Medicinal and Aromatic Plant Science and Biotechnology ©2007 Global Science Books

Processed Black Garlic (*Allium sativum*) Extracts Enhance Anti-Tumor Potency against Mouse Tumors

Jin-ichi Sasaki[1*] • Chao Lu[1] • Einosuke Machiya[2] • Mami Tanahashi[3] • Katsunori Hamada[4]

[1] Department of Medical Technology, Hirosaki University School of Health Sciences, Honcyou 66-1, Hirosaki 036-8565, Aomori Japan
[2] Tenma-Bayashi Ryutsu-Kako Co. Ltd, Morinoue 284-11, Shichinohe 039-2827, Aomori Japan
[3] Nisshin Honey Co. Ltd, Anpachi 3133-1, Anpachi 503-0125, Gifu Japan
[4] Yahata Bussan Co. Ltd, Nihongi 498-2, Yonago 689-3541, Tottori Japan

Corresponding author: * **jnsasaki@tk2.so-net.ne.jp**

ABSTRACT

Black garlic (*Allium sativum*) was produced from ordinary white garlic (*Allium sativum*) clove by processing it in a temperature (70°C)- and humidity (75%)-controlled room for a month. The final product by this procedure was soft and sweet with a less irritating odor and fruity taste. The heat-extracts of black garlic were chemically and bio-functionally analyzed and compared with those of ordinary fresh garlic extracts. The aged black garlic contained an increasing amount of amino acids, and organo-sulfur substance, *S*-allyl-L-cysteine (SAC), which probably contributed much to the enhancement of anti-tumor potency. The tumor cure rate by black garlic extracts attained 50% against Meth A fibrosarcoma of BALB/c mouse by intra-tumor injection of 1 mg extracts, three times every other day. By contrast, fresh garlic extracts used as a reference failed to induce tumor-free animals, even though they reduced tumor size to 60% to compare with the tumor mass in non-treated control mouse. Additionally, the black garlic extracts showed anti-bacterial activity against medically important bacteria such as MRSA (methicillin-resistant *Staphylococcus aureus*), enterohemorrhagic *Escherichia coli* O157:H7, *Pseudomonas aeruginosa*, and *Candida albicans*, however its potency was less than that of fresh garlic extracts.

Keywords: antibacteria activity, anti-tumor activity, *S*-ally-L-cysteine, γ-glutamly-*S*-allyl-L-cysteine

INTRODUCTION

Lately, an increasing concern of citizens is toward the novel functions of foods expected to improve or strengthen their health conditions by blending their inherent functions into their daily food styles (Mazza1998; Bratman 2000; Sasaki 2006).

Under these social requirements, we initiated bio-functional research of food stuffs to clarify novel functions included using squid fish (Sasaki *et al*. 1997; Naraoka *et al*. 2000), garlic (Sasaki *et al*. 1999, 2003), mushroom (Sasaki *et al*. 2000), and sweet corn (Sasaki *et al.* 2003). In these studies we reported the beneficial effects hoping to use them to improve the health of individuals and to prevent diseases. Among those food stuffs tested, garlic extracts demonstrated a variety of bio-functions such as to reduce blood pressure and high cholesterol, prevent heart attacks and cancer, and inhibit microbes (Bratman 2000).

The chemical constituents of garlic are very complex containing over 100 different compounds which are regarded as contributing to beneficial biological effects. The most important and unique feature is the high content of organo-sulfur substance, which are at least four times more than other high sulfur-containing vegetables such as onion, broccoli and cauliflower (Garlic and Health Group 2007).

Recently a new type of the black garlic was developed in Japan by processing ordinary fresh garlic under the control of temperature and humidity. The processed garlic product is black in color with less irritating odor and fruity tasty, and is therefore readily edible without any further treatments. In the present study aged black garlic showed an increased anti-tumor action with a high curative rate against Meth A fibrosarcoma in mouse, which fresh garlic extracts failed to cure.

In this paper, we described bioactivities achieved by aged black garlic extracts by focusing on anti-tumor and antibacterial functions compared with those of fresh garlic extracts and discuss these in relation to their chemical constituents.

MATERIALS AND METHODS

Processed (aged) black garlic

Black garlic was created by mainataing fresh garlic clove in a temperature (70°C)- and humidity (75%)-controlled room for 30-40 days without any additional treatments and additives. Garlic cloves naturally aged in the controlled room changing color from white to gray and eventually black a month later (**Fig. 1**). The nature of black garlic is soft and with a non-irritating odor and sweet fruit taste.

Black garlic extraction

The aged black garlic was smashed, and moderately heated at 100°C for 2 hours to extract water-soluble compounds, then filtrate was centrifuged at 4,000 rpm (2220 × *g*) for 20 min. The supernatant was frozen at -80°C overnight, and lyophilized (Freeze Dryer, BFD-2, Nihon Freezer Co LTD, Japan) for powder preparations.

Chemical analysis

Extracts was analyzed by an amino acid analyzer (JLC-500/v, Nihondenshi Co, LTD, Japan) for amino acid content, and liquid chromatography (Alliance 2696, Japan) for SAC and γ-glutamyl-*S*-allyl-L-cysteine (GSAC).

Received: 16 July, 2007. Accepted: 31 August, 2007.

Original Research Paper

Fig. 1 Processed black garlic clove. Fresh white garlic changed its color to black by processing in a temperature- and humidity-controlled room for a month.

Anti-oxidant activity

Antioxidant potency of samples was measured by the DPPH method, and expressed by mg used to reduce 50% of 1.1-diphenyl-2-picrylhydrazzyl (RS50%).

Anti-tumor test

Meth A fibrosarcoma in BALB/c mouse, having been maintained by serial intra-peritoneal transplantation, was used for anti-tumor test of garlic extracts. Briefly, tumor cells adjusted at 5.0×10^6/mL/mouse in GIT cell culture medium (Nihonseiyaku Co. Ltd, Tokyo, Japan) were intradermally transplanted, then the extract dissolved in disinfected saline was injected into the tumor on days 2, 4, 6 after tumor transplantation. The anti-tumor potency of the garlic extracts was evaluated three weeks later from tumor transplantation by measuring tumor size (longitude × latitude mm) (Takaya *et al*. 1994).

Cytotoxicity test

The cytotoxic action of the aged black garlic extracts was tested against Meth A tumor cells at 5.0×10^7/mL suspended in GIT medium, and incubated together with the extracts by mixed culture under 5% CO_2-air (Takaya *et al.* 1994). Living cells were microscopically counted by the trypan blue dye exclusion test at 3, 6, 9, and 24 hours after incubation.

Anti-bacteria test

Anti-bacteria potency of the extracts was studied by the nutrient agar plate method (Sasaki *et al*. 1999) against representative pathogenic bacteria such as MRSA (methicillin-resistant *Staphylococcus aureus*), enterohemorrhagic *Escherichia coli* O157:H7, *Pseudomonas aeruginosa*, and *Candida albicans*. They are causative agents in the medical field or society and provoke food poisoning and infection.

Statistic analysis

Statistic analysis (χ^2) was applied to evaluate the significance between experimental and control groups in animal experiments.

RESULTS AND DISCUSSION

Chemical composition of black garlic extracts

Chemical constituents in the aged black and fresh white garlic clove are listed in **Table 1**. Carbohydrate in the aged black garlic increased in amount, but others did not change much in amount comparing with those of fresh garlic extracts.

Amino acid amount, especially cysteine, phenylalanine, tyrosine, leucine, valine, alanine, glycine, glutamic acid, and aspartic acid, greatly rose in the aged black garlic (**Table 2**).

The most important organo-sulfur substances in black garlic are considered to be the water soluble *S*-allyl-compounds. After aging of garlic, SAC drastically increased in the processed black garlic, and its amount reached 194.3 μg/g after 40 days aging (23.7 μg/g before aging began), while GSAC dropped from 748.7 μg/g to 248.7 μg/g after aging (**Table 3**).

Table 1 Chemical constituents of black and fresh garlic bulbs.

	Aged black garlic	Fresh garlic
Energy (kcal/100 g)	227.1	138
Water conc. (%)	45.1	60.3
Protein (%)	9.1	8.4
Lipid (%)	0.3	0.1
Carbohydrate (%)	47.0	28.7
Ash (%)	2.1	ND
Na (mg)	4	ND
Ca (mg)	24	ND
Lactobacillus (№ *Lactobacillus*/g)	300<	ND

ND: not determined

Table 2 Constituents (mg/100 g) of amino acid in aged black and fresh garlic.

Amino acid	Aged black garlic	Fresh garlic
Cysteine	60	100
Ricin	230	290
Histidine	110	130
Phenylalanine	300	190
Tyrosine	340	170
Leucine	460	260
Isoleucine	250	150
Methionine	90	70
Valine	410	250
Alanine	410	220
Glycine	360	180
Proline	210	180
Glutamic acid	1670	960
Serine	330	210
Threonine	270	190
Aspartic acid	930	630
Tryptophane	80	94
Arginine	970	1300

Table 3 Content of S-allyl-L-cysteine (SAC) and γ-glutamyl-S-allyl-L-cysteine (GSAC).

	SAC	GSAC
Aged black garlic	194.3 μg/g	248.7 μg/g
Fresh garlic	23.7	748.7

Table 4 Increased antioxidant potency of aged black garlic.

Sample	RS50%[a]
Aged black garlic	
Japanese	4.1
Chinese	7.3
Fresh garlic	
Japanese	114.9
Chinese	88.5

[a] mg used to reduce 50% of 1.1-diphenyl-2-picrylhydrazzy

Antioxidant potency

The bio-functional activity test indicated that antioxidant potency greatly increased in aged black garlic extracts and its activity reached 25-fold more compared with that of fresh garlic (**Table 4**).

Table 5 Anti-tumor potency of aged black garlic extracts against Meth A tumor. Extract was injected into tumor on day 2, 4, 6 after tumor transplantation, and its activity was evaluated three weeks later from tumor transplantation.

Sample	Dosage	No cured/No mice used	Percentage non-cured against control
Exp. (1)			
Aged	1 mg	2/5	40
Control	(–)	0/5	100
Exp. (2)			
Aged	1 mg	3/5	55
Control	(–)	0/5	100
Total			
Aged	1 mg	5/10 ($p<0.05$)	47.5
Control	(–)	0/10	100
Reference			
Fresh	5 mg	0/5	64
Control	(–)	0/5	100

Table 6 Cytotoxicity test of aged black garlic extracts against Meth A tumor cells. One percent aged black garlic extracts was mixed with tumor cells for cultivation under 5% CO_2-air condition. Viability of cells were tested by dye exclusion test by counting 200 cells.

Incubation time (hrs)	Aged black garlic	Control (medium)
	(% viability of tumor cells)	
0	97	97
3	95	96
6	90	92
9	83	81
24	61	62

Anti-tumor activity

Anti-tumor potency in aged black garlic was strengthened and its curative rate in the mouse model reached 50% ($p < 0.05$) in the 1.0 mg treatment after three injections (**Table 5**). The average tumor size in non-cured mice (5/10) was half that of the non-treated control group. Fresh garlic extracts used as a reference failed to induce the tumor-free mice despite an increase in the amount of dosage applied, i.e. 5.0 mg, in three times injections (3 × 5 mg).

Cytotoxicity test against tumor cells

One percent of the aged black garlic extracts did not show cytotoxicity against Meth A tumor cells in mixture cultivation test (**Table 6**). Therefore another mechanism probably exists such as the enhancement of the immune system in anti-tumor activity.

Anti-bacteria test

Aged black garlic extracts at 5% inhibited the growth of all bacteria, while 1% solution did not exhibit bacteria killing activity (**Fig. 2**). The activity of aged black garlic extract was less than that of fresh garlic extract reported previously (Sasaki *et al*. 1999, 2003).

DISCUSSION

From Roman antiquity through to World War I, garlic clove was used to prevent wound infections, and was called Russian Penicillin during World War II. After running out of antibiotics, the government used this ancient remedy for treatment of the wounded soldiers. In Europe, garlic has come to be seen as an all-around treatment for preventing atherosclerosis, the cause of heart disease and strokes. Studies have found that certain forms of garlic can lower total cholesterol levels by about 9 to 12%. Garlic also appears to slightly improve hypertension, protect against free radicals, and slow blood coagulation. Joining all these benefits, garlic may be a broad-spectrum treatment for arterial disease (Bratman 2000).

Fig. 2 Bacteria killing potency of aged black garlic extracts. From left to right: MRSA (methicillin-resistant *Staphylococcus aureus*), *Pseudomonas aeruginosa*, enterohemorrhagic *Escherichia coli* O157:H7, *Candida albicans.* Five percent garlic extracts (left) inhibited the growth of bacteria, but one percent did not (right).

Recently, garlic researchers have been focusing on the benefits of a variety of sulfur-containing compounds instead of allicin, which has been long time believed to play a central role in a variety of activities of garlic. Sulfur-containing compounds are classified as an odorous oil soluble and odorless water-soluble composite. The oil-soluble compounds include diallyl-sulfide (DAS), diallyl-disulfide (DADS), diallyl-trisulfide and allyl-methyl-trisulfide, dithiins, and ajoene. The water-soluble compounds consist of cysteine derivatives, such as SAC, *S*-allyl-mercaptocysteine (SAMC), *S*-methyl-cysteine, and γ-glutamyl-cysteine derivatives. The water-soluble compounds are more stable and there are less undesirable effects than the oil-soluble compounds (Imada 1990).

During aging of garlic, biologically important composites such as SAC are synthesized and increasingly stored. In contrast, odorous and irritating compounds gradually change into much milder, gentle and non-odorous constituents. The odor and irritating side effects of fresh garlic are almost eliminated, while various beneficial properties are increasingly produced.

Black garlic was recently developed in Japan by processing (aging) ordinary fresh white garlic in a temperature- and humidity-controlled climate for 30-40 days. During aging, a chemical cascade of reactions (Maillard and Browning reactions) occurred within garlic cloves, whose color gradually changed from white to brown and finally black accompanied with a qualitative change in constituents (**Fig. 1**). The final product has a fruity taste with less irritating odor, and is rich in the sulfur-containing compound, SAC.

Preliminary evidence suggests that regular use of garlic may help prevent cancer, and eating garlic is known to raise immunity (Bratman 2000). Actually, our data also demonstrated that the extracts of aged black garlic induced an increase in anti-tumor potency with 50% curative rate in tumor-bearing mouse ($p < 0.005$). Further, the tumor mass of non-cured mice in the extracts-treated (therapy) group was half the size of that of the non-therapy control group (**Table 5**). In contrast, fresh garlic extracts could not cure tumor-bearing mice. The difference in curative rate between the black and fresh garlic extracts may be a difference in the amount of SAC, which increases significantly in the aged black garlic during aging.

Since the aged black garlic extracts showed no cytotoxicity against Meth A tumor cells in the mixture cultivation test, the participation of an extracts-enhanced immune system is considered to play a possible key role to eradicate tumors *in vivo*. Actually, Ishikawa *et al*. (2006) applied aged garlic extracts to patients with advanced cancer (liver, pancreatic, colon), and the effectiveness of the extracts was proved when the decline of NK (natural killer) cell number declined. Besides, NK cell activity increased significantly in the aged garlic extracts-treated patients.

The aged garlic extracts they used were different from

our aged black garlic extracts in the manner in which they were processed. Their specimens were prepared by slicing and keeping garlic in water or alcohol for up to two years before being turned into a dietary supplement while our extracts were made from black garlic aged in a controlled room for a month. However a definite comparative study has not yet been carried out and remains as a future work. Our aged black garlic extracts and theirs are the only two existent products created by two different processing criteria.

One of the beneficial activities of aged black garlic is to have high antioxidant potency, which is much more efficient to fight oxidant-related DNA damage. In a study to assess the antigenotoxic activity of several garlic organo-sulfur compounds (allicin, dially sulfide, dially disulfide, *S*-allyl cystein, mercaptan) in the human hepatoma cell line Hep G2, it was demonstrated that garlic organo-sulfur compounds tested (5-100 μM) worked to protect against DNA damage (Belloir *et al*. 2006).

Further, it has been reported that aged garlic extracts fed to rats significantly reduced the number of colon tumor and aberrant crypt foci, suggesting the presence of chemo-preventative effects on carcinogenesis through suppression of cell proliferation (Katsuki *et al*. 2006). More interesting is that SAC was detectable in serum obtained from the aged garlic extracts-treated rats. These studies surely support the effectiveness of the garlic organo-sulfur compounds to prevent DNA damage or carcinogenesis, and tumor cell growth.

Additionally one important bio-function is anti-bacterial activity in the aged black garlic extracts. The bactericidal capacity of garlic has been well known for centuries, and lately new data have been added to its activity using the following pathogenic bacteria: MRSA (methicillin-resistant *Staphylococcus aureus*), enterohemorrhagic *Escherichia coli* O157:H7, *Pseudomonas aeruginosa*, *Candida albicans*, and *Bacillus anthracis*, which are causative agents of food poisoning and bio-terrorism (Sasaki 1999, 2003).

The black garlic extracts also showed bactericidal activity just as fresh garlic. Its potency was heat-stable and could resist 100°C for 2 hours. In the past, bactericidal activity has been generally illustrated by allicin alone, which is known to be very unstable and is rapidly degraded. Our data showed that the heat stable element(s) also exists as bactericidal agent(s) in garlic.

Bactericidal activity also exists in the odor (volatiles) released from crushed garlic bulbs (Sasaki 2006). The odor oriented-anti-bacterial activity was not only a specific phenomenon observed in garlic but a generalized phenomenon that commonly occurred in a variety of plants (vegetables). This phenomenon is presumably an acquired defense system in plants to survive in harmful surroundings thus avoiding the attacks from bacteria and parasites (Sasaki, in preparation).

Aged black garlic has only just recently begun to be developed in Japan by devising the appropriate processing procedures. As such, several issues still remain unsolved such as developing of additional novel beneficial effects, effect on the immune system, among others. These issues will soon be solved as one way of contributing more to improve human health care.

ACKNOWLEDGEMENTS

The authors acknowledge Ryutsu-Kako (Japan) and Yahata Bussan Co. Ltd (Japan) for the financial support in 2006.

REFERENCES

Belloire C, Singh V, Daurat C, Siess MH, le Bon AM (2006) Protective effects of garlic sulfur compounds against DNA damage induced by direct- and indirect-acting genotoxic agents in Hep G2 cells. *Food and Chemical Toxicology* **44**, 827-834

Bratman S (2000) *Your Complete Guide to Herbs*, Prima Publishing, California, pp 67-75

Garlic and Health Group (2007) Collection of six papers. *Medicinal and Aromatic Plant Science and Biotechnology* **1**, 1-36

Imada O (1990) Toxic aspects of garlic. In: First World Congress on the Health Significance of Garlic and Garlic Constituents, p 47 (Abstract)

Ishikawa H, Saeki T, Otani T, Suzuki T, Shimoura H, Nishino H, Fukuda S, Morimoto K (2006) Aged garlic extract prevents a decline of NK cell number and activity in patients with advanced cancer. *Journal of Nutrition* **136**, 816S-820S

Katsuki T, Hirata K, Ishikawa H, Matsuura N, Sumi S, Itoh H (2006) Aged garlic extract has chemopreventive effects on 1,2-dimethylhydrazine-induced colon tumor in rats. *Journal of Nutrition* **136**, 847S-851S

Mazza G (1998) *Functional Foods: Biochemical Processing Aspects*, Technomic Publishing Co. Inc., Canada, pp 1-460

Naraoka T, Chung HS, Uchisawa H, Sasaki J, Matsue H (2000) Tyrosinase activity in anti-tumor compounds of squid ink. *Food Science and Technology Research* **6**, 171-175

Sasaki J (2006) Bioactive phytocompounds and products traditionally used in Japan. In: Ahmad I, Farrukh A, Mohammad O (Eds) *Modern Phytomedicine – Turning Medical Plants into Drugs*, Wiley VCH, Weinheim, pp 79-96

Sasaki J, Ishita K, Takaya Y, Uchisawa H, Matsue H (1997) Anti-tumor activity of squid ink. *Journal of Nutrition, Science, Vitaminology* **43**, 455-461

Sasaki J, Ishita K, Uchisawa H, Matsue H (1999) Antibacterial activity of garlic powder against *Escherichia coli* O157. *Journal of Nutrition, Science, Vitaminology* **45**, 785-790

Sasaki J, Kita J (2003) Bacteriocidal activity of garlic powder against *Bacillus anthracis*. *Journal of Nutrition, Science, Vitaminology* **49**, 297-299

Sasaki J, Soga H, Shinohara C, Itoh Y, Sasahara R, Yoshida K (2003) Anti-tumor activity of sweet corn phytoglycogen and ground powder of sweet corn. *Annals of the Society of BCG and Biological Response Modifier Immunotherapy* **27**, 27-31

Sasaki J, Uchisawa H, Matsue H, Kita T, Ishita K, Sugawara C, Takahashi K, Nohtomi T, Yoshida K (2000) Anti-tumor activity of Maitake mushroom extracts. *Annals of the Society of BCG and Biological Response Modifier Immunotherapy* **24**, 25-28

Takaya Y, Uchisawa H, Matsue H, Okuzaki B, Narumi F, Sasaki J, Ishita K (1994) An investigation of anti-tumor peptidoglycan fraction from squid ink. *Biological Pharmaceutical Bulletin* **17**, 846-849