

Fractionated Aged Black Garlic Extracts Enhance Growth of Anti-My-10 Hybridoma Cells and Production of IgG1 Antibody

Ji Young Lee · Namhyun Chung* · Yong Kwon Lee*

Received: 30 July 2013 / Accepted: 23 September 2013 / Published Online: 31 March 2014

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Abstract Aged black garlic (ABG) was extracted with 20% ethanol and water (crude extracts) and fractionated into three categories (>10, 3-10, and <3 kDa). The effect of crude extract supplements on anti-My-10 hybridoma cell growth and IgG1 antibody production was investigated in suspension culture with a chemically defined protein-free medium. We observed that supplementation of ABG to the cell culture medium stimulated anti-My-10 hybridoma cell growth and production of IgG1 antibody, particularly with fractionated ABG of low molecular weight. The stimulation depended upon the concentration and the size of the fractionated ABG. We also found that the growth-promoting activity was not correlated with high antibody production. These results suggest that fractionated ABG is a novel and promising alternative as an animal cell culture supplement.

Keywords aged black garlic · fractionation · hybridoma cell line · mouse IgG1 production · suspension culture

Animal cells have been widely used as a host for the production of pharmaceutical proteins such as cytokines, vaccines, monoclonal antibodies (mAb), and other therapeutic proteins. Cell culture-related pharmaceutical antibody production is currently a \$70 billion/year business (Lager, 2009). Considering the increasing market potential of pharmaceutical antibodies, development of an

efficient antibody manufacturing process may be needed to meet the demand.

Optimization of culture medium is one of the key steps for the production of any biopharmaceutical product. For many years, mammalian cell culture was performed with supplementation of serum, but serum-supplemented medium can be a source of contamination during the production of biopharmaceuticals. Serum- and protein-free medium have been developed to avoid these risks, but they have several disadvantages compared with serum supplementation (Van der Valk et al., 2004). To overcome these disadvantages, medium additives that can modulate cell metabolism in favor of enhanced and continuing proteosynthesis were introduced to increase productivity of biopharmaceuticals (Butler, 2005; Keenan et al., 2006). An alternative to a serum-supplemented medium can be a serum-free medium supplemented with various raw materials (Lee et al., 2009). Recently, a number of studies have shown that differentially ultrafiltered fractions of media additives (such as protein hydrolysates) may replace crude types and exert positive effects on cell culture. Ultrafiltration or nanofiltration stages avoid high molecular size peptides and lead to an easier purification of recombinant proteins (Franek et al., 2000; Farges-Haddani et al., 2006; Chun et al., 2007).

Garlic (*Allium sativum*) has been considered a valuable healing agent by people for thousands of years (Amagase et al., 2001). Garlic contains unique organosulfur compounds, which provide its characteristic flavor, odor, and most of its potent biological activity. During aging, the lipid-soluble volatile organosulfur compound allicin is converted into stable and odorless compounds including S-allyl cysteine, the organosulfur compound with a potent antioxidant effect (Borek, 2001). In this present study, we evaluated the supplementation effect of various aged black garlic (ABG) fractions to serum-free culture media. We found that ABG fractions have a significant effect on anti-My-10 hybridoma cell growth and antibody production capacity.

A mouse hybridoma cell line (anti-My-10) was cultured in 125-mL Erlenmeyer flasks (Corning Inc., USA) containing a 7:3 mixture of CDM4MAb (Hyclone, USA) and DMEM medium (JBI, Korea). The initial cell density was 2.5×10^5 cells/mL. The

J. Y. Lee
Research Center for Radiotherapy, Korea Institute of Radiological and Medical Sciences, Seoul 139-706, Republic of Korea

N. Chung
Department of Biosystems and Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul 136-713, Republic of Korea

Y. K. Lee
Department of Food and Nutrition, Yuhan College, Bucheon 422-749, Republic of Korea

*Corresponding authors
(N. Chung: nchung@korea.ac.kr; Y. K. Lee: yklee@yuhan.ac.kr)

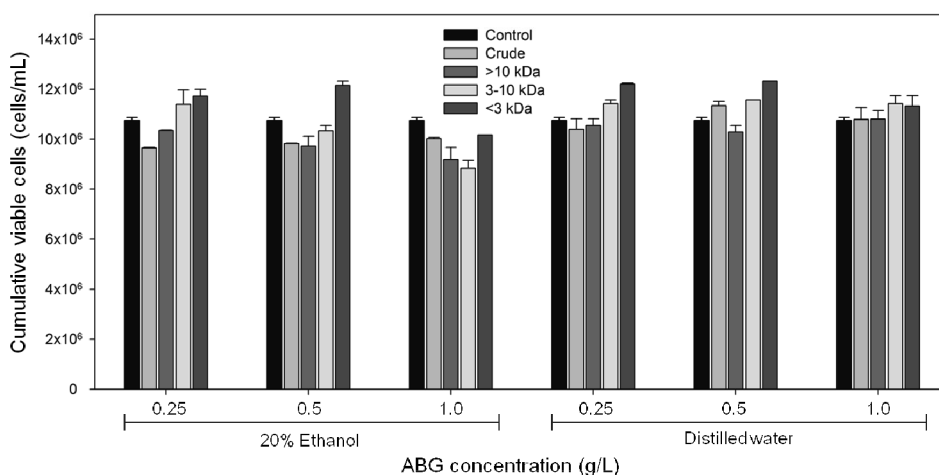


Fig. 1 Cumulative cell number during a cell culture period of 7 days. Values are represented as the mean \pm standard error of 3 replicates. No ABG was added to the control media.

flasks were placed on a rotary shaker at 80 rpm. During the cultivation period, samples were obtained periodically from the cell culture. Cell viability was determined by trypan blue dye exclusion using a hemocytometer.

ABG was purchased from Uiseong Blackgalic Agricultural Association (Korea). The aged black garlic was manufactured by the following steps. The garlic was incubated 3 h at 90°C under 100% humidity. Then, the temperature was reduced to 75°C and incubated for an additional 198 h. The pieces were further incubated with decreasing temperature to 65°C for an additional 35 h. The temperature was then reduced to 55°C and incubated for 51 h. Finally, the pieces were incubated for 168 h at 0–5°C. ABG was peeled, ground, and extracted with 20% ethanol and distilled water. The extracted solution was centrifuged at 1900 \times g for 30 min to isolate supernatants. The supernatant of ABG extracts were fractionated according to nominal molecular weight of 3 and 10 kDa using Centricon Plus centrifugal filter units (Millipore Co., USA). The fractionated and non-fractionated ABG extracts were freeze-dried and pulverized. Fractions and non-fractionated ABG extracts were adjusted to 200 g/L (w/v; nominal) with phosphate buffered saline, filtered through a 0.2- μ m Durapore filter (Millipore Co.), and added to the cell culture medium to final concentrations of 0.25, 0.5, and 1 g/L.

IgG1 concentrations were assayed using an ELISA kit (Bethyl Laboratories, USA). In brief, 96-well immunoplates were coated with goat anti-mouse IgG1 and incubated for 60 min. After incubation, plates were blocked for 30 min using postcoat solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0). Prior to the addition of samples, plates were washed twice with wash solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0). Samples were added to the well in triplicate and incubated for 2 h. After an additional wash, HRP-conjugated goat anti-mouse IgG1 was added and incubated for 1 h. Plates were then washed and the substrate (3,3',5,5'-tetramethylbenzidine) was added. Reactions were stopped with 2 M H₂SO₄. All incubation steps were performed at room temperature. Plates were read at 450 nm using an EL 800

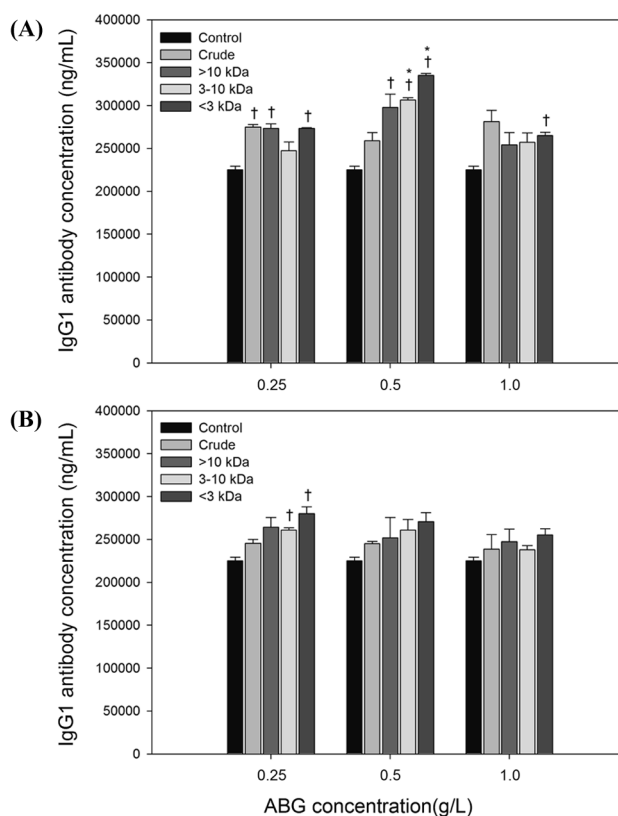


Fig. 2 Effect of crude extracts and fractionated ABGs on the production of IgG1 antibody by anti-My-10 hybridoma cells on day 7. The ABG was extracted with 20% ethanol (A) and distilled water (B). Each ABG was fractionated by molecular weight into 3 size categories: <3, 3–10, and >10 kDa. No ABG was added to the control media. $\dagger p < 0.05$ compared to the control medium; $* p < 0.05$ compared to the crude extract of ABG.

ELISA reader (Bio-Tek Inc., USA). IgG1 antibody levels were determined using a standard curve. The data were expressed as the mean \pm standard error. p values of less than 0.05 were considered

significant. All statistical analyses were performed using Prism 3.02 (Graph Pad Software, USA).

The growth-promoting activity of ABG crude extracts and their fractions was investigated using anti-My-10 hybridoma cells. Fig. 1 shows the cumulative viable cell number for a period of 7 days with various supplementations. Cumulative viable cell number was higher than with control, with fractions of 3–10 kDa and/or <3 kDa when 0.25 and 0.5 g/L of 20% ethanol extract and their fractions was added. All cumulative viable cell numbers from 1.0 g/L crude 20% ethanol extract and its fractions were lower than with control. Cumulative viable cell number was higher than with the control, with fractions of 3–10 kDa and <3 kDa, when 0.25, 0.5, and 1.0 g/L of water extract and their fractions were added. The 0.5 g/L crude water extract also had a higher cumulative cell number than the control. In particular, fractions of <3 kDa had the highest cumulative cell number among the crude extracts and all three fractions, except a single exception. These results showed that fractions of <3 kDa have a strong growth-promoting activity on anti-My-10 hybridoma cells.

Figure 2 shows the yield of mouse IgG1 antibodies after 7 days of culture with various supplementations. In the case of 20% ethanol extraction, IgG1 antibody concentration with crude extracts and all fractions was higher than with the control. In particular, all three fractions from 0.5 g/L of 20% ethanol extraction had higher IgG1 antibody levels than all other fractions and crude extracts. The concentration of IgG1 antibody with the three fractions was in the order of (<3 kDa; 335,000 ng/L) > (3–10 kDa; 306,500 ng/L) > (>10 kDa; 298,000 ng/L). In the case of water extraction, IgG1 antibody concentration with crude extract and all fractions was also higher than the control. With water extraction, the overall concentration of IgG1 antibody was higher with 0.25 g/L than with the other two concentrations. The concentration of IgG1 antibody with 0.25 g/L was in the order of (<3 kDa; 280,200 ng/L) > (>10 kDa; 264,200 ng/L) > (3–10 kDa; 261,000 ng/L) > (crude; 245,500 ng/L) > (control; 225,300 ng/L).

Animal cell culture medium must be rich in nutrients and supplemented with key substances in order to optimize cell growth and maximize biopharmaceuticals production capabilities. To satisfy the two standards and to meet the demand for a safer medium, it is necessary to identify new supplements. In this present study, we demonstrated that ABG fractions may represent a novel and promising alternative as an animal cell culture supplement. We assessed the efficacy of differentially extracted and fractionated BAG to examine anti-My-10 hybridoma cell growth and IgG1 antibody production in suspension culture of a chemically defined protein-free medium. We found that, whether ABG was extracted with 20% ethanol or water, some fractionated ABG, the <3 kDa fraction in particular, supported anti-My-10

hybridoma cell growth to a greater extent than the other fractions and crude extracts. We also determined that a high concentration (1.0 g/L) of crude extract and fractionated ABG did not have a positive effect on the cell growth. It is worth mentioning that growth-promoting activity was not correlated with high antibody production. For example, at 0.5 g/L of 20% ethanol extraction, cumulative viable number was lower with crude extract, >10, and 3–10 kDa than with the control; however, antibody production was higher than with the control. It is possible that this supplementation methodology may be applicable to other types of cell growth and production of useful biopharmaceuticals.

We provide the first evidence that supplementation of the cell culture medium with ABG, particularly fractionated ABG, stimulates cell growth of anti-My-10 hybridoma animal cells and the production of secreted IgG1 antibody. Stimulation depends on the concentration and size of the fractionated BAG. We expect that the use of fractionated ABG <3 kDa as a medium supplement may provide the appropriate small size for easy downstream processing.

Acknowledgment The present research was funded by the research fund of Yuhan College in 2009.

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