Effects of Chinese yam storage protein on formation of aberrant crypt foci in 1,2-dimethylhydrazine-treated mice

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Summary
The effects of Chinese yam (*Dioscorea opposita* Thunb.) dietary storage protein on the formation of 1,2-dimethylhydrazine (DMH)-induced aberrant crypt foci (ACF) were investigated in large intestines of mice. Feeding with Chinese yam storage protein resulted in significant suppression of DMH-induced ACF formation in all mice examined. Dietary yam and yam storage protein suppressed aberrant crypt (AC) growth and significantly increased terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) positive cell numbers in large intestines. An in vitro study showed that the digestion products of yam storage protein induced cellular apoptosis. In agreement with this finding, the present study also clearly demonstrated that DMH-induced ACF formation was inhibited by dietary yams. Hence, daily ingestion of yams may suppress colon carcinomas in humans.

Keywords
yam (Nagaimo); aberrant crypt foci; colon cancer; 1,2-dimethylhydrazine; storage protein

Yam (*Dioscorea* spp., *Dioscoreaceae*) is classified as monocotyledonous, but it is considered to be closely related to dicotyledonous plants because a second cotyledon remains undeveloped in the embryo [1]. Yam tubers (*Dioscorea* spp.) are widely consumed in Asia, Africa and Central America. Chinese yam (*Dioscorea opposita* Thunb.) is highly nutritious and possesses numerous functional components including diosgenin, allantoin, choline, polyphenol oxidases and proteins. The plant is cultivated and consumed in Japan. Dried Chinese yam tubers have been also used in herbal medicines since ancient times [2].

Dioscorin, the storage protein of yam plants, accounts for 85% of total protein in yam plant tubers. Several food and biological functionalities of dioscorin have been reported, including inhibitory effects on angiotensin-converting enzymes (ACE) [3] and trypsins [4], as well as antioxidant properties [5, 6]. Lectin is a major component of yam storage protein, its amino acid sequence being similar to that of dioscorin [7].

Rates of colorectal cancer have increased in Japan in recent years, reflecting changes in diet and lifestyle. Previously, we observed greater suppression of aberrant crypt foci (ACF) by Chinese yam than potato or sweet potato in 1,2-dimethylhydrazine (DMH)-treated mice [8, 9]. Moreover, DNA microarray analyses indicated a relationship between Chinese yam consumption and elevated expression of apoptosis-related genes in the large intestine. These observations suggested that yam storage protein potently suppresses the formation of ACF. In the present study, we investigated the effects of Chinese yam (*Dioscorea opposita* Thunb.) storage protein on formation of aberrant crypt foci in 1,2-dimethylhydrazine-treated mice.

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MATERIALS AND METHODS

Preparation of Chinese yam storage protein

Chinese yam tubers were purchased from Tokachi Kawanishi Agriculture (Hokkaido, Japan). Skin was removed by scraping and the tubers were freeze-dried and crushed into fragments. The resulting yam powder was mixed with distilled water at 4 °C. After 24 h, the residue was removed by centrifugation (7000 ×*g*, 4 °C, 40 min). Protein was precipitated from the supernatant by addition of ammonium sulphate to saturation. The resulting precipitate was dialysed and freeze-dried. A part of this freeze-dried product was lysed with buffer containing sodium dodecylsulphate (SDS), electrophoresed on 12.5% SDS-polyacrylamide gels under reducing conditions and stained with Coomassie Brilliant Blue 2.5 $g \cdot l^{-1}$ [10].

Digestion of Chinese yam storage protein

Freeze-dried protein samples were dissolved in water (40%) and protease A (Amano Enzyme, Nagoya, Japan) was added to a final enzyme concentration of 2.4 $g \cdot l^{-1}$. After incubation for 6 h at 37 °C, the solution was heated at 65 °C for 30 min to inactivate proteases and was freeze-dried [11]. A portion was lysed in a buffer containing SDS, electrophoresed on 12.5% SDS-polyacrylamide gels under reducing conditions and stained with Coomassie Brilliant Blue 2.5 $g \cdot l^{-1}$.

Animals and diet

Four-week-old male Institute of Cancer Research (ICR) mice were obtained from Japan Clea (Tokyo, Japan) and housed in isolator cages (7 mice per cage) at 20 °C under a 12 h light–dark cycle. Mice were randomly divided into 4 groups. After acclimation to the test diet for 10 days, each animal was given 1,2-DMH-HCl by intraperitoneal administration (i.p.) once a week for 8 weeks. Experimental diets for mice were based on the AIN-93 diet [12]. This diet was supplemented with storage protein (10 $g \cdot kg^{-1}$) or freeze-dried yam powder (250 $g \cdot kg^{-1}$). All protocols involving animals were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the Obihiro University Guidelines.

Identification of aberrant crypt foci

Aberrant crypt foci (ACF) in large intestinal villi were identified and quantified as described previously [13, 14]. Following excision of the large intestine from mice under ether anaesthesia, a section between the cecum and the vent was removed and rinsed in cold saline. Subsequently, cells were fixed overnight in phosphate-buffered saline containing 4% paraformaldehyde and were stained for 30 min at room temperature using 3% methylene blue in saline. ACF were counted throughout the large intestine using a microscope.

Quantitative analysis of apoptosis in the large intestine

In order to observe apoptotic cell formation, samples of large intestine were fixed in phosphate-buffered saline containing 4% paraformaldehyde, dehydrated using increasing concentrations of ethanol and embedded in paraffin. Paraffin sections were stained using a solution containing 10 $mmol \cdot l^{-1}$ Tris-HCl (pH 7.4), 10 $mmol \cdot l^{-1}$ EDTA, 100 $mmol \cdot l^{-1}$ NaCl and 500 $ng \cdot ml^{-1}$ 4',6-diamidino-2-phenylindole (DAPI) and were used in terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) experiments. Apoptotic cells were counted by a method described in our previous studies [15, 16].

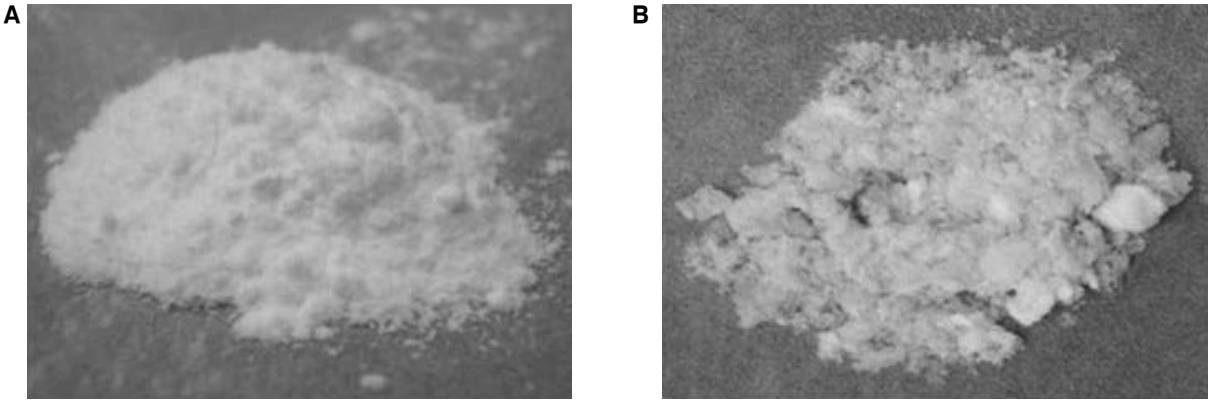

Fig. 1. Fractionation of storage proteins from Chinese yam.

A – freeze-dried sample of Chinese yam, B – freeze-dried sample of storage protein from Chinese yam.

Cell cultures

Caco-2 cells from the Riken Gene Bank (Tsukuba, Japan) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mmol·l^{-1} glutamine and 0.1 mmol·l^{-1} nonessential amino acids. For routine maintenance, Caco-2 cells were passaged before becoming confluent [17].

Apoptotic cell characterization and quantification

Cells were cultured on LAB-TEK chamber slides (Thermo Scientific Nunc, Rochester, New York, USA) in the presence or absence of storage protein digestion products in DMEM containing 0.1% bovine serum albumin (BSA) for the indicated periods. Subsequently, cells were fixed in phosphate buffered saline (PBS) containing 3% paraformaldehyde for 20 min and stained with DAPI for 10 min at room temperature. Apoptotic cells were identified by characteristic fragmented DAPI-stained nuclei under a fluorescent microscope [16]. To identify apoptotic cells, TUNEL staining was performed using a TACS 2TdT-Fluor In Situ Apoptosis Detection Kit (Trevigen, Gaithersburg, Maryland, USA).

Statistical analysis

Statistical analyses were performed using ANOVA and Scheffe's test. Differences were considered significant when $p < 0.05$.

RESULTS

Initially, we analysed the contents of the major component of freeze-dried Chinese yam powder (Fig. 1). Freeze-dried yams had a protein content of approximately 10%. Fig. 1 and Fig. 2 show a freeze-dried yam sample and SDS-PAGE patterns of the water-soluble protein fractions, respectively. A major band of water-soluble protein was observed to have a molecular weight of 29–30 kDa (Fig. 2), which was essentially the same as that of the yam tuber storage protein dioscorin [10]. These observations indicated that Chinese yams from northern Japan contain dioscorin as the major water-soluble protein.

Throughout the experimental feeding period, the body and liver weights of animals fed AIN-93 plus yam or 1% storage protein were essentially

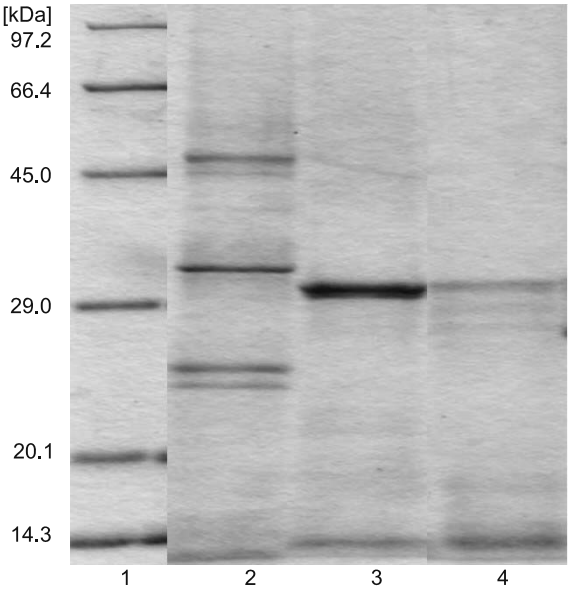

Fig. 2. SDS-polyacrylamide gel electrophoresis profiles of storage protein from Chinese yam.

1 – molecular weight marker, 2 – protease, 3 – storage protein from Chinese yam, 4 – protease-digested Chinese yam storage protein.

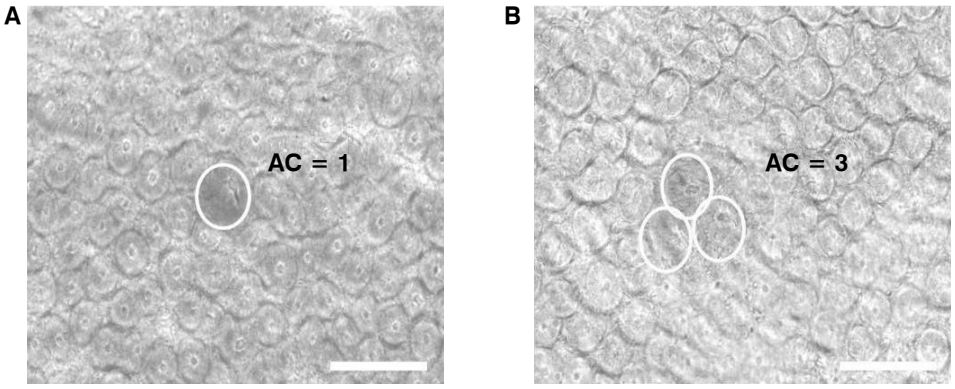

Fig. 3. Effects of dietary storage protein from Chinese yam on ACF formation in DMH-treated mice.

A, B – formation of aberrant crypt foci (ACF) induced by DMH in large intestinal villi (indicated by circle; ×40; the bar indicates a distance of 125 μm).

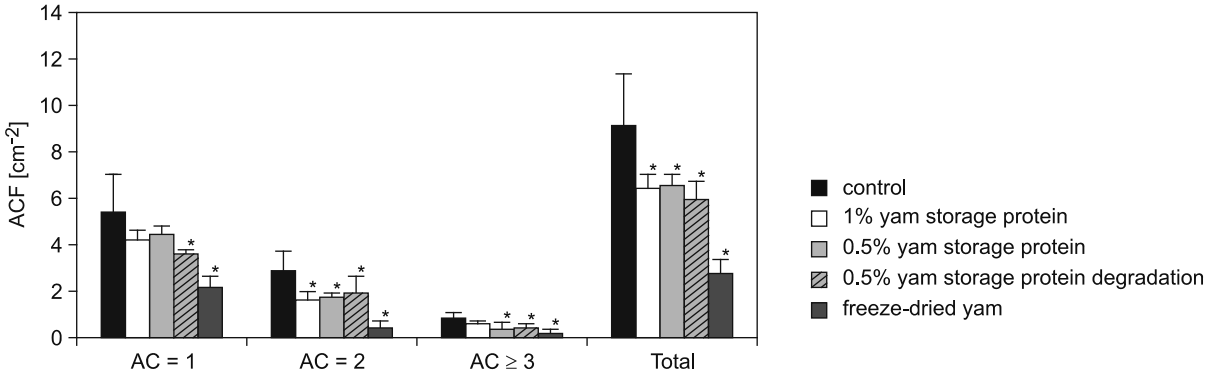

Fig. 4. Effects of dietary storage protein from Chinese yam on ACF formation and growth of AC in DMH-treated mice.

Numbers of ACF and AC. Data are presented as mean ± standard deviation of 7 experiments.
* – significant differences ($p < 0.05$) from the control.

identical to those of animals fed AIN-93 alone. After 8 weeks of feeding, DMH-induced ACF was found in the large intestines of control animals. Staining of ACF was more intense than normal intestinal tissue, and indicated a three-dimensional structure (Fig. 3). Formation of ACF in animals fed the AIN-93 diet containing yams or storage protein was almost 40% less than that in the control group (Fig. 4). These results indicated that Chinese yam and its storage protein potently inhibited formation of ACF.

In general, the foci of aberrant crypts varied from single crypts to plaques of multiple crypts as the DMH-induced phenotypes progressed. Therefore, we divided ACF into three levels according to the number of aberrant crypts (AC 1, 2 and 3). Dietary yam and yam storage protein significantly suppressed the growth of aberrant crypts (Fig. 4). Samples of extracted intestines were stained with DAPI and apoptotic cells were identified using TUNEL assays (Fig. 5). Numbers of TUNEL-positive cells in the large intestine were significant-

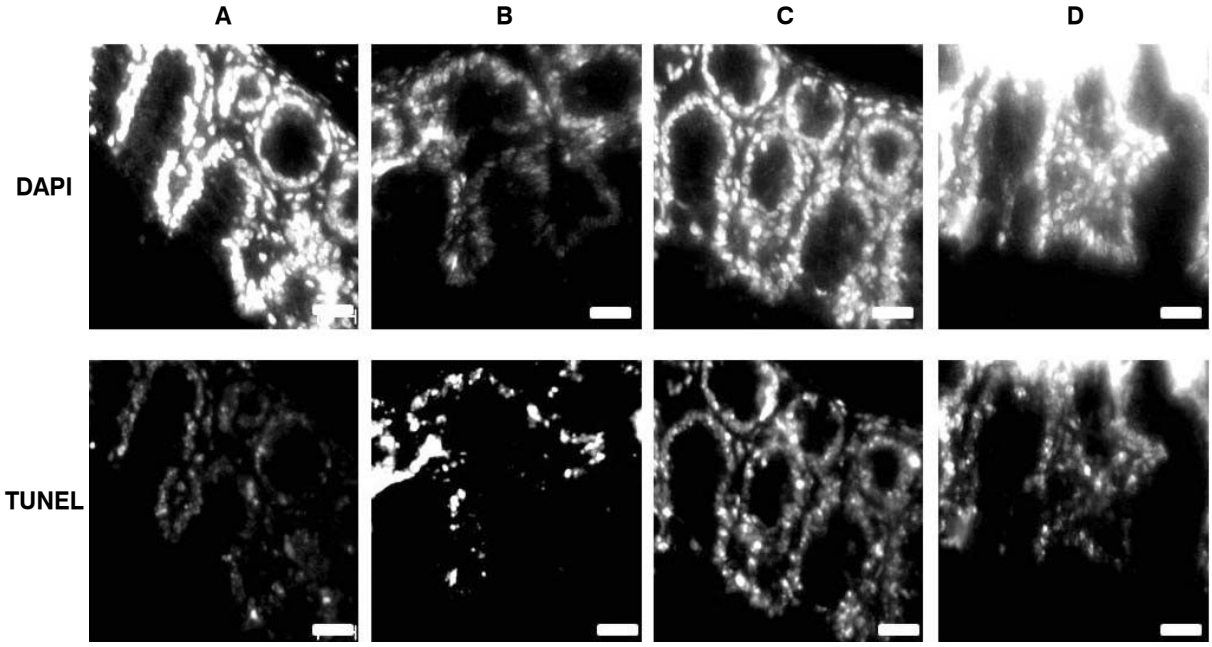

Fig. 5. Effects of storage protein on large intestinal apoptosis in DMH-treated mice.

Intestines stained with 4',6-diamidino-2-phenylindole (DAPI) and TUNEL assay. The bar indicates a distance of 25μm.
A – control, B – 1% yam storage protein, C – 0.5% degraded yam storage protein, D – freeze-dried yam.

ly increased in yam storage protein-supplemented animals (Fig. 6).

Because it is difficult to accurately quantify apoptosis in vivo, we investigated the effects of storage protein digestion products on cellular proliferation and apoptosis in Caco-2 human colon cancer cells. Fig. 2 shows the SDS-PAGE profile of protease-digested yam storage protein, indicating decomposition after treatment with a serine protease. Digestion products of the yam storage protein significantly suppressed cell proliferation in a time-dependent manner (Fig. 7). Nuclear morphology of DAPI-stained Caco-2 cells was characterized by aggregated and fragmented nuclei after treatment with storage protein digestion products. Subsequent TUNEL staining revealed only fragmented DNA in these cells (Fig. 8). Thus, treatment with storage protein digestion products induced apoptosis in Caco-2 cells.

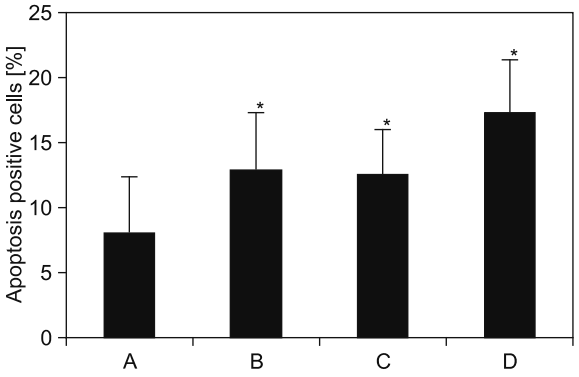

Fig. 6. The percentage of apoptotic cells on large intestinal apoptosis in DMH-treated mice.

A – control, B – 1% yam storage protein, C – 0.5% degraded yam storage protein, D – freeze-dried yam.
* – a significant difference ($p < 0.05$) from the control.

DISCUSSION

The presented data show that the yam storage protein dioscorin suppressed the formation of ACF in DMH-treated mice. Several research groups have investigated functions of dioscorin. In in vivo studies, dioscorin was shown to have both antioxidant and radical-scavenging activities [18–20]. Humans are reportedly exposed to the potent carcinogen DMH and other hydrazines through both environment and diet [21]. Alarmingly, DMH has been shown to induce colon cancer in experimental animals [22]. DMH is metabolized in the liver to diazonium ions, which are known to elicit oxidative stress [23]. Hence, dietary dioscorin may prevent formation of ACF through its antioxidant properties.

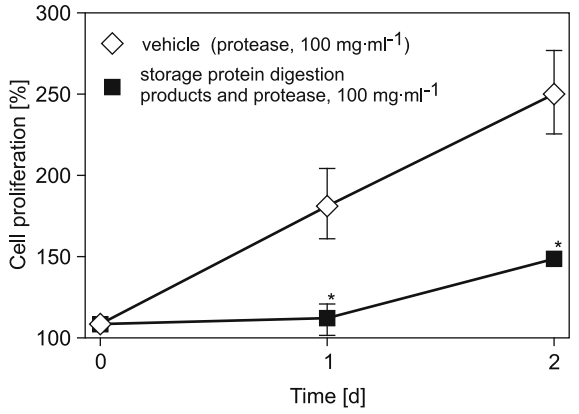

Fig. 7. Effects of storage protein digestion products on cell proliferation in Caco-2 cells.

Data are presented as mean ± standard deviation of 2 independent experiments conducted in triplicate.
* – a significant difference ($p < 0.05$) from the vehicle (same day).

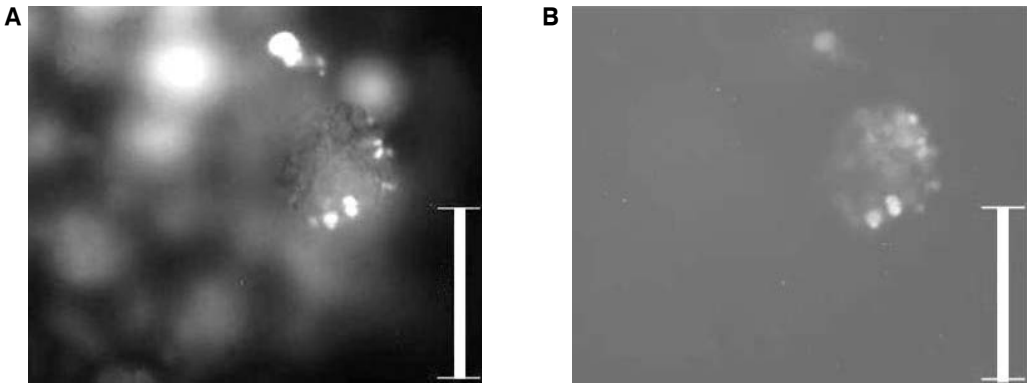

Fig. 8. Effects of storage protein digestion products on apoptosis in Caco-2 cells.

A – cells stained with 4',6-diamidino-2-phenylindole (DAPI), B – TUNEL assay. The bar indicates a distance of 25μm.

Alternatively, as treatment with DMH has been shown to trigger WNT/β-catenin [24] signal and mitogen-activated protein (MAP) kinases cascades that promote formation of ACF [25], dioscorin may also act on these pathways. However, no marked change in the expression of related genes were observed in our previous study [8] and further studies are required to determine whether dietary yam or its storage protein dioscorin affect these pathways.

Injection of DMH into mice induces early AC, which may progress to advanced ACF and tumours. In the present study, dietary administration of yam or its storage protein suppressed development and growth of AC. Other researchers have suggested that dioscorin exhibits systemic and mucosal immunomodulatory activities after ingestion in vivo. In particular, dioscorin was shown to induce Peyer's patches [26] and secretion of immunoglobulin A (IgA) from mucosa [27]. Because IgA deficiency has been associated with increased incidence of colon cancer, future investigations of the relationship between yam storage protein and immune function may provide important insights in the problem.

In the present study, the yam storage protein fraction induced colonic cell apoptosis in DMH-treated mice. This observation supports the results of our previous DNA microarray study, which suggested that dietary yam induces colonic cell apoptosis in DMH-treated mice through immune and apoptotic mechanisms. Further studies are required to elucidate the detailed mechanisms.

In the present in vitro experiments, digestion products of the storage protein inhibited Caco-2 cell proliferation. Previous studies identified antioxidant and antiproliferative properties of these protein products and have revealed that they act as dietary supplements. Although the mechanisms by which antioxidants inhibit cancer cell proliferation are unclear, induction of the tumour suppressor p53 by antioxidants has been reported [28]. Moreover, expression of p53 induced apoptosis in colon cancer cells. Caco-2 cells express low levels of p53 under normal culture conditions [28]; therefore, the present data may reflect induction of p53 by yam storage protein digestion products, and subsequent suppression of Caco-2 cell proliferation. Although the relationship between formation of ACF and suppression of colon cancer proliferation is unclear, yam protein products that inhibited DMH-induced ACF formation also inhibited cancer cell proliferation and induced apoptosis. In future studies, we will investigate whether p53 is involved in the effects of yam storage protein.

In conclusion, the present study demonstrates potent anti-colon cancer activities of dietary yam storage protein, indicating that daily ingestion of yams or yam storage protein extracts may suppress colon carcinomas in humans.

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