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Effect of *Lentinus edodes* on the Growth of Intestinal Lactic Acid Bacteria

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As the growth factor of lactic acid bacteria, LD (trehalose) was isolated from *Lentinus edodes* by using silica gel column chromatography. LD induced the growth of *Bifidobacteria breve* and *Lactobacillus brevis*, which were isolated from human feces. LD selectively induced the growth of lactic acid bacteria among total microflora. When total intestinal microflora were cultured in the medium containing LD, it stimulated the growth of lactic acid bacteria and inhibited harmful enzymes, β-glucosidase, β-glucuronidase, and tryptophanase, of intestinal bacteria. LM, which was a monosaccharide from *L. edodes*, induced the growth of lactic acid bacteria but it seems to be invaluable *in vivo*. LH isolated from *L. edodes* by Sephadex G-100 column chromatography was not effective for the growth of lactic acid bacteria.

Key words : *Lentinus edodes*, Intestinal lactic acid bacteria

INTRODUCTION

Dietary habits has been dramatically changed in Korea since 1970. The death rate due to colon cancer in Korea has been increased rapidly year by year. It was reported that the rapid increase of colon cancer has been due to environmental changes, especially nutrition, rather than heredity. The relationship between dietary fat and the risk of colon cancer has been suggested from the results of epidemiological studies (Weisburger and Reddy, 1977).

Higher fat intake increases the growth of colon bacteria, capable of converting primary bile acid to the carcinogenic substance, as well as the induction of colon bacterial enzymes, β-glucuronidase, nitroreductase and tryptophanase, capable of converting procarcinogen, such as amines and glucuronic acid conjugates of xenobiotics, to carcinogen (Goldin *et al.*, 1977; Kinoshita and Gelvoin, 1978). Therfore, colon bacteria may have an important role in carcinogenesis. Indigestible oligosaccharides are able to influence not only stool bulk but also intestinal bacterial metabolism. The supply of indigestible oligosaccharides is an important factor for the growth of intestinal microflora in human colon (Huges *et al.*, 1993; Ishibashi *et al.*, 1993). The balance of these intestinal bacterial flora is closely related to human health conditions: soluble dietary fibers and indigestible oligosaccharides are fermented in the large intestine by intestinal bacteria and effective for improving the intestinal environment (Modler *et al.*, 1990; Tomomatsu *et al.*, 1994).

Because mushrooms were used as a health food for a long time in our country and Asian countries, we investigated the effect of mushrooms on the growth of lactic acid bacteria.

Among the mushrooms, *Lentinus edodes* is most effective for the growth of lactic acid bacteria (Han *et al.*, 1996). In the present paper, we isolated the component stimulating the growth of intestinal lactic acid bacteria from *L. edodes* and investigated the effect of this component on some intestinal bacterial enzymes converting procarcinogen to carcinogen.

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MATERIALS AND METHODS

Materials

The basidiocarps of *L. edodes* was purchased at Kyung Dong Market in Seoul, Korea. *p*-Nitrophenyl-β-D-glucuronide, *p*-nitrophenyl-β-D-glucopyranoside and tryptophan were purchased from Sigma Chem. Co. (U. S.A.). General anaerobic medium [GAM(-glu)] waspurchased from Nissui Seiyaku Co., Ltd. (Tokyo, Japan). The other chemicals were analytical reagent grade. *Bifidobacterium breve* JCM 1192 and *Lactobacillus brevis* II-46 were purchased from Japan Collection of Microorganisms. Total intestinal microflora were collected from a healthy Korean male (age, twenties).

Extraction and isolation of growth factor of lactic acid bacteria

L. edodes was extracted with distilled water at 80°C for 4h. The extract was filtered. The resulting supernatant was concentrated. The above procedure was repeated once more for the residue. The combined extract was applied to silica gel column chromatography using $CHCl_3$/MeOH (1:1) as an elution solvent: two fractions, LD and LM, were obtained. The fraction LD was applied to the silica gel column chromatography again (elution solvent, BuOH:pyridine:D.W. =85:10:10, v/v), and pure LD was isolated, which was a disaccharide. LD had the following spectral properties: FAB-MS m/z (negative ion): 343(M+). ^{1}H-NMR (500 MHz, D_2O): δ 3.54 (1H, t, 9.4), 3.73 (1H, dd, J= 3.8, 9.9), 3,84 (1H, dd, J=5.0, 11.9), 3.90-3.96 (3H, m), 5.28(1H, d, J=3.8). ^{13}C-NMR (125 MHz, D_2O) of LD: 63.2 (C6, C6'), 72.4 (C4, C4'), 73.7 (C2, C2'), 74.9 (C3, C3'), 75.2 (C5, C5'), 95.95 (C1, C1'). The fraction LM was applied to the same column chromatography, and pure LM was isolated, which was a monosaccharide. The combined water extract was applied to Sephadex G-100 and fractions containig molecules with M.W. higher than 10 kdaltons were collected and freeze-dried. This freeze-dried fraction was named as LH.

Culture of lactic acid bacteria or total microflora

Bifidobacterium breve JCM 1192, *Lactobacillus brevis* II-46 or total microflora of human or rat were inoculated into GAM(-glu) broth containing LD. Each medium was incubated at 37°C for 12 h and 20 h, and centrifuged at 3000 rpm for 10 min. The precipitate was suspended in 0.1M phosphate buffer and then measured enzyme activities. pH was measured for the resulting supernatant (Han *et al.*, 1993)

Enzyme assay

β-Glucosidase was assayed as follows: 1.2 ml each reaction mixture consisting of 0.4 ml of 2 mM p-nitrophenyl-β-D-glucopyranoside, 0.6 ml of 0.1 M phosphate buffer (pH 7.0) and 0.2 ml of enzyme solution was incubated for 30 min at 37°C and then stopped by adding 0.8 ml of 0.5 N NaOH. The stopped reaction mixture was centrifuged at 3000 rpm for 10 min and assayed the activity by measuring an absorbance at 405 nm.

β-Glucuronidase was assayed as follows: 1 ml each reaction mixture consisting of 0.2 ml of 2 mM *p*-nitrophenyl-β-D-glucuronide, 0.6 ml of 0.1 M phosphate buffer (pH 7.0) and 0.2 ml of enzyme solution was incubated for 30 min at 37°C. Incubation was terminated by adding 1 ml of 0.5 N NaOH. The reaction mixture was then centrifuged at 3000 rpm for 10 min and the activity was assayed by measuring an absorbance at 405 nm.

Tryptophanase was assayed as follows: the reaction mixture containing 0.2 ml of complete reaction mixture (2.75 mg pyrophosphate, 19.6 mg disodium EDTA dihydrate and 10 mg bovine serum albumin in 100 ml of 0.05 M potassium phosphate, pH 7.5), 0.2 ml of 20 mM tryptophan and 0.1ml of enzyme solution was incubated for 1 h at 37°C. The incubation was stopped by adding 2 ml of color reagent solution (14. 7 g *p*-dimethylaminobenzaldehyde, 52 ml H_2SO_4 and 948 ml of 95% ethanol) followed by centrifugation at 3000 rpm for 10 min. The enzyme activity was assayed by measuring an absorbance at 405 nm.

Assay of short chain fatty acid

For short chain fatty acid determination, the cultured broth was treated according to the method of Ueno *et al.* (1982). A 5 μl aliquot of extracts with internal standard pyridine was injected into a Hewlett Pakard Model HP5900A gas chromatography equipped with a flame ionization detector and 3 mm×20 m capillary column packed with HP 20M (carbowax 20M). The carrier gas was nitrogen, with a flow rate 12 psi. The temperatures of injector and detector were 155°C and 165°C, respectively. Initial and final column temperature were 60°C and 160°C, respectively. Temperature program rate was 10°C/min. The retention times of acetic, propionic, butyric and lactic acids were 3.00, 4.35, 6.13 and 4.32 min, respectively

RESULTS AND DISCUSSION

Isolation of growth factor for lactic acid bacteria from *L. edodes*

To isolate the growth factor for lactic acid bacteria from *L. edodes*, water extract of *L. edodes* was fractionated on the basis of polarity: ether, ethylacetate, butanol and residual (water) fractions. Growth activity and pH lowering activity of the cultured medium were measured. Effect of the residual (water) fraction on these activities was the best, followed by the butanol fraction. However, the ether fraction was not effective. The residual (water) extract of *L. edodes* was applied to silica gel column chromatography using $CHCl_3$/MeOH (1:1) as an elution solvent. We obtained the active fraction containing two compounds, Rf=0.12 and Rf=0.52 (deveolping solvent system, BuOH: pyridine: D.W=85:10:10). This active fraction was applied to silica gel column chromatography again and two compounds, LM which was a monosaccharide and LD which was a disaccharide, were isolated purely. LD was α-trehalose by carbon-13 and proton nuclear magnetic resonnance and FAB-mass. The extract of *L. edodes* contained 5% trehalose. We did not examine here the effect of LM on the growth of lactic

acid bacteria, because LM was a monosacchride which was, we thought, ineffective *in vivo*. In addition, the water extract of *L. edodes* was applied to Sephadex G-300 column chromatography. The fraction containing high molecular weight (>10,000 daltons) components only was isolated. The activity of lactic acid bacteria growth was measured for the LH fraction. However, the LH fraction was not effective.

Inhibitrory effect of LD from *L. edodes* on enzyme activities of intestinal microflora

L. brevis, B. breve or total microflora of human or rat were cultured in the medium containing 0.5% and 1% of LD or LH and then the final pH of these media was measured (Fig. 1). pH-lowering activity of LD was similar to that of lactulose in all tested bacteria. However, LH was not effective. *L. brevis, B. breve* or intestinal microflora of humans or rats were cultured for 12 and 20 h in the medium containing LD which was excellent on pH-lowering activity and then the enzyme activity (β-glucosidase, β-glucuronidase and tryptophanase) was measured.

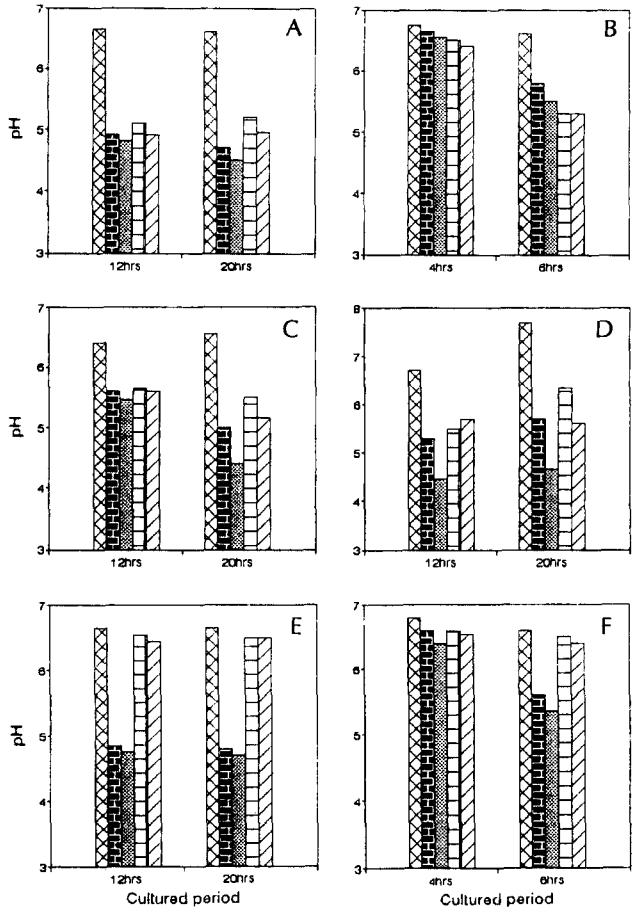

Fig. 1. Effect of LD or LH isolated from *L. edodes* on the final pH of the medium: A, *B. breve* was cultured in GAM(-glu) containing lactulose or LD; B, *L. brevis* was in GAM(-glu) lactulose or LD; C, rat intestinal microflora were in GAM(-glu) containing lactulose or LD; D, human intestinal microflora were in GAM(-glu) lactulose or LD; E, *B. breve* was in GAM(-glu) lactulose or LH; F, *B. breve* was in GAM(-glu) lactulose or LH. , control; , 0.5% lactulose; , 1% lactulose; , 0.5% LD or LH; , 1% LD or LH.

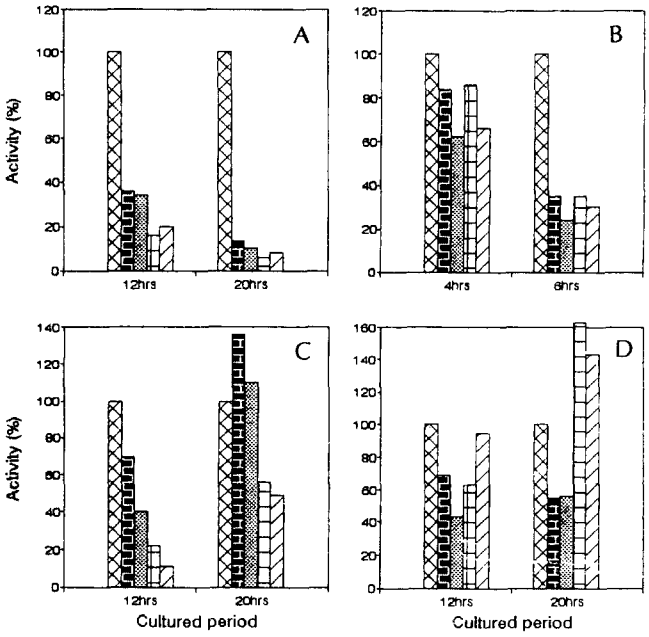

Fig. 2. Effect of lactulose or LD on β-glucosidase activity of *B. breve* (A), *L. brevis* (B), rat intestinal microflora (C) and human intestinal microflora (D). , control; , 0.5% lactulose; , 1% lactulose; , 0.5% LD or LH; , 1% LD or LH.

When *L. brevis, B. breve* or intestinal microflora of humans or rats were cultured for 12h by adding 0.5% or 1% of LD into the medium, β-glucosidase activities of *L. brevis* and *B. breve* were inhibited potently (Fig. 2). However, β-glucosidase activity of intestinal bacteria of human was not inhibited, although that of rat intestinal bacteria were weakly inhibited. When intestinal microflora of humans or rats were cultured for 12h in the medium containing LD and lactulose, β-glucuronidase activity was inhibited to 90 and 45%, respectively (Fig. 3). After 20h cultivation, the enzyme activity was inhibited to 97 and 83%, respectively. Thus, by the addition of LD or lactulose, the productivity of β-glucuronidase activity was inhibited effectively. LD was more effective than lactulose. When intestinal bacteria were cultured in the medium containing LD, tryptophanase activity was also inhibited to 87 and 92% (Fig. 4). Whether SCFA produced by intestinal lactic acid bacteria was induced by LD or not, SCFA concentrations in the cultured media were determined. As shown in Fig. 5, by adding LD to the medium, the concentration of lactic acid was 3 to 4-fold higher than that of the medium without LD. However, the concentration of acetic acid was not significantly in-

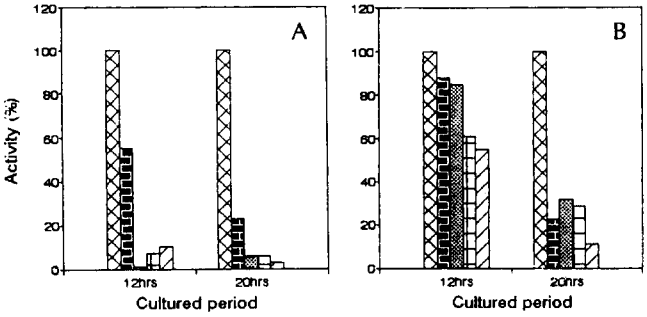

Fig. 3. Effect of lactulose or LD on β-glucurondase activity of rat intestinal microflora (A) and human intestinal microflora (B). , control; , 0.5% lactulose; , 1% lactulose; , 0.5% LD or LH; , 1% LD or LH.

duced. The other SCFA, butyric acid and propionic acid, were nearly not produced in the medium with and without LD.

β-Glucosidase, β-glucuronidase and tryptophanase were associated with conversion of procarcinogen to carcinogen. And there was epidemiological evidence that the polulation with high fecal β-glucuronidase and tryptophanase activity had a greater risk of colon cancer than the population with low fecal enzyme activity (Kim *et al.*, 1994). In addition to these evidences, Kim *et al.* (1992) reported that high colonic pH induces these enzymes. Conclusively, high colonic pH may induce these enzymes of intestinal bacteria and be related to the incidence of colon cancer.

Because indigestible oligosaccharides are not absborbed from stomach and small intestine, they are able to influence not only stool bulk but also intestinal bacterial metabolism. The supply of indigestible oligosaccharides, such as fructo-ologosaccharide, are the important factor for the growth of intestinal microflora in human colon (Huges *et al.*, 1993; Ishibashi *et al.*, 1993). In the present study, LD isolated from *L. edodes* lowered pH of the medium as well as inhibited the activity of some harmful enzymes, i.e. β-glucosidase, β-glucuronidase and tryptophanase. This result

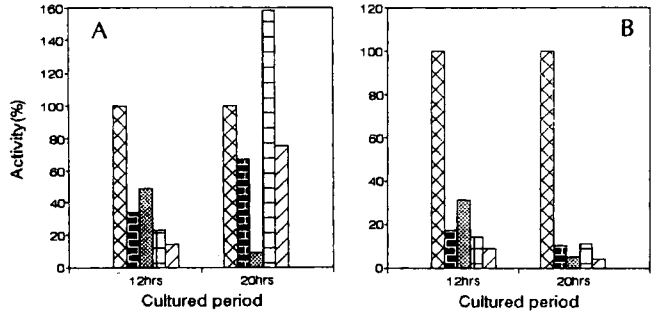

Fig. 4. Effect of lactulose or LD on tryptophanase activity of rat intestinal microflora (A) and human intestinal microflora (B). , control; , 0.5% lactulose; , 1% lactulose; , 0.5% LD or LH; , 1% LD or LH.

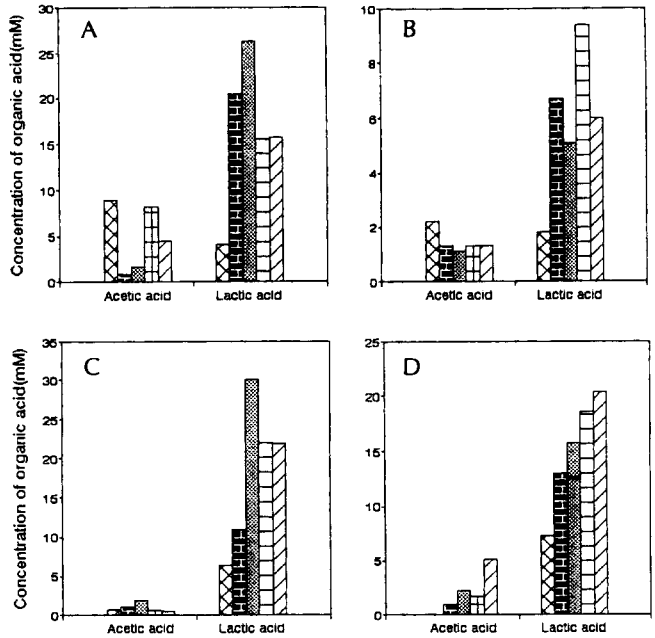

Fig. 5. Effect of lactulose or LD on short chain fatty acids produced by culturing *B. breve* (A), *L. brevis* (B), rat microflora (C) and human microflora (D). , control; , 0.5% lactulose; , 1% lactulose; , 0.5% LD or LH; , 1% LD or LH.

suggested that LD were not absorbed from the stomach and the small intestine of human, stimulated the growth of lactic acid bacteria and lowered the production of β-glucosidase, β-glucuronidase and tryptophanase of intestinal bacteria. These enzyme activities were induced by the ingestion of meat products, which might be the risk factor of colon cancer. Mushrooms are frequently consumed with meat in Korean diet and also known to have having an antitumor effect. Therefore, we suggest that *L. edodes* containing a bifidus factor like LD may be used for the improvement of intestinal condition. Also, this food containing a bifidus factor may have a beneficial role to decrease the harmful bacterial enzymes in intestine, which may have inhibitory effect of colon cancer.

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REFERENCES CITED

Goldin, B. and Gorbach, L., Alterations in faecal microflora enzymes related to diet, age, Lactobacillus supplements, and dimethylhydrazine. *Cancer*, 40, 2421-2426 (1977).

Han, M. J., Im, H.-Y., and Kim, D.-H., Rapid detection of growth facter of intestinal lactic acid bac-

teria. *Korean J. Fd. Hygiene*, 8, 91-95 (1993).

Han, M. J., Bae, E.-A., Rhee, Y.-K. and Kim, D.-H,. Effect of mushrooms on the growth of intestinal lactic acid bacteria. *Korean J. Fd. Sci. Technol.* 28, 947-952 (1996).

Hughes, D. B., and Hoover, D. G., Bifidobacteria, Activity and potential benefits. *Fd. Technol.* Jun., 120-124 (1993).

Ishibashi, N. and Shimamura,S, Bifidobacteria, Research and development in Japan. *Fd. Technol.,* Jun., 128-135 (1993).

Kim, D.-H., Kang, H.-J., Kim, S.-W., and Kobashi, K., pH-Inducible β-glucosidase and β-glucuronidase of intestinal bacteria. *Biol. Pharm. Bull.*, 40, 1667-1669 (1992).

Kim, D.-H., Kang, H.-J., Park, S.-H., and Kobashi, K., Characterization of β-glucosidase and β-glucuronidase of alkalotolerant intestinal bacteria. *Biol. Pharm. Bull.*, 17, 423-426 (1994).

Kinoshita, K. and Gelvoin, H. V., β-Glucuronidase catalized hydrolysis of benze-α-pyrene-glucuronide and binding to DNA. *Science*, 199, 307-309 (1978).

Modler, H. W., McKellar, R. C., and Yaguchi, M., Bifidobacteria and bifidogenic factors. *Can. Inst. Fd. Sci. Technol. J.,* 23, 29-41 (1990).

Sugawara, M., Charactristics of oligosaccharides and their physiological effects. *Bifidus*, 7, 1-12 (1993).

Tomomatsu, H., Health effects of oligosaccharides. *Fd. Technol.,* Oct., 61-65 (1994).

Ueno, K., Watanabe B. and Kino, M., Analysis of final products by gas chromatography. In Ueno, K. (Ed.). *Isolation and identification of anerobic bacteria.* Siane Press (Japanese), pp.45-62, 1982.

Weisbuger, J.H. and Reddy, B.S., Colon cancer: its epidemiology abd exoerimental production. *Cancer*, 40, 2414-2420 (1977)