isolate was cultivated in a liquid medium supplemented with L-tyrosine, it began to produce pigments only after cell growth stopped. The tyrosinase enzyme was extracted and the enzyme activity was measured by using L-tyrosine and 3-hydroxytyrosine (L-dopa) as substrates. The crude enzyme preparation porduced pigments at rates of 2.1×10^{-3} and 5.0×10^{-3} optical density units/min measured at 490*nm* for tyrosine and dopa, respectively. Possible content of L-tyrosine in a soybean paste formula was calculated.

Introduction

Fermented soybean products, especially soy sauce and soybean paste, are browned during koji preparation, aging in salt brine, and storage. There have been many works[1-7] to study browning reactions of fermented soybean products. Excessive browning deteriorates not only appearance[3,5] but also taste, aroma and nutritional quality.[3]

It is generally accepted that Maillard reaction is the primary browning reaction of fermented soybean products and that other minor reactions may add to the browning.[3,8,9] However, there have been reports opposing the conventional Maillard reaction hypothesis of the browning of fermented soybean products. Other than Kim et al.[5] who reported that different strains of koji mold contribute differently to the browning of soybean paste, Okuhara et al.[6] and Okuhara et al.[7] reported that pentoses contributed only 10-20% of total browning of soy sauce and that solutions containing sugars and amino acids browned at much slower rates than actual soy sauce did. They concluded that Maillard reaction may not be the most important browning reaction and that peptides and other unknown factors were more important. Hashiba[4] who also worked with simulated soy sauce (sugar-amino acid mixture) found that the browning rate of the mixture was approximately 10% of that of actual soy sauce.

Amino acids such as aspartic acid and glutamic acid, even though they involve in the Maillard browning with reducing sugars[10], effectively inhibit the Maillard reaction between other amino acids and sugars. Nafici and Markakis[11] reported that when they added 0.01-0.04 *M* aspartic acid and glutamic acid into glucose and fructose lysine browning systems, 74-78% and 60-87% reductions in browning, respectively, were occurred. The two amino acids are abundant in soybean protein (glutamin acid = 18.82 *g* 16*g*N and aspartic acid = 11.04/16*g*N)[12] and in wheat protein (glutamic acid = 29.0% and aspartic acid = 3.8%)[13] and consequentially in soy sauce (glutamic acid = **16.74-32.80** *g*/16 *g*N and aspartic acid = 5.87-7.20 *g*/16 *g*N).[12] It is also well known that soy sauce has its peculiar good taste-enhancing effect due to its high content of glutamic acid. Therefore Maillard browning should be effectively inhibited in soy sauce as it was in model systems of Nafici and Markakis.

Nevertheless the conventional and most prevailing

hypothesis for browning of fermented soybean products has been the Maillard reaction, while some workers as aforementioned maintain that Maillard reaction may not be the primary browning reaction when they studied with simulated soy sauce systems.

Th objective of this paper was to isolate browning bacteria in the presence of L-tyrosine and to study their pigmenting characteristics under the assumption that bacteria produce L-tyrosinase which oxidizes L-tyrosine originated from raw materials to cause browning of fermented soybean products.

Materials and Methods

Bacterial source

Home-made soybean paste was obtained from a household in Seoul area. The outer surface of the sample was darkened during storage in a refrigerator.

Culture media

Yeast extract (0.3%)-malt extract (0.3%)-peptone (0.5%)-glucose (1.0%) agar (YMPGA) supplemented with 0.1% L-tyrosine (YMPGTA) was used to isolate browning bacteria from the soybean paste sample. Yeast extract (0.1%)-peptone (0.2%)-glucose (0.1%) agar (YPGA) and YPGA with 0.1% L-tyrosine (YPGTA), potato glucose agar, nutrient agar (Difco Laboratories, Detroit, M1), were used to observe the pigmentation of the pimentforming isolates on solid media.

Isolation and identification of pigmenting bacteria

Soybean paste sample was streak-plated on YMPGTA and incubated at 28°C for 7 days. Colonies with brown to brownish black pigmentation around them were chosen for further studies.

Bergey's manual of determinative bacteriology[14] was used to identify the bacterial isolates.

Bacterial gowth and pigment formation

Samples were taken from the shaked 500ml flask containing 100 *ml* of YMPGTB. Whenever samples were taken, diluted eliquots were pour-plated on plate count agar (Difco Laboratories, Detroit MI) for enumeration of the bacterium and approximately 5 *ml* of growth medium was centrifuged to remove bacterial cells and debries before the supernatant was used to measure optical densites (OD) at 490 *nm*.

Production and extraction of tyrosinase

A pigmenting isolate (strain 8) was grown on2000*ml* of YMPGA (approximately) 100 plates) at 28°C for 7 days and tyrosinase was extracted from the coarsely cut agar pieces by 2000 *ml* of 0.1 *M* glycine buffer solution (pH 6.2) at 4° for 60 hr. The extract was centrifuged to remove nonsoluble solids and the enzyme was precipitated with 80% saturated $(NH_4)_2SO_4$ at 4°C for 2 days. The precipitate was obtained by filtration and dialyzed against distilled water for 3 days at 4°C. The supernatant was kept at 4°C and used as crude enzyme.

Measurement of enzyme activity

Tyrosinase activity was measured by a spectrophotometer (Sequoia-Turner Corp., CA) with L-tyrosine and L-dopa as substrates. Optical density at 490 *nm* was taken from the shaken mixture of 3.0 *ml* of 0.1 *M* phosphate buffer (pH 6.2), 0.5 *ml* of enzyme preparation, and 0.002 *M* substrates at 40°C at 5 min intervals.

Results and Discussion

Pigment forming bacteria

Twenty six strains of brown pigment-forming bacteria were isolated from a single home-made fermented soybean paste sample. They all were identified as strains belonging to *Bacillus subtilis*. The isolates had different pigmenting characteristics showing yellowish light brown, light brown, pinkish brown, darkish brown, and brownish black. Those bacteria producing dark pigments revealed lighter coloring in the early phase of the pigmentation. The pigmenting characteristics on various solid media are as in Table. 1.

Home-made (i.e., microbiologically uncontrolled) soybean paste/soy sauce koji contains different kinds of molds including *Aspergillus oryzae* and bacteria originated from the raw materials and acquired from its environments. Cho and Lee[15] who studied the distribution of microorganisms in Korean home-made soy sauce koji reported that there were 10^4-10^{10} bacteria/g of koji and that *B. subtilis* and *Bacillus pumilis* were the dominant ones. Bacteria were found to be mostly in the form of dormant spores. Chung[16] reported that *Bacillus* was the most frequently found bacteria in Korean home-made soy sauce koji. Watanabe et al.[17] reported that industrially prepared Japanese soy sauce koji inoculants contained 10^4-10^6 bacteria/g and were sure that these

contaminated bacteria propagated as mold grew during the preparation of koji. They, however, did not mention the bacterial populations in the koji itself. Therefore it seemed to be universal that soy sauce koji was contaminated with an appreciable number of bacteria whether it was industrially prepared or home-made.

Tyrosine as the probable substrate for browning

Fig. 1 shows YPGA and YPGTA plates inoculated with strains producing brown and brownish black pigments in the presence of L-tyrosine. Pigmentation around the colonies is apparent only in the medium supplemented with L-tyrosine.

Chung[16] who studied bacterial profile of home-made soy sauce koji reported that a strain of *Bacillus* which was identified as *B. subtilis* produced dark brown pigment on nutrient agar, yeast extract-peptone-glucose agar and koji extract agar. He simply reported the phenomenon without further comment on the probable cause. None of the pigmenting isolates of this report produced dark brown pigments on nutrient agar, but it was light brown to brown. A pigmenting strain of *B. subtilis* (*B. subtilis*

Table 1. The pigmenting characteristics of the isolates

Strain	Pigmentation on common laboratory media after 5 days of incubation		Pigmentation on YPGTA (hours of incubation)		
I.D	PDA	NA	24	48	72
1.	B	LB	PB	B	DB
2.	B	LB	PB	B	DB
3.	LB	LB	PB	B	DB
4.	LB	LB	LB	B	BB
5.	LB	YLB	LB	B	BB
6.	B	YLB	PB	PB	DB
7.	B	YLB	PB	PB	BB
8.	LB	LB	LB	B	BB
9.	LB	LB	LB	B	BB
10.	B	LB	LB	B	BB
11.	LB	LB	Y	YB	BB
12.	LB	LB	YB	B	BB
13.	LB	LB	YB	B	BB
14.	LB	YLB	YB	B	BB
15.	B	LB	PB	B	DB
16.	LB	YLB	YB	B	BB
17.	B	LB	YB	B	BB
18.	B	LB	PB	B	DB
19.	B	LN	PB	B	DB
20.	B	LB	PB	B	DB
21.	B	YLB	PB	B	DB
22.	BB		LB	YB	LB
23.	B	LB	PB	B	DB
24.	LB	LB	YB	B	BB
25.	B	LB	PB	B	DB
26.	B	Y	YB	B	BB

B = brown, LB = light brown, YLB = yellowish light brown, Y = yellow, PB = pinkish brown, DB = darkish brown, BB = brownish black

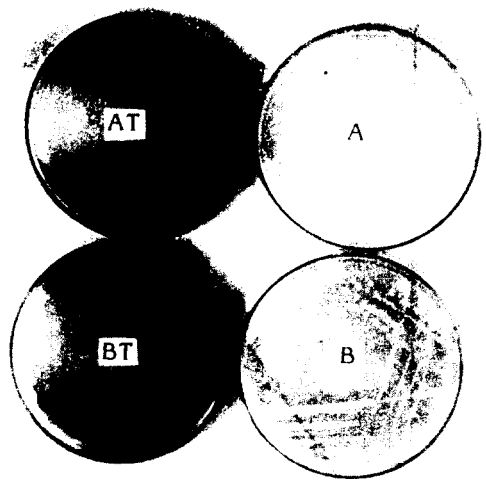

Fig. 1. Pigment formation on YPGA with (AT and BT) and without (A and B) supplemented tyrosine by a brownish black pigment-forming isolate (A: strain 8) and a brown pigment-forming isolate (B: strain 13) after 72 hours of incubation at 28°C.

var. *niger*) in the presence of L-tyrosine was also described elsewhere.[14] The brown pigmentation on PDA and nutrient agar should be due to the indigenous tyrosine in the medium components.

Kim et al.[5] reported that different strains of koji mold showed different browning characteristics. Ebine[3] maintained that more efficient protein-hydrolyzing koji mold caused darker pigmentation than less efficent protein hydrolyzers and explained that it was due to increased amounts of reactants for Maillard reaction produced from proteins. It, however, can be explained in a different manner that increased amounts of L-tyrosine were released from proteins. Under the assumption of this work, the L-tyrosine so released serve as the substrate of L-tyrosinase to produce brown pigments.

Lee[12, 18] who analyzed amino acid composition of soy sauce and soybean paste reported that tyrosine was in low concentration in soy sauce compared to that in soybean paste. 30-40% of all other amino acids (threonine, valine, isoleucine, leucine, phenylalanine, methionine, cystine, tryptophan) analyzed were found in soy sauce whereas only 15% of tyrosine was found in it. The % content of all amino acids including tyrosine were the same in soybean paste. This clearly proved that only tyrosin disappeared during aging process in salt brine. It is doubtless that tyrosine, after it is made free by protein hydrolyzing enzymes, underwent specific reaction(s). Chang[1] studied the change in amino acid composition of soy sauce during aging and reported that the content of tyrosine decreased rapidly while that of other amino acids did not change appreciably. His explanation for the phenomenon was that tyrosine and pentoses reacted to generate pigments by Maillard reaction. This, however, was not probable because those amino acids which react with sugars at a similar or ever faster rate[10] did not decrease as tyrosine did. Soy sauces are known to contain high concentration of glutamic acid, which was reported to inhibit Maillard browning very efficiently.[11]

In this communication, brown pigment-forming bacteria were isolated and characterized to support our hypothesis that bacteria in the presence of tyrosine causes browning of fermented soybean products by using Korean home-made soybean paste as a model. On the other hand evidences that Maillard reaction may not be the primary browning reaction were provided. In addition the total content of possible tyrosine in a soybean paste formula was calculated (see appendix) to be 0.936% (W/W).

Growth of pigmenting bacteria and pigment formation.

One (Strain 8) of the isolated pigmenting bacteria was chosen to observe cell growth and pigment formation in a shaken YMPGTB. Maximum viable cell number was about 1.0×10^9 colony forming units per *ml* at stationary phase of growth curve (Fig. 2). Microscopic observation revealed that as soon as the bacterium reached stationary phase it began to sporulate and that spores were liberated from the parent cells following the sporulation.

The final pigmentation in YMPGTB was 8.63 in OD units and that in YMPGB was 1.23 (Fig. 2). The difference in OD (7.4 units) should be due to supplemented tyrosine. The apprarent OD in YMPGB is believed to be due to tyrosine indigenous to the components of the medium. Pigmentation increased rapidly only after cell growth reached the stationary phase and continued to increase linearly for the following 70 hours until pigment formation did not increase any further (Fig. 2).

Tyrosinase activity

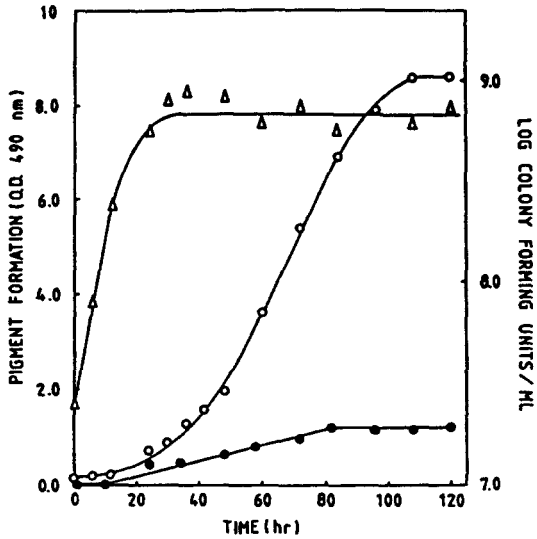

Fig. 2. Growth(△) and pigmentation of a strain of *B. subtilis* in YMPGT broth with (○) and without (●) tyrosine

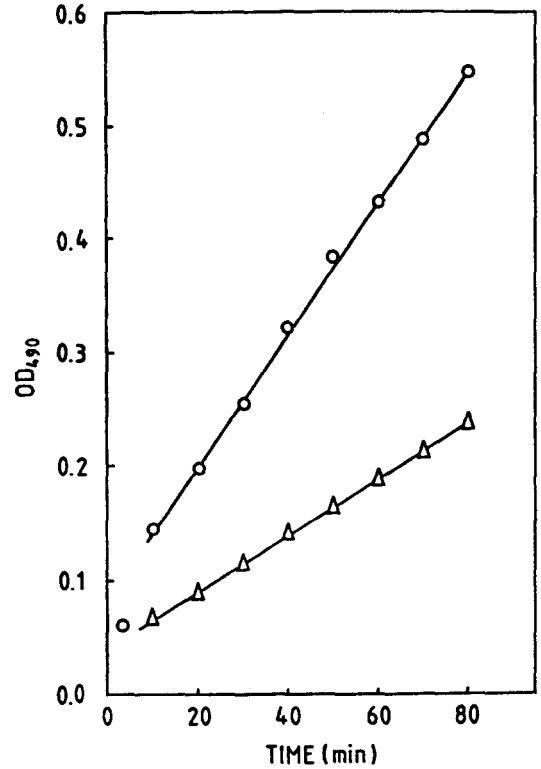

Fig. 3. Activity of crude tyrosinase extracted from YMPGA grown a strain of *B. subtilis* on L-dopa (○) and L-tyrosine (△)

Approximately 30ml of crude enzyme preparation was obtained from 2000 *ml* equivalent of YMPGA. No effort was made to liberate the enzyme from the bacterial cells because they sporulate right after active growth ceased followed by autolysis of cell wall to liberate spores and intracellular enzymes.

L-dopa was oxidized (5.0×10^{-3} OD/min) more than twice faster than L-tyrosine was (2.1×10^{-3} OD/min) as shown in Fig. 3. The faster oxidation of L-dopa by the enzyme can be easily deduced because L-tyrosine should be oxidized first to L-dopa before melanin formation.[13, 19]

Conclusions

It is advocated that bacteria play an important role in the browning of fermented soybean products and that Maillard reaction may not be as important as it has heen believed to be. The abundance of bacteria in Korean home-made soy sauce/soybean paste koji, the existence of browning bacteria among them and the existence of plenty of tyrosine in raw materials were all proved.

Calculation

Tyrosine is the most found phenolic material in soybean and wheat as one of the constituting amino acids of proteins. Soybean contains 35% protein (*w/w*) of which 4.1% is tyrosine. Consequently, 35/100 × 4.1/100 = 0.01435, that is, 1.435% of total weight of soybean is tyrosine. Wheat contains 12.9% of protein of which 4.0% is tyrosine. In the same manner, 12.9/100 × 4.1/100 = 0.00516, that is, 0.516% of total weight of wheat is tyrosine. Based on a soybean paste formula (soybean 2790 *g*, wheat 178 *g*, salt 390 *g*, water 1020 *ml*) by Kim et al [5], it is calculated that soybean constitutes 63.73% of total formula and wheat 4.07%. Therefore 63.73 *g* of soybean and 4.07 *g* of wheat are in 100 *g* of the formula mix. Consequently tyrosine originated from soybean is 63.73 × 1.435/100 = 0.915 *g* and that from wheat 4.07 × 0.516/100 = 0.021 *g*. Therefore the total amount of tyrosine theoretically found in 100 *g* of soybean paste formula mix is 0.936 *g* (= 0.915 + 0.021).

References

1. Chang, C.H.: *Korean J. Agric. Chem. Soc.*, 9, 9 (1968)

2. Chung, K.M., Cho, S.H. and Kim, J.U.: *J. Korean Agic. Chem. Soc.*, **24**, 200 (1981)
3. Ebine, H.: *J. Breu. Soc.* (Jap.), **75**, 145 (1980)
4. Hashiba, H.: *Agric. Biol. Chem.*, **37**, 871 (1973)
5. Kim, S.S., Kim, S.K., Ryu, M.K. and Cheigh, H.S.: *Kor. J. Appl. Microbiol. Bioeng.*, **11**, 67 (1983)
6. Okuhara, A., Nakajima, T., Tanaka, T., Saito, N. and Yokotsuka, T.: *J. Ferment. Technol.*, **47**, 57 (1969)
7. Okuhara, A., Saito, N. and Yokotsuka, T.: *J. Ferment. Technol.*, **49**, 272 (1971)
8. Motai, H.: *Nippon Shokuhin Kogyo Gakkaishi.*, **23**, 372 (1976)
9. Yomo, H.: *J. Breu. Soc.* (Jap.), **75**, 149 (1980)
10. Ashoor, S.H. and Zent, J.B.: *J. Food Sci.*, **49**, 1209 (1984)
11. Nafici, K. and Markakis, P.: *J. Agric. Food Chem.*, **31**, 1117 (1983)
12. Lee, C.H.: *Kor. J. Food Sci. Technol.*, **5**, 210 (1973)
13. Kim, D.H.: *Food Chemistry*, Tamgudang, Seoul, p. 539 (1971)
14. Buchanan, R.E. and Gibbons, N.E.: *Bergey's manual of deteminative bacteriology*, 8th ed. Williams and Wilkins Co., Baltimore, MD. pp. 529-550 (1974)
15. Cho, D.H. and Lee, W.J.: *J. Korean Agric. Chem. Soc.*, **13**, 35 (1970)
16. Chung, Y.S.: *Kor. J. Microbiol.*, **1**, 30 (1963)
17. Watanabe, D., Ebine, H., and Ooda, D.: *Soybean Foods*, Kwangrim, p. 148 (1982)
18. Lee, C.H.: *Kor. J. Food Sci. Technol.*, **8**, 19 (1976)
19. Ohba, T., Kato, H., Kurata, T., and Fujimaki, M.: *Agr. Biol. Chem.*, **39**, 139 (1975)

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대두발효식품의 갈변과 관련된 티로신산화 세균에 관한 연구

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마이야르반응이 대두발효식품의 주갈변반응이라는 종래의 주장을 부정하는 증거와 함께 특히 한국재래식 대두발효식품의 갈변현상은 주로 오염된 세균 때문일 수 있다는 가설을 제시하고 이를 증명하기 위해 가정에서 만든 된장시료로부터 티로신이 첨가된 배지에 갈변색소를 생성할 수 있는 세균 26균주를 분리하여 이들 세균들이 모두 *Bacillus subtilis*임을 밝혔다. 이 갈변세균들은 0.1% 티로신이 첨가된 yeast extract-peptone-glucose 배지에 짙은 갈색 내지는 암갈색 색소를 생성하였으나 티로신이 첨가되지 않았을 때는 갈변색소를 생성시키지 않았다. 분리세균중 임의로 선택된 갈변세균을 티로신이 함유된 액체배지에 진탕 배양했을 때 균체증식 곡선의 정상기 이후부터 갈색색소를 왕성하게 생성하였으며 0.1% 티로신이 첨가된 배지와 같은 조성으로 티로신만 제외된 배지에서의 변색의 차이는 OD_{490}이 7.4 정도였다. 또한 갈변세균을 배양한 배지로부터 추출한 조효소액은 티로신을 2.1×10^{-3} OD/min로 도파는 5.0×14^{-3} OD/min의 속도로 갈변시켰다. 참고로 된장원료중의 티로신 함량을 계산한 결과 0.936%가 있다는 것이 밝혀졌다.