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Degradation of Cholesterol by *Bacillus subtilis* SFF34 in Flatfish during Fermentation

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***Bacillus subtilis* SFF34 degrading cholesterol was applied to reduce residual cholesterol content in fermented flatfish. When the bacterial cells were inoculated as a start culture, a maximal level (1.7 U/g) of cholesterol oxidase was obtained after 10 days, which was two times higher than that (0.8 U/g) without inoculation. Residual cholesterol contents with and without inoculation of the cells were 0.5 mg/g and 0.8 mg/g after 12 days of fermentation, respectively. Cholesterol derivatives including cholesterol-5α, 6α-epoxide, 4-cholesten-3-one and 7β-hydroxycholesterol were detected in raw flatfish as well as fermented flatfish. Campesterol and 25-hydroxycholesterol were detected only after fermentation. However, no significant differences in their contents were observed regardless of inoculation.**

Key words: *Bacillus subtilis*, cholesterol, cholesterol oxides, degradation, fermented flatfish

Cholesterol (5-cholesten-3-ol) and its oxides have been detected in a variety of foods and foodstuffs, especially, eggs, milks, meats, seafood and their processed products (Paniangvait *et al.* 1995). Among the processed seafood, fermented anchovy, clam, crab, fish gills, flatfish, herring roe, octopus, oyster, pollack roe, squid, sea squirt, sea urchin and shrimp, have been widely consumed in Korea as side dishes for a long period of time. Since some of the cholesterol oxides have been hypothesized to be associated with colon cancer and to have potent angiotoxic effects suggesting that they have a likely role in cardiovascular diseases (Kauntiz, 1978), it was suggested that the bacterial degradation of cholesterol in cholesterol-containing foods may be useful for human health (Watanabe *et al.*, 1986).

The degradation and assimilation of cholesterol by various microorganisms is well known in natural environments. Cholesterol oxidase (cholesterol: oxygen oxidoreductase, EC 1.1.3.6) is known to be a key enzyme, which catalyses the oxidation of cholesterol to 4-cholesten-3-one and the reduction of oxygen to hydrogen peroxide (Smith and Brooks, 1976; Sakodinskaya, 2000). The enzyme, especially originating from bacterial cells, plays an important role in the degradation of cholesterol in fermented foods (Smith 1987; Paniangvait *et al.* 1995).

A great number of microbial strains have been reported to produce cholesterol oxidase, including *Brevibacterium* (Uwajima *et al.*, 1974), *Corynebacterium* (Shirokano *et al.*, 1977), *Pseudomonas* (Aono *et al.*, 1994), *Rhodococcus* (Kreit *et al.*, 1994; Sojo *et al.*, 1997; Watanabe *et al.*, 1986), *Schizophyllum* (Fukuyama and Miyake, 1979), and *Streptomyces* spp. (Tomioka *et al.*, 1976). *Nocardia erythropolis* produced cholesten-3-one from cholesterol (Turfitt 1944). A similar reaction has also been found in a great number of microorganisms, such as *Arthrobacter* (Arima *et al.* 1969; Liu *et al.* 1983), *Mycobacterium* (Stadtman *et al.* 1954), *Nocardia* (Turfitt 1944), *Pseudomonas* sp. (Talalay and Dobson 1953) and *Bacillus sphaericus* (Suh *et al.* 1993). However, knowledge of the cholesterol degradation pathway remains limited (Gilliland *et al.* 1985).

Previously, we isolated a bacterial strain, producing a high level of cholesterol oxidase, from Korean traditional fermented flatfish, which was identified to be a strain of *Bacillus subtilis* (Kim *et al.*, 2002). Its two extracellular cholesterol oxidases were characterized and their reaction products with cholesterol were identified as 4-cholesten-3-one (Rhee *et al.*, 2002). The purpose of this study was to ascertain whether the bacterial strain participates in the degradation of cholesterol in fermented flatfish. Therefore, we used the bacterial cells in the fermentation of flatfish, and investigated the degradation of cholesterol, the production of cholesterol oxidase, and the formation of its oxidized derivatives during fermentation.

Materials and Methods

Bacterial strain

B. subtilis SFF34 used in this study has been described in detail previously (Kim *et al.*, 2002). Bacterial cells were

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grown at 30°C with shaking at 150 rpm in nutrient broth. The cells were harvested by centrifugation and resuspended in 0.9% NaCl solution, and then stored at 4°C for inoculation.

Preparation of fermented flatfish

Fermented flatfish was prepared using 500 g of flatfish, 400 g of Chinese radish, 185 g of cooked millet, 26 g of malt extract, 14 g of ginger, 30 g of garlic, 20 g of red pepper and 35 g of salt as recommended (Jung *et al.*, 1992). Flatfish and radish were sliced before use. The other raw materials were mashed together and mixed with the sliced flatfish and radish. *B. subtilis* SFF34 cells were inoculated to 1×10^6 cfu/g, when needed. Fermentation was carried out statically at 30°C for 12 days in a 2-liter capped jar.

Cholesterol oxidase activity assay

During the fermentation, flatfish was extracted at 4°C for 16 h with 0.1 M sodium phosphate buffer (pH 7.0) after homogenizing. The extract was filtered and the filtrate was subjected to cholesterol oxidase assay, which was carried out using the method of Richmond (1973). The reaction mixture was composed of 3 ml of 0.1 M sodium phosphate buffer (pH 7.0)-0.05% Triton X-100, 0.05 ml of 6 mM cholesterol solution in isopropanol and 0.05 ml of the crude enzyme solution. The enzyme reaction was carried out at 30°C for 1 min in a 10 mm light path cuvette, suitable for use in a thermostat double-beam spectrophotometer. After incubation, the increase in absorbance resulting from the oxidation of cholesterol was measured at 240 nm. The enzyme unit of cholesterol oxidase was defined as the amount of enzyme oxidizing 1 μmole of cholesterol to 4-cholesten-3-one per min at 30°C.

Determination of the content of cholesterol and its oxides

Lipid was extracted from fermented flatfish by the method of Marmer and Maxwell (1981). The fermented flatfish (40 g) was mixed well with 80 ml of chloroform-ethyl ether (1:1, v/v) and 0.5 ml of 5α-cholestane (2 mg/ml in chloroform) was added as an internal standard. After being stirred vigorously for one hour, the mixture was filtered and the filtrate was rinsed with 10 ml of ethanol-chloroform (1:1, v/v). The organic solvent of the filtrate was, then, evaporated under vacuum and the dried extract was resuspended in 15 ml of diethyl ether. The resuspension was then filtered and mixed with 20 ml of 0.5 M KOH in methanol, and saponified by stirring at 60°C for 1 h. After saponification, 20 ml of distilled water was added and the unsaponified fractions were extracted twice with 25 ml of diethyl ether. The organic fractions were pooled and washed with distilled water until the washings were neutral.

Residual cholesterol was determined by an enzymatic method using a CHOL-TEST kit (Boehringer Mannheim Gmbh Co., Germany). After reaction, increased absorbance was measured at 405 nm using a spectrophotometer (Shimadzu UV161, Japan). Cholesterol contents were calculated from the absorbances using cholesterol as a standard (Sigma-Aldrich Co., USA).

Cholesterol derivatives were analyzed using a gas chromatograph (Hewlett Packard 6890A, USA) by the method of Pie *et al.* (1991). A flame ionization detector and a 30 m fused silica capillary DB5 column with a film thickness of 1.0 μm (J & W Scientific, USA) were used. The GC conditions were as follows: flow rate, 1.5 ml/min; pressure, 26.1 psi; oven temperature, 280°C; injector temperature, 290°C; detector temperature, 300°C. Helium was used as the carrier.

Results and Discussion

Production of cholesterol oxidase and degradation of cholesterol by B. subtilis sff34 during fermentation of flatfish

Cholesterol oxidase production and cholesterol degradation in flatfish were monitored during fermentation at 30°C for 12 days with or without inoculation of *B. subtilis* SFF34 cells (Fig. 1). Cholesterol oxidase activity increased according to culture time in both cases. Without inoculation, the maximal level of enzyme activity was obtained after 10 days, which was 0.8 U/g. This is most likely because some of the indigenous bacteria in the flatfish participate in the production of cholesterol oxidase. The enzyme activity in the flatfish mixture to which the bac-

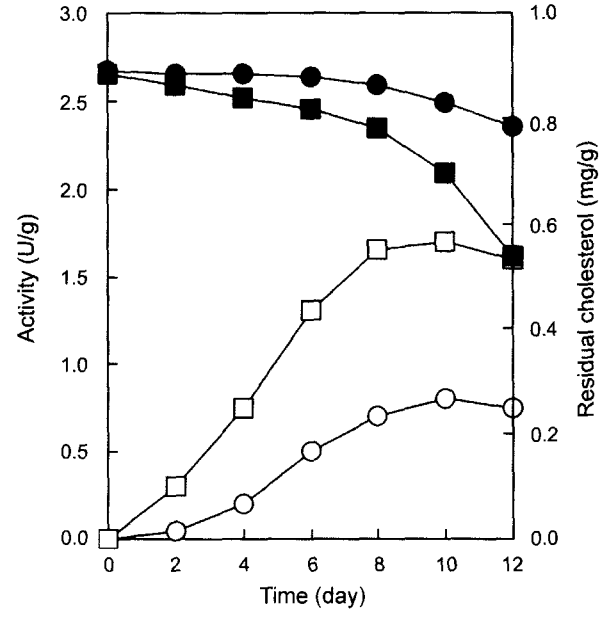

Fig. 1. Time course of cholesterol degradation during the fermentation of flatfish. The flatfish mixture inoculated with *B. subtils* SFF34 was fermented at 30°C for 12 days. During the fermentation, cholesterol oxidase activity (open symbols) and residual cholesterol content (closed symbols) were monitored every two days (square, inoculated; circle, not inoculated).

terial cells were inoculated increased more drastically than that without inoculation, and reached a maximal level (1.7 U/g) 10 days after fermentation, which was twice as high as that without inoculation.

The cholesterol content decreased according to the fermentation time in both cases. During the early fermentation period, no significant reduction in the cholesterol content was observed. After 6 days of fermentation, the residual cholesterol content decreased rapidly. Much greater degradation was observed when *B. subtilis* SFF34 cells were inoculated. The cholesterol content in the fermented flatfish without inoculation decreased from 0.9 mg/g of the original content of 0.8 mg/g after 12 days of fermentation. However, it decreased to 0.5 mg/g after the same period of fermentation time when cells were inoculated. This result suggests that *B. subtilis* SFF34 play an important role in the degradation of cholesterol in fermented flatfish (Fig. 1).

Detection of various cholesterol derivatives in fermented flatfish

Major cholesterol degradation products in the fermented flatfish were separated using a GC (Fig. 2). Before fermentation, six major peaks were detected. And, three more major peaks were observed after fermentation regardless of the inoculation of bacterial cells (designated x to z in Figs. 2B and 2C). However, the GC pattern with inoculation of bacterial cells was similar to that observed without inoculation of bacterial cells, except in the more rapid decrease of cholesterol content.

A mixture of standard cholesterol oxides and the extract of the fermented flatfish was also analyzed by a GC (Fig. 3). Standard cholesterol derivatives such as 5α-cholestane, cholesterol, campesterol, cholesterol-α, 6α-epoxide, 4-cholesten-3-one, 7β-hydroxycholesterol and 25-hydroxycholesterol were well separated as seven peaks, as shown in Fig. 3A. The mixture of standard cholesterol derivatives and the extract of fermented flatfish showed higher peaks. However, their patterns were similar except for the 7β-hydroxycholesterol and 25-hydroxycholesterol peaks (note asymmetric peaks 5, 6 and z in Fig. 3B). The asymmetric peak was thought to be due to a compound in the fermented flatfish (designated 6) unresolved from 7β-hydroxycholesterol (designated 5) and 25-hydroxycholesterol (designated z) (Fig. 3B). Compounds 6 and x have yet to be analyzed in detail, as have several minor peaks in the fermented flatfish (Fig. 3).

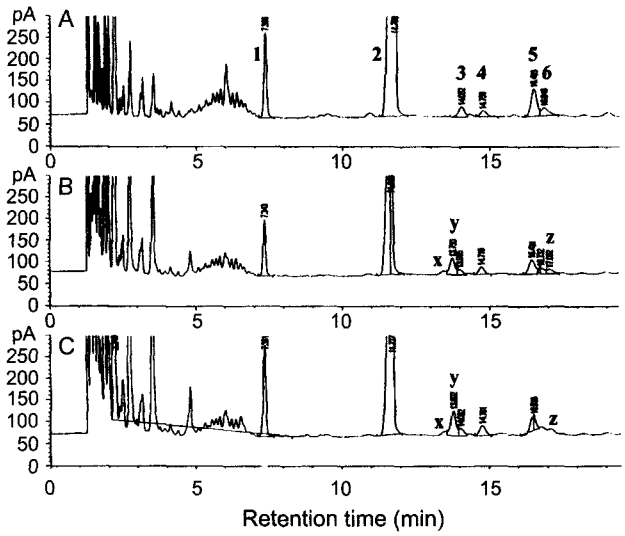

Fig. 2. Typical gas chromatograms of cholesterol and its derivatives in fermented flatfish. Gas chromatographic analyses were carried out before (A) and after fermentation at 30°C for 12 days with (C) or without (B) inoculation of *B. subtilis* SFF34 cells. The major peaks before fermentation are designated 1 to 6 in panel A, and the peaks newly formed after fermentation as x to z in panels B and C.

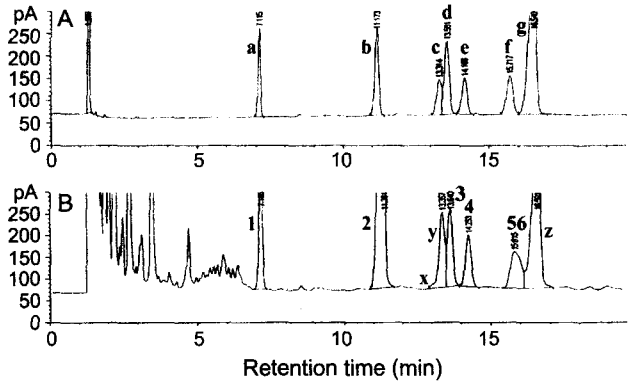

Fig. 3. Detection of cholesterol derivatives in fermented flatfish. Panel A represents the GC chromatogram of standard cholesterol and its derivatives including 5α-cholestane as internal standards (designated a), cholesterol (b), campesterol (c), cholesterol-5α, 6α-epoxide (d), 4-cholesten-3-one (e), 7β-hydroxycholesterol (f) and 25-hydroxycholesterol (g). Panel B represents the GC chromatogram of a mixture of the fermented flatfish extract and standard cholesterol derivatives. Designation 1 to 6 and x to z in panel B are the same as those used in Fig. 2.

Changes in the contents of cholesterol derivatives in fermented flatfish

After fermentation with and without inoculation of *B. subtilis* SFF34 cells, the levels of cholesterol derivatives were calculated (Table 1). Significant amounts of campesterol and 25-hydroxycholesterol were detected after fermentation, although they were not detected in raw materials. 4-Cholesten-3-one levels increased slightly but cholesterol-5α, 6α-epoxide levels decreased as the fermentation proceeded. However, no significant changes in the 7β-hydroxycholesterol content, the most abundant cholesterol oxide, were observed.

In this study, *B. subtilis* SFF34, a bacterial strain producing cholesterol oxidase (Kim *et al.*, 2002; Rhee *et al.*, 2002) was applied to the fermentation of flatfish in order to reduce the cholesterol level. When bacterial cells were not inoculated, the cholesterol level in the flatfish reduced very slowly, which is thought to be due to natural bacterial

Table 1. Cholesterol derivative content before and after 12 days of fermentation of flatfish with or without of *B. subtilis* SFF34 inoculation

Compound	Retention time (min)	Content (μg/g)			
		Without inoculation		With inoculation	
		Before	After	Before	After
Campesterol	13.3	–[a]	10.4±1.1	–	10.6±1.0
Cholesterol-5α, 6α-epoxide	13.6	37.2±3.5	24.3±1.9	37.2±3.5	23.5±2.2
4-Cholesten-3-one	14.2	15.5±1.2	21.0±2.0	15.5±1.2	22.4±1.9
7β-Hydroxycholesterol	15.7	199.1±9.3	185.6±8.1	199.1±9.3	205.4±9.8
25-Hydroxycholesterol	16.5	–	22.6±2.1	–	20.3±2.2

[a]Not detected.

flora in the raw materials. When the *B. subtilis* cells were inoculated, cholesterol levels reduced more rapidly, indicating that the bacteria play an important role in the degradation of cholesterol in flatfish during fermentation (Fig. 1).

It has been reported that many cholesterol oxides are produced when cholesterol is degraded in the presence of oxygen. Regarding the oxidized cholesterol products, several derivatives such as 7α-hydroxycholesterol, 7β-hydroxycholesterol, α-epoxide, β-epoxide, cholestanetriol and 7-ketocholesterol have been identified in processed marine food products (Osada *et al.*, 1993). It has also been reported that trace amounts of cholesterol oxides were detected in fresh seafood, but several oxides, such as 7β-hydroxycholesterol, α-epoxide and 7-ketocholesterol, were detected at a significant level in processed seafood (Paniangvait *et al.*, 1995). A typical gas chromatogram of salted and dried anchovies showed many unidentified peaks, and, therefore, side chain cholesterol oxide derivatives other than 25-hydroxy cholesterol were probably present (Oshima *et al.*, 1993).

When cholesterol derivatives were analyzed during the fermentation, significant amounts of the major cholesterol oxides, including campesterol, cholesterol-5α, 6α-epoxide, 4-cholesten-3-one, 7β-hydroxycholesterol and 25-hydroxycholesterol, were detected (Fig. 3). However, no significant increase in cholesterol oxides was observed during the fermentation of flatfish, although cholesterol degradation increased when the bacterial cells were inoculated (Table 1). This result suggests that the bacteria also degrade the oxidized cholesterol derivatives. Therefore, it is thought that *B. subtlis* SFF34 has a great potential for reducing the cholesterol content of foods without increasing the levels of oxidized derivatives. *B. subtilis* is useful in food industries because it produces several extracellular hydrolytic enzymes such as amylases (Lee *et al.*, 2002; Baysal *et al.*, 2003), lipases (Eggert *et al.*, 2003) and proteases (Park *et al.*, 2003a). The bacteria have been widely used for the production of fermented foods in many countries (Kiers *et al.*, 2000; Beaumont 2002; Omafuvbe *et al.*, 2002; Sarkar *et al.* 2002; Park *et al.*, 2003b). They are also used as a host strain for the production of industrially important enzymes and many foreign proteins (Lee *et al.*, 2002; Sa-Pereira *et al.* 2002; Chiang *et al.* 2003; Moers *et al.* 2003).

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