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# Protective Effect of Antler in Experimental Colon Carcinogenesis

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**Abstract** – There are epidemiological evidences that the population with high fecal β-glucuronidase activity has greater risk of colon cancer than the population with low fecal β-glucuronidase. This relationship was investigated by using the mouse-dimethylhydrazine colon carcinogenesis model and the extract of antler which was a β-glucuronidase inhibitor. Mice with low fecal β-glucuronidase activity induced by administration of water and Folch's fraction of antler had significantly fewer aberrant crypts after injections of 1,2-dimethylhydrazine (DMH) than mice treated with DMH alone. The result supports the hypothesis that the inhibitor of β-glucuronidase such as antler extract can protect an animal against the induction of colon cancer.
**Key words** – β-glucuronidase, antler, colorectal cancer, aberrant crypt.

## Introduction

In order to improve the welfare of mankind in modern society, the eradication of disease is important. For thousand of years in Korea, oriental herbal medicines have played a key role in maintaining our well-being. Among these oriental medicines, the unossified velvet antler of *Cervus spp.* has been widely used as a valuable drug. From ancient times in China velvet antler has mainly been prescribed for the treatment of anaemia, exhaustion, tumors and for immunostimulation.

Epidemiological studies suggest that the dietary factors such as high animal fat and protein are prime factors in etiology of colon cancer (Goldin and Gorbach, 1976; Weisburger, 1977). The following hypothesis has been suggested by many researchers (Hill, 1975; Reddy *et al.*, 1975; Wynder and Reddy, 1974). Dietary fat changes bile acid and cholesterol metabolites quantitatively and qualitatively, as well as the concentration and metabolic activity of bacteria in the colon, which may produce carcinogens or carcinogenic compounds from bile acid and cholesterol metabolites. Intestinal bacteria may play an important role in liberating active key intermediates with chemical carcinogens inducing colon tumors in experimental animals (Goldin and Gorbach, 1976; Weisburger, 1971). We are currently engaged in the study of bacterial enzymes, especially β-glucuronidase, and the metabolism of DMH. It is thought that DMH injected into the rat may be conjugated with glucuronic acid immediately in the liver and secreted via the bile to the intestine. The glucuronic acid conjugate would be hydrolyzed by bacterial β-glucuronidase to free compound, producing a relatively highly localized concentration of this compound in the colonic mucosa. This active carcinogen causes the colonic cancer (Fiala, 1975 and 1977). If fecal β-glucuronidase or its related enzymes could be inhibited, the postulated glucuronic acid-conjugated carcinoma by DMH should be prevented. Although there are many reports of diversified pharmacological activities of extract from velvet antler of different *Cervus spp.*, few substantiated results have been obtained. Therefore, we investigated the effect of antler extracts on the carcinogenicity of DMH in the colon.

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## Experiment

**Chemicals** – 1,2-Dimethylhydrazine (DMH), *p*-nitrophenyl α-L-rhamnopyranoside, *p*-nitrophenyl β-

D-glucopyranoside, *p*-nitrophenyl β-D-glucuronide and methylene blue were purchased from Sigma Chem. Co. (U.S.A.). Brain heart infusion broth was from Difco Co. (U.S.A.)

**Extraction and fractionation of antler** – Antler (4 kg) was extracted with water (10 liter) at 80°C. Then the water extract (350 g) was fractionated sequentially with ethylether, ethylacetate and butanol; ethylether fraction (0.5 g), ethylacetate fraction (1. 1g), butanol fraction (1.2 g) and residual fraction (61.5 g). In addition, antler powder (500 g) stood at room temperature for 24h with 3 liter of $CHCl_3$:MeOH (2:1), filtered and evaporated (37 g, Folch's fraction).

**Culture of lactic acid bacteria or total microflora** – Total microflora of human were inoculated into GAM(-glu) broth containing several kinds of antler extracts. Each medium was incubated at 37°C for 20h and centrifuged at 3000 rpm for 10 min. The precipitate was suspended in 0.1M phosphate buffer and then measured some enzyme activities.

**Assay of enzyme activity** (Kim and Kobashi, 1986; Kim *et al.*, 1994) – All preparatory procedures for the enzyme determination were carried at 0-4°C. For the assay of β-glucosidase, the reaction mixture consisting of 0.4 ml of 2 mM *p*-nitrophenyl β-D-glucopyranoside, 0.6 ml of 0.1M phosphate buffer, pH 7.0, and 0.2 ml of enzyme solution was prepared. For the assay of β-glucuronidase, the reaction mixture consisting of 40 μl of 10 mM *p*-nitrophenyl β-D-glucuronide, 0.76 ml of 0.1M phosphate buffer, pH 7.0, and 0.2 ml of enzyme solution was prepared. For the assay of α-rhamnosidase, the reaction mixture consisting of 0.4 ml of 2 mM *p*-nitrophenyl α-L-rhamnopyranoside, 0.6 ml of 0.1M phosphate buffer, pH 7.0, and 0.2 ml of enzyme solution was prepared. Each reaction mixture was incubated at 37°C for 30 min and reaction mixture was stopped by adding 0.25N NaOH. The stopped reaction mixtures were centrifuged at 1000×g for 20 min. Then, the absorbance of the reaction mixture was measured at 405 nm within 10 min.

Tryptophanase activity was determined as follows (Chung *et al.*, 1975): 0.4 ml of reaction mixture (2.75 mg pyrophosphate, 19.6 mg disodium EDTA dihydrate and 10 mg bovine serum albumin in 100 ml of 0.05M potassium phosphate, pH 7.5), 0.4 ml of 0.02M L-tryptophan and 0.1 ml of the enzyme solution were incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.1 ml of 60% trichloroacetic acid and the precipitated protein was removed by centrifugation at 5000×g for 10 min. The supernatant was assayed for indole. Indole was determined as follows: 0.5 ml of the supernatant was assayed colorimetrically at 550 nm for indole with 2.0 ml of the color reagent (14.7 g *p*-dimethylaminobenzaldehyde, 52 ml $H_2SO_4$ and 948 ml of 95% ethanol).

**Partial purification of β-glucuronidases** – *E. coli* HGU-3 β-glucuronidase was purified as follows. *E. coli* HGU-3, alkalotolerant intestinal bacterium of human, was cultured in 10L BHI broth and collected at 3000×g for 3 min at 4°C. The precipitate was suspended with 50 mM phosphate buffer, pH 7, and sonicated. The resulting supernatant was fractionated with ammonium sulfate and then applied to DEAE-cellulose column chromatography and saccharic acid 1,4-lactone-legand-affinity column chromatography. The specific activity of the purified enzyme was 0.125 μmol/min/mg protein.

Lysosomal β-glucuronidase was partially purified from rat liver according to Brand and Hess (1983) and the specific activity was 0.132 μmol/min/mg protein.

**Animal** – The animals were housed in plastic cages with wire tops. Unless otherwise stated, the animals were fed Samyang *ad libitum* and had free access to water.

The mouse (ICR male, 15 g) was divided to 6 group. Three groups (DMH-treated group (II), DMH +Folch's fraction-treated group (III), DMH+water Ex-treated group (IV)) were given weekly *s.c.* injection of DMH (20 mg per kg of body weight per week) for 10 weeks. The other groups (normal control group) was given weekly *s.c.* injections of saline not containing DMH. Group I and Group II fed the usual powdered laboratory diet (10 g). Group III fed the powdered diet containing the Folch's fraction of antler (20 mg/mouse/day). Group IV fed the powdered diet containing the water extract of antler (20 mg/mouse/day). After last injection, all animals were fed 5 weeks and then sacrificed.

**Visualization and quantification of aberrant crypts (AC)** – After sacrifice of animals, the colon was removed immediately, flushed with Kreb's Ringer, slit open from caecum to anus, and fixed in 10% buffered formalin. Following the previously cited protocol (McLellan and Bird, 1988: Rao *et al.*, 1993), the fixed colons were stained with methylene blue and the colons were assessed for AC by using the light microscope. The parameters used to assess

**Table 1.** Inhibitory effect of the antler extracts on β-glucuronidase of intestinal bacteria

| Extract | $IC_{50}$ (mg/ml) | |
|---|---|---|
| | β-glucosidase | β-glucuronidase |
| Ethylether-extracted Fr. | >2 | >2 |
| Ethylacetate-extracted Fr. | >2 | >2 |
| Butanol-extracted Fr. | >2 | 0.48 |
| Residual Fr. | >2 | >2 |

Enzyme activity was 0.125 μmole/min/mg protein.

the colons were occurrence and distribution of AC. The occurrence was measured by quantitating the mean number of foci of AC per colon. The number of AC per focus (ACF) was also recorded. To determine the distribution of AC, the colon was divided into two sections. Rectum (R) represented the first 2 cm from the rectal end. Sigmoidal (S) and descending (D) colons were the next 2.5cm from the rectum.

## Results

**Inhibition of some enzymes by the antler extracts** – The inhibitory effect of water soluble fractions of antler against β-glucuronidase of *E. coli* HGU-3 is shown in Table 1. Butanol-extracted fraction of velvet antler only inhibited HGU-3 β-glucuronidase. Its $IC_{50}$ was 0.48 mg/ml. However, the other solvent-extractable fractions of velvet antler did not inhibit it.

**Inhibitrory effect of velvet antler extracts on the production of some enzymes of intestinal microflora** – To investigate the effect of velvet antler extracts on pH-lowering activity and the activities of some harmful enzymes of intestinal bacteria, total microflora of human were inoculated into the medium containing 0.25% or 0.5% velvet antler extracts (Table 2). On pH-lowering activity, ether-extracted fraction was the best, followed by ethylacetate-extracted fraction and residual fraction. Most of the antler extracts induced β-glucosidase of intestinal bacteria: Ethylacetate-extracted fraction and water extracts were the highest. However, most of antler extracts inhibited the activity of β-glucuronidase of intestinal bacteria: Ether-extracted fraction and water-extracted fraction were the best, followed by ethylacetate-extracted fraction and residual fraction. α-Rhamnosidase also was inhibited by most of antler

**Table 2.** Effect of velvet antler extracts on final pH and the harmful enzymes activities of intestinal bacteria

| Group | pH | Enzyme activity (%) | | | |
|---|---|---|---|---|---|
| | | β-Glucosidase | β-Glucuronidase | β-Rhamnosidase | Tryptophanase |
| Control | 6.2±0.04[1)] | 100±15.5 | 100±7.5 | 100±12.5 | 100±18.9 |
| Lactulose | | | | | |
| 0.25% | 5.1±0.08[a] | 141±12.1[a] | 40±2.3[a] | 145±15.9[a] | 104±16.4 |
| 0.5% | 4.9±0.06[a] | 126±8.5 | 16±1.5[a] | 38±6.3[a] | 121±21.1 |
| Water extract | | | | | |
| 0.25% | 5.8±0.06[a] | 169±12.3[a] | 68±5.5[a] | 74±16.9 | 115±15.5 |
| 0.5% | 5.8±0.06[a] | 139±15.5[a] | 63±12.1[a] | 91±18.5 | 125±11.4 |
| Ethylacetate | | | | | |
| extract | 5.5±0.04[a] | 168±15.9[a] | 72±5.2[a] | 80±20.5 | 120±12.1 |
| 0.25% | 5.5±0.04[a] | 187±14.3[a] | 73±5.5[a] | 82±18.9 | 123±13.5 |
| 0.5% | | | | | |
| Ether extract | | | | | |
| 0.25% | 5.4±0.06[a] | 118±9.8 | 57±4.3[a] | 65±19.5[a] | 154±23.2[a] |
| 0.5% | 5.5±0.04[a] | 131±10.2[a] | 56±7.4[a] | 79±15.5 | 147±18.5[a] |
| BuOH extract | | | | | |
| 0.25% | 5.8±0.06[a] | 211±41.4 | 95±8.2 | 69±16.2[a] | 145±25.5 |
| 0.5% | 5.7±0.04[a] | 94±15.5 | 64±21.5[a] | 76±17.2 | 146±15.4[a] |
| Residue | | | | | |
| 0.25% | 5.6±0.07[a] | 113±16.8 | 80±5.5[a] | 86±14.5 | 136±12.1[a] |
| 0.5% | 5.6±0.05[a] | 129±12.4 | 58±15.8[a] | 105±12.1 | 104±15.5 |

The activities of β-glucosidase (0.13 μmole/min/ml medium), β-glucuronidase (0.043 μmole/min/ml medium), α-rhamnosidase (0.02 μmole/min/ml medium) and tryptophanase (0.004 μmole/min/ml medium) were taken as 100%.
[1)]Mean±SD

**Table 3.** Effect of antler extract on intestinal bacterial enzymes of mice treated with DMH

β-Glucosidase

| Group | Enzyme activity(mole/min/g wet feces) | | |
|---|---|---|---|
| | before[1)] | during | after |
| Normal | 1.55±0.61[2)] | 1.36±0.28 | 1.84±0.66[b] |
| DMH-treated | 1.28±0.14 | 2.22±0.14[a] | 1.57±0.08[a,b] |
| DMH+Folch's Fraction-treated | 1.28±0.06 | 1.61±0.79 | 1.62±0.81 |
| DMH+Water Ex-treated | 1.45±0.38 | 1.65±0.03 | 1.41±0.48 |

β-Glucuronidase

| Group | Enzyme activity (mole/min/g wet feces) | | |
|---|---|---|---|
| | before | during | after |
| Normal | 0.43±0.15 | 0.55±0.33 | 0.70±0.13[a] |
| DMH-treated | 0.49±0.10 | 1.17±0.31[a] | 1.07±0.28[a] |
| DMH+Folch's Fraction-treated | 0.40±0.06 | 1.27±0.12[a] | 0.58±0.17[a,b] |
| DMH+Water Ex-treated | 0.40±0.15 | 0.91±0.03[a] | 0.43±0.04[b] |

Tryptophanase

| Group | Enzyme activity (mole/min/g wet feces) | | |
|---|---|---|---|
| | before | during | after |
| Normal | 0.008±0.002 | 0.013±0.011 | 0.018±0.004[a] |
| DMH-treated | 0.018±0.011 | 0.013±0.011 | 0.023±0.004 |
| DMH+Folch's Fraction-treated | 0.013±0.004 | 0.025±0.007[a] | 0.015±0.007[b] |
| DMH+Water Ex-treated | 0.018±0.004 | 0.038±0.004[a] | 0.013±0.004[a,b] |

[1)] before, before the administration of DMH or DMH+antler extract; during, during the administration of DMH or DMH+antler extract; after, after the administration of DMH or DMH+antler extract.
[2)]Mean±SD
[a]statistically significant, compared to that of before (enzyme activity) of each group ($p<0.05$).
[b]statistically significant, compared to that of during (enzyme activity) of each group ($p<0.05$).

extracts: butanol-extracted fraction and ether-extracted fraction were the highest. However, tryptophanase was not affected.

**Inhibitory effect of velvet antler extract on fecal bacterial enzymes in mice** – The effect of antler, which inhibited bacterial β-glucuronidase *in vitro*, on fecal bacterial enzymes in mice was investigated *in vivo* by using the mouse-DMH colon carcinogenesis model (Table 3). By injection of DMH to mouse weekly for ten weeks, fecal β-glucuronidase was induced 2-fold and fecal β-glucosidase was weakly induced. However, tryptophanase was not induced. During administering velvet antler extract, the β-glucuronidase activities of the group treated with DMH were not affected, compared to the group treated with DMH alone. However, The enzyme activities were slowly decreased from 5 weeks administeration of velvet antler and was decreased to 70% of the group treated with DMH alone 10 weeks after administering it. On the case of mice untreated with DMH, the enzyme activity was not affected by administering antler extract except tryptophanase (Data not shown).

**Effect of antler extracts on colon carcinoma** – Whether the population with high fecal β-glucuronidase activity had greater risk of colon cancer than the population with low fecal β-glucuronidase or not was investigated by using the mouse-dimethylhydrazine colon carcinogenesis model and Folch's fraction and water fractions of velvet antler, which was a β-glucuronidase inhibitor *in vitro* (Table 4). The incidence of AC in the 4 groups of mice was as follows: Those of group I, II, II and IV were 0%, 90%, 50% and 40%, respectively. The average number of ACF were 0, 8.12, 1.14 and 1.40 AC/colon, respectively. Particularly, mice with low fecal β-glucuronidase activity, produced by consumption of the water frac-

**Table 4.** Effect of antler extract on formation of AC in mouse colon induced by DMH

| Group | Incidence | No. ACF/Colon | No. AC/Focus | Distribution | |
|---|---|---|---|---|---|
| | | | | R | S & D |
| Normal | 0/10 | 0 | 0 | 0 | 0 |
| DMH-treated | 9/10 | 8.123±.32[1)] | 2.40±0.92 | 61.6 | 38.4 |
| DMH+Folch's Fraction-treated | 5/10 | 1.14±0.34[a] | 1.17±0.37[a] | 24.0 | 76.0 |
| DMH+Water ex-treated | 4/10 | 1.40±0.52[a] | 2.2±0.54[a] | 4.7 | 95.3 |

[1)]Mean±SD
[a]statistically significant, compared to that of DMH-treated group ($p<0.05$).

tion or Folch's fraction of velvet antler, had significantly fewer AC after injections of DMH than mice treated with DMH only. The of inhibition mode was similar to that of β-glucuronidase *in vitro*.

## Discussion

In human intestine, the absorption of ammonia and some compounds is affected by intestinal pH. Low pH of intestinal environment decreases the absorption of ammonia. This low pH, it was suggested, could inhibit the colon cancer promotion. Therefore, pH lowering activity basically is important from the prevention of colon cancer. The mechanism of the prevention of lactic acid bacteria against colon cancer, we thought, may be similar to it. The good pH-lowering activity of ether-extracted fraction may be corelated with the growth of lactic acid bacteria. Furthermore, these velvet antler extracts inhibited β-glucuronidase activity, which is the important enzyme associated with conversion of procarcinogen to carcinogen and causing the colon cancer.

There are epidemiological evidences that the population with high fecal β-glucuronidase activity had a greater risk of colon cancer than the population with low fecal β-glucuronidase. This relationship was investigated by using the mouse-DMH colon carcinogenesis model and the water extract of velvet antler as a β-glucuronidase inhibitor. The mouse colon carcinoma model was made by the injection of DMH according to the method of Reddy *et al.* (1975). By using this model, ACs on the colon of mouse were produced well and bacterial β-glucuronidase was induced about 2-fold. Therefore, formation of ACs was related to bacterial β-glucuronidase. This result supported epidemiological evidence of colon cancer. The antler extract was used because the antler was the good inhibitor *in vitro*. Mice with low fecal β-glucuronidase activity, produced by consumption of the water extract of velvet antler had significantly fewer AC after injections of DMH than mice treated with DMH alone. The average number of AC, size of AC and mortality were improved by treating the antler which was a good inhibitor of β-glucuronidase. The inhibitory effect of the water extract of antler was potent. It is suggested that the antler extract could reduce the risk factor of colon cancer as well as liver damage (Kim *et al.*, 1994) by inhibiting the hydrolysis to glucuronides of proximate metabolism.

This result supports the hypothesis that the population with high β-glucuronidase activities had a greater risk of colon cancer than those with low β-glucuronidase and its inhibitors can protect against the induction of colon cancer.

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