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ORIGINAL ARTICLE

Anti-oxidizing Functional Effect of Polyamine as a Bioconversion Starter Using Microbes Isolated from Fluke babsikhae

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Abstract Polyamines are well known as important molecular compounds for many biochemical processes in animals and plants. The function of biogenic polyamine has been especially well studied for its role in the oxidative system and the protection of membranes from peroxidation in cells. We investigated the polyamine contents of fluke babsikhae and isolated polyamine-producing lactic acid bacteria (LAB) to improve the probiotic antioxidative function of the Korean traditional fermented food, fluke babsikhae. In the present study, first we confirmed that fluke babsikhae possesses more antioxidative molecules compared to the other antioxidative vegetable (Korean radish) and ascorbic acid by using the method of peroxide value (POV). Secondly polyamines, which are considered as antioxidative molecules, were detected in fluke babsikhae using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). In order to improve the probiotic function of fluke babsikhae, LABs were isolated from the fermented food, and these isolated microbes were tentatively identified as *Lactobacillus brevis* strain bh3 and *Lactobacillus* sp. using 16S rRNA sequencing. Polyamine was produced from these two strains and also was confirmed to have antioxidative activity. Especially *Lactobacillus* sp. was shown to produce high amounts of putrescine. Therefore, this strain can be considered as a starter to improve the probiotic function of fluke babsikhae. According to the results, fluke babsikhae will be a good candidate for a healthy functional food and also isolated LAB will be considered as a starter for probiotics by bioconversion.

Keywords antioxidant · fermented food · fluke babsikhae · lactic acid bacteria · polyamine

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Introduction

Biogenic amines, including tyramine, histamine, putrescine, cadaverine, spermine, and spermidine, can be produced and degraded by the normal metabolic activities of animals, plants, and microorganisms. These amines are mainly produced by microbial decarboxylation of amino acids in foods (Brink et al., 1990).

Polyamine is known classically by the names of putrescine, spermidine, and spermine, and they are present in all organism cells. Polyamine plays an important role in regulating cell growth and proliferation, the stabilization of negative charges of DNA, RNA transcription, protein synthesis, apoptosis, and the regulation of the immune response (Elvira et al., 2007).

Polyamine is mostly obtained from dietary sources, and can be expected to have cell regeneration and antioxidant effects in foods containing polyamine (Elvira et al., 2007).

Babsikhae is a well-known fermented food eaten as a side dish or snack with Kimchi along the east coast of Korea. Babsikhae is composed of mainly squid, fluke, and sculpin mixed together with rice and other materials. Fish proteins in babsikhae are broken down into essential amino acids such as glutamic acid, lysine, threonine, ornithine, and polyamine during fermentation. Polyamine

was reported to be found in carp roe by different preservation methods including fermentation (Martin et al., 2011).

Squid sikhae is known to lower blood pressure and other functional properties (Choi et al., 2001), and pollock sikhae is known to have an antioxidant effect (Cha et al., 2002). In the present study, we investigated the presence and effect of the antioxidative activity of polyamine as a bioconversion starter using microbes isolated from fluke babsikhae and confirmed the anti-oxidant effects of polyamine from fluke babsikhae and polyamine produced from isolated lactic acid bacteria (LAB). Experiments were also carried out to determine the polyamine contents (putrescine, spermidine, and spermine) of fluke babsikhae compared to Korean radish (Elvira et al., 2007). This study demonstrated that isolated microbes can be used as bioconversion starters in fluke babsikhae.

Materials and Methods

Sample preparation. The fluke babsikhae materials used (a mixture of fluke, rice, radish, garlic, and ginger) were obtained from Jukdo market in Pohang, and were ground with 80% methanol and freeze-dried. The freeze-dried extract was stored in a freezer (Hwang et al., 2001).

Isolation of LAB. Extracts from fluke babsikhae were inoculated on MRS Agar (Difco, USA), incubated for 3–4 days at 30°C, and LAB were isolated from the incubated MRS Agar plates. The isolated strains (*Lactobacillus brevis* strain bh3 and *Lactobacillus* sp.) were checked for polyamine production and identified by 16S rRNA sequencing.

Dansylation for thin layer chromatography (TLC). One gram of the freeze-dried sample was added to 10 mL of distilled water, followed by addition of 0.5 mL of $HClO_4$ to the sample for TLC, and then centrifuged at 15,000 g for 30 min. The supernatant (300 μL) was mixed with 600 μL of dansyl chloride and 300 μL of saturated Na_2CO_3. The mixture was incubated at 60°C for 1 h in a dark condition. After reaction, 5–10 μL of proline was added to the sample, and the upper part was then discarded. Benzene was then added to the sample, and the benzene layer was evaporated (Gros and Labouesse, 1969).

Benzoylation of high performance liquid chromatography (HPLC). Two milliliters of the sample was mixed with 6 mL of 6M-NaOH and 5 μL of benzoyl chloride. After reaction for 20 min, the sample was mixed with 2 mL of saturated NaCl and 2 mL of chloroform, and centrifuged at 3000 rpm for 5 min. The chloroform layer was evaporated and then dissolved in 1 mL of methanol. The final sample was prepared by filtration through a 0.45-ìm pore filter cloth (Hwang et al., 1997; Özogul et al., 2002).

Analysis of polyamine content by HPLC. Polyamine contents were analyzed by HPLC (Ultimate 3000, Dionex, USA) with ultra violet (UV) detection at 254 nm wavelength. Mobile phases were acetonitrile and deionized water, and the shed was 40:60.

Analysis of polyamine content by TLC. Polyamine content was investigated by TLC (silica gel 60 F254, Merck, USA) with a mixture of dichloromethane, diethyl ether, and triethylamine at a ratio of 4:1:1 as a mobile phase. The samples were prepared by dansylation. Two microliters of the sample was spread on TLC plate and developed for 85 min. TLC results were checked by UV detection at wavelength of 365 nm.

Measurement of super oxide dismutase (SOD) activity. SOD activity was measured by the peroxide value (POV) method using a SOD test kit by applying cytochrome C reduction NO_2-TB (Nitro-Blue tetrasolium) with Japan's Wa-Ko (Japan). The absorbance was measured at 560 nm (Spectrophotometer, DU 730, Beckman).

Results and Discussion

In the present study, we focused on the antioxidant function of fluke babsikhae in relation to polyamine. The presence of polyamine in fluke babsikhae was confirmed by TLC and HPLC methods. To determine that polyamine has antioxidant properties, SOD-test was carried out, and the antioxidant activity was compared to Korean radish, which has known antioxidant activity (Hwang et al., 2001), and the standard control was ascorbic acid.

Measurement of SOD activity. We prepared 10 mg/mL each of fluke babsikhae extract and radish extract to investigate their antioxidant effects The standard control was 0.1 mg/mL of ascorbic acid, which has 10% SOD activity. The SOD activity of Korean radish and fluke babsikhae was measured at about 31 and 45%, respectively. The antioxidant activity rate of babsikhae was higher than the Korean radish that already has a proven antioxidant effect. To confirm the presence of polyamines known to have an antioxidant function, HPLC method was used on fluke babsikhae.

Polyamine analysis by HPLC. The polyamine content in fluke babsikhae was compared with those in the Korean radish extract using standard polyamines, such as putrescine, spermidine, and spermine (Sigma, USA). The contents of putrescine in fluke babsikhae were much higher than those in Korean radish (Fig. 1). Peak (1) is the expected ornithine, a precursor of polyamine. In a previous study (Elvira et al., 2007), radish was shown to have various polyamines such as putrescine, spermidine, and spermine, whereas fluke babsikhae has mainly putrescine.

Polyamine analysis by TLC. Polyamine contents were checked by TLC using the UV colorization method after danslyation of the sample. TLC was performed with dichloromethane, diethyl ether, triethylamine, at 4:1:1, and checked by a UV 365 nm wave (Fig. 2). In the results, P, D, and M, are putrescine, spermidine, and spermine standards, respectively. The putrescine in fluke babsikhae had a higher content, but the spermidine was not detected by both TLC and HPLC.

Isolation of polyamine-producing microbe in fluke babsikhae. We expected that a bioconversion of polyamine produced by LAB was capable of making safe, functional food. Twenty LABs were

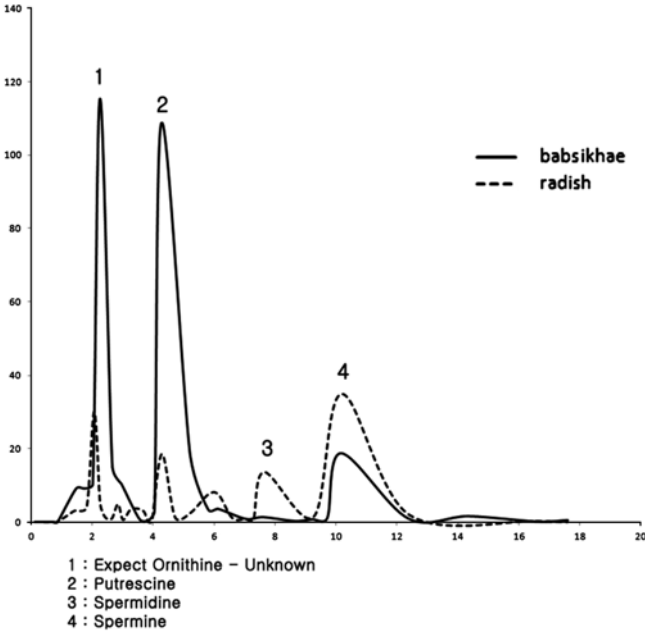

Fig. 1 Polyamine in fluke babsikhae and Korean radish by HPLC. The black and dotted lines are the fluke babsikhae and Korean radish, respectively. All polyamines (putrescine, spermidine, and spermine) were detected in Korean radish, but in fluke babsikhae only two polyamines (putrescine and spermine) were detected. The quantity of putrescine in fluke babsikhae was much higher than that in Korean radish.

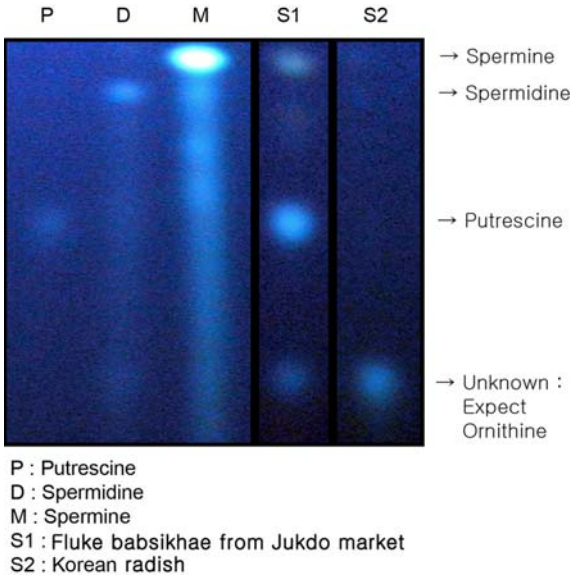

Fig. 2 Polyamine contents in fluke babsikhae and Korean radish by TLC. Spots of polyamine contents from Korean radish were weak, whereas all polyamines were detected. Babsikhae had two clear spots of polyamine contents (putrescine and spermine). Thus, an antioxidant effect can be expected not only from Korean radish but also from babsikhae.

isolated from babsikhae. Polyamine production was checked using 20 strains, among which two strains were confirmed to

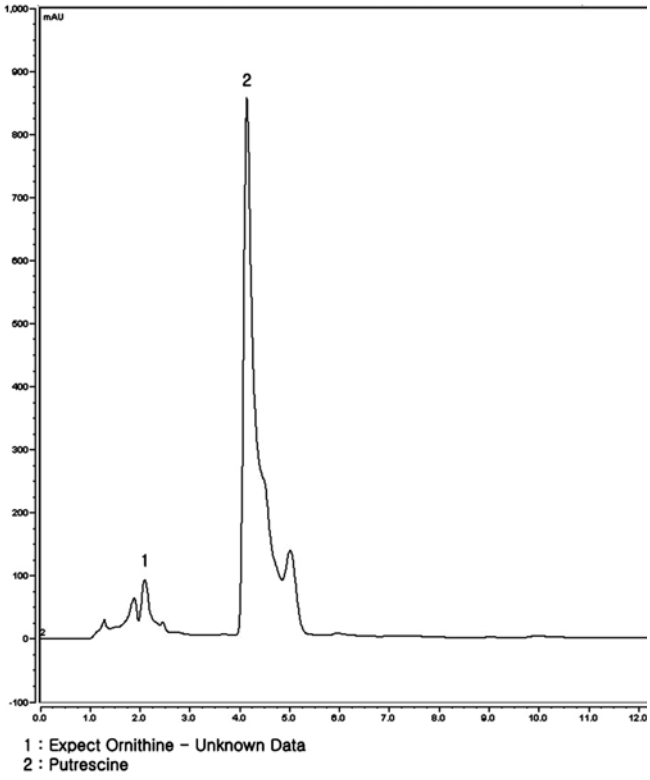

Fig. 3 Putrescine in LAB No. 2 strain by HPLC. Many putrescines were detected by HPLC and they are expected to be used for bioconversion. The unknown peak (1) is thought to be ornithine, a precursor of polyamine

produce a high amount of polyamine. The two polyamine-producing bacteria were tentatively identified as *L. brevis* strain bh3 (No. 1) and *Lactobacillus* sp. (No. 2).

Polyamine analysis of two strains (No. 1 and No. 2) by HPLC and TLC. Two strains cultured for 72 h were used for HPLC analysis as described in 'Materials and Methods'. Putrescine was highly detected in the No. 2 strain (Fig. 3), whereas the contents of putrescine, spermidine, and spermine in the No. 1 strain were weakly detected (data not shown). Each unknown peak in both strains (peaks 1), represents the expected ornithine, a precursor of polyamine (Fig. 3). Putrescine was detected clearly in the No. 2 strain but spermine was only detected in the No.1 strain by TLC (Fig. 4).

SOD activity test of polyamine-producing LAB. To investigate the SOD activity of polyamine-producing strains, two strains were cultured for 72 h. The SOD activities of both strains were almost the same at 53.92%, although a high amount of putrescine was detected in the No.2 strain. We expected that the more powerful antioxidative activity of spermine than that of putrescine, would result in the same SOD activities (Lovaas, 1991).

A high amount of polyamine was produced in 72 h of cultivation. Hence, when using a bioconversion starter, culturing the strains for 72 h is a suitable condition.

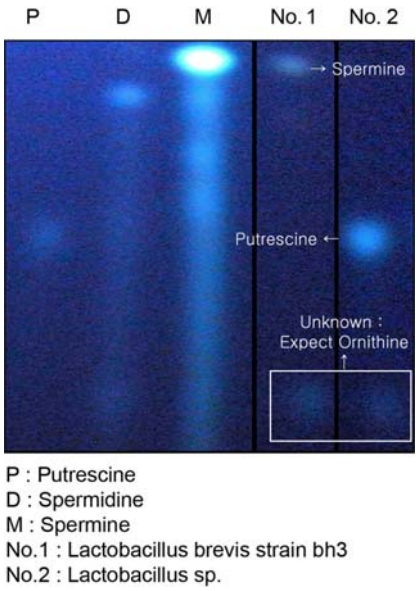

Fig. 4 Comparison of polyamine contents at LAB No. 1 and No. 2 strains by TLC. The No. 1 strain produced more spermine than the No. 2 strain, but the putrescine was more prominent produced in the No. 2 strain than that of No. 1 strain.

Conclusion. The present study suggests that polyamine has many functions, and is present in the Korean traditional fermented food, fluke babsikhae, from the east coast of Korea. We isolated two LABs that have a known antioxidant effect. Results showed that fluke babsikhae is a good candidate for a functional food and as a bioconversion starter in the future. Especially, we determined that polyamines in fluke babsikhae have a stronger antioxidant effect than in radish. Nevertheless, although fluke babsikhae has been eaten for a long time along the localized east coast as a good, traditional fermented food, the level of research is still poor. Through our study, we can expect that fluke babsikhae will be developed into a high business value as a functional food with optimal bioconversion. However, further studies, including additional test on the functional activity and polyamine producing process of the strain, should be carried out.

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