Effect of Non-dialyzable Extract of Amaranth Seeds on Immunoglobulin Production and Proliferation of a Human-human Hybridoma Cell Line, HF10B4

Yasuto SASAKI*, Kyoko NAKAMURA* and Kazuki SHINOHARA**

* Central Research Laboratory, Nitto Flour Milling Co., Ltd.,
6-2-1, Tokai, Ohta-ku, Tokyo 143
** National Food Research Institute, the Ministry of Agriculture, Forestry, and Fisheries, Tsukuba, Ibaraki, 305

The effects of a non-dialyzable extract of amaranth seeds on the immunoglobulin M (IgM) production and proliferation of a human-human hybridoma cell line, HF10B4, were examined. The extract of amaranth seeds promoted the IgM production of HF10B4 cells at concentrations ranging from 0.25mg/ml to 2.5mg/ml. The proliferation of HF10B4 cells was slightly activated at concentrations ranging from 0.007mg/ml to 0.01mg/ml. The IgM production-stimulating (IPS) activity of the extract remained after heating it at 40, 60 and 80°C for 20 min. The IPS activity of the extract was found to exist in the fractions with a molecular weight of over 30000 on Sephacryl S-100 HR column chromatography of the extract. On DEAE-Sepharose ion exchange chromatography of the active fraction, the IPS activity was found in both unadsorbed and adsorbed fractions.

The grain amaranth (genus *Amaranthus*) originated with the Aztecs is considered to be one of the most promising crops because it has superior nutritional value. Amaranth seeds contain 12~18% protein with a well-balanced amino acid composition and are rich fats and minerals. The seeds are now consumed as a popcorn-like confection "Joy" or "Pop" in Latin America and India or as a snack food and breakfast cereal in the United States and Japan. Some of the physicochemical properties of the extracts of amaranth seeds, such as emulsifying properties, gel formation and water binding capacity, have been clarified[1)~3]). On the other hand, it is expected that amaranth seeds can be utilized as a dietary source of nutritious and tasty hypoallergenic foods for persons with allergies to such foods as wheat, corn, and other cereals, milk, eggs, nuts and yeast[4]). SHINDO et al.[5) evaluated that breads baked with amaranth seed flour were suitable for allergic patients. CALDERON De LA BARCA and VASQUEZ-MORENO[6] purified physiologically active lectin from *Amaranthus cruentus* seeds, which recognizes glycoconjugates in the proliferative region of the normal human colonic epithelium and neoplastic lesions of the colon[7]). It is thus expected that amaranth seeds may have some physiological functions. Among the physiological functions, immunological activation is noteworthy. To study the effect on an *in vitro* system, hybridoma cell lines which have immunoglobulin-production ability are useful tools. In this paper, we examined the effects of a non-dialyzable extract of amaranth seeds on the immunoglobulin M (IgM) production and proliferation of a human-human hybridoma cell line, HF10B4.

**Materials and Methods**

**Preparation of amaranth seeds extract**

Amaranth (*Amaranthus hypochondriacus* Linne) seeds purchased from the Krumaicho Amaranth Production Union were ground in a Cyclotec mill (Tecator, Sweden) to pass through a 30-mesh screen and were then...
defatted with acetone. The defatted powder (150 g) was extracted in 1.5 l of phosphate-buffered saline solution (PBS, pH 7.4) with a Labo-stirrer Model-LR41C (Yamato). The extract was centrifuged at 13 000 g for 20 min at 10°C. The supernatant was dialyzed against cold deionized water in seamless cellulose tubing (Viskase Sales Corp., U.S.A) with a molecular weight cutoff of 12 000~14 000, for 60 h at 5°C, and the inner solution was lyophilized. The obtained dried extract was dissolved in PBS and sterilized by filtration through a Millipore filter (0.22 μm) before administration to the cultured cells.

**Cells and cell culture**

The cell line used in this study was a human-human hybridoma cell line, HF10B4, which was established by fusing human lymphocytes of a lung cancer patient with human fusion partner NAT-30 cells. HF10B4 cells produce monoclonal antibody against human lung cancer. The cells were maintained in eRDF-ITES medium composed of enriched RDF medium (Kyokuto Pharmaceutical Kogyo Co.) supplemented with 5 μg/ml of insulin (I), 10 μg/ml of transferrin (T), 153 μg/ml of ethanolamine (E) and 4.3 pg/ml of selenite (S).

The cells during the exponential growth phase were collected, washed with PBS, and then plated at the cell density of 2 × 10^5 cells/ml for IgM production determination and 5 × 10^4 cells/ml for proliferation assay in 24-well microplates. To the cells, different amounts of the extract of amaranth seeds were added and cultured in a CO₂ incubator with a humidified 5% CO₂-95% air atmosphere at 37°C.

**Determination of amount of IgM**

The amount of IgM produced by HF10B4 cells was measured by an enzyme-linked immunosorbent assay (ELISA) with peroxidase-labeled sheep anti-human IgM antibody. Determination of IgM amount by the ELISA method was carried out as described previously. The reaction was stopped by addition of 1.5% oxalic acid, and the absorbance at 405 nm was measured. The amount of IgM production was determined from the calibration curve for the standard human IgM (Tago, U.S.A). As a positive compound which stimulates the immunoglobulin production of hybridoma cells, chicken egg-yolk lipoprotein (YLP; Q.P.Co) was used.

**Cell proliferation assay**

The effect of the non-dialyzable extract of amaranth seeds on the proliferation of HF 10 B4 cells was examined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay according to the method of SHINOHARA et al. and MOSMANN. The tetrazolium salts are attractive candidates for measuring living cells, because they measure the activity of various dehydrogenase enzymes. In the MTT assay, the tetrazolium ring is cleaved in active mitochondria, and so the reaction occurs only in living cells. One hundred μl of cultured cell suspension was incubated with 20 μl MTT dissolved in PBS in 96-well plates for 4 h in a CO₂ incubator at 37°C.

The insoluble formazan was dissolved in HCl-isopropanol, and the absorbance at 545 nm was measured with a Wellreader (Seikagaku Industrial Co.).

**Heat treatment of non-dialyzable extract of amaranth seeds**

The extract (0.1 g/ml) was heated at 40, 60 or 80°C for 20 min and then centrifuged at 1840 g for 10 min at room temperature. The supernatant was filtered through a Millipore filter (0.22 μm) before utilizing in the experiments.

**Chromatography**

Gel-filtration chromatography was performed on a Sephacryl S-100HR column (1.6 cm i.d. × 85 cm). Elution was achieved with degassed 50 mM phosphate buffer (pH 7.5) containing 0.15 M NaCl at a flow rate of 48 ml/h at 5°C. The active fraction obtained by gel-filtration was then applied to a DEAE-Sepharose FF gel column (2.6 cm i.d. × 45 cm) equilibrated with 0.1 M phosphate buffer (pH 6.8) and eluted with phosphate buffer with a linear gradient from 0 to 1.0 M NaCl concentration at a flow rate of 120 ml/h at 5°C. Effluent fractions (10 ml) were analyzed for protein by absorbance at 280 nm and total carbohydrates by the phenol-sulfuric acid method.
**SDS-gel electrophoresis**

Electrophoresis was performed on a 10% sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE) using ExcelGel™, gradient 8-18 and Multiphor II (Pharmacia LKB Biotechnology Co.). Gels were stained with coomassie brilliant blue R-250. Molecular weight standards were a product of Oriental Yeast Co. Ltd. which consists of cytochrome c monomer (12.4 KD), cytochrome c dimer (24.8 KD), cytochrome c trimer (37.2 KD), cytochrome c tetramer (49.6 KD), and cytochrome c hexamer (74.4 KD).

**Results**

**Effect of non-dialyzable extract of amaranth seeds on IgM production and proliferation of HF10B4 cell line**

Figure 1 shows the amount of IgM in the culture medium and the viability of HF10B4 cells after the cells were cultured in the presence of different amounts of the extract of amaranth seeds. The IgM production of HF10B4 cells was clearly stimulated by the extract of amaranth seeds at concentrations ranging from 0.25 mg/ml to 2.5 mg/ml. At those concentrations, the number of viable HF10B4 cells tended to decrease, but at low concentrations of the extract such as 0.007 and 0.01 mg/ml, the number of viable cells increased. The amount of IgM produced by HF10B4 cells in the presence of 0.25 mg/ml of amaranth seeds extract was almost the same as that in the presence of 0.2 mg/ml of YLP which has already been reported as an immunoglobulin-production stimulating (IPS) factor.

**Heat stability of IPS activity of non-dialyzable extract of amaranth seeds on HF10B4 cells**

Heat stability of the IPS activity of the non-dialyzable extract of amaranth seeds on HF10B4 cells was examined. The extract (0.1 g/ml) in PBS was heated at 40, 60 or 80°C for 20 min. Fifty µl of the heated extract was added to the culture of HF10B4 cells (2×10⁶ cells/ml) in 24-well microplates, and the cells were cultured for 3 h. The amount of IgM produced in the culture medium is shown in Fig. 2. The IPS activity of the non-dialyzable extract remained even after the heating treatment. Figure 3 shows the effect of different amounts of non-dialyzable extract heated at 80°C for 20 min on the proliferation of HF10B4 cells. Addition of 10 µl of the heated extract to the cells was found to increase the number of viable cells of HF10B4 slightly.

**Gel filtration of non-dialyzable extract of amaranth seeds on a Sephacryl S-100 HR column**

The non-dialyzable extract of amaranth seeds was filtrated through a Sephacryl S-100 HR column (Fig. 4). The major fraction of protein was distributed in the molecular weight region of approximately 55 000. Three fractions containing carbohydrates also appeared over a molecular weight of 10 000.

**IPS activity of gel-filtrated fractions of**
Fig. 2 Heat stability of IgM production-stimulating activity of the non-dialyzable extract of amaranth seeds

The non-dialyzable extract (0.1 g/ml) of amaranth seeds was heated at 40, 60 or 80°C for 20 min, and 50 μl of the extract was added to the culture medium of HF10B4 cells (2 × 10^4 cells/ml) for 3 h and the amount of IgM was determined by the ELISA method. Each value represents the mean ± SD (n=3).

non-dialyzable extract of amaranth seeds

With the Sephacryl S-100 HR gel filtration of the non-dialyzable extract of amaranth seeds, the fractions with molecular weights of over 30 000 and below 30 000 were collected to examine the effects of the protein fractions. After dialyzing them against 50 mM phosphate buffer (pH 7.5), the effects of each fraction on the IgM production and proliferation of HF10B4 cells were examined. Figure 5 shows the amount of IgM produced in the culture medium after culture for 3 h and the growth of cells after culture for 2 days. The IPS activity was found only in the fraction with a molecular weight of over 30 000, although a decrease in the number of viable HF10B4 cells was observed. The active fractions also showed strong cell agglutinating activity toward HF10B4 cells (Fig. 6). The fraction with a molecular weight of below 30 000 had no IPS activity.

DEAE ion-exchange Sepharose chromatography of the fractions with molecular weight of over 30 000 and the IPS activity of the separated fractions

The fraction with a molecular weight of over 30 000 of which the IPS activity was confirmed was then chromatographed on a DEAE ion-exchange Sepharose fast flow column, and the IPS activity of the separated fractions on HF10B4 cells was examined. Figure 7 shows the elution profile and the IPS activity of the separated fractions after HF10B4 cells (2 × 10^5 cells/ml) were cultured with 50 μl of the fraction for 3 h. With the DEAE ion-exchange Sepharose chromatography, the fractions with molecular weight of over 30 000 were found to be separated into non-adsorbed and two adsorbed fractions from the protein and carbohydrate assays. IPS activity on HF10B4 cells was found in both fractions.

Analysis by SDS-gel electrophoresis

Figure 8 shows SDS-PAGE profiles of the non-dialyzable extracts of amaranth seeds. The SDS-PAGE analysis of the non-dialyzable extract of amaranth seeds and its IPS active
Fig. 4 Sephacryl S-100HR gel filtration profile of the non-dialyzable extract of amaranth seeds

The non-dialyzable extract of amaranth seeds (0.25g/ml) was charged on a Sephacryl S-100 HR column (1.6 cm i.d. x 83 cm) equilibrated with 50mM phosphate buffer containing 0.15M NaCl (pH 7.0) and the elution was developed with the same buffer.

Fraction showed similar profiles of which clear bands appeared at molecular weights of about 12000, 27000, 32000 and 41000. However, the bands with molecular weights of 27000, 32000 and 41000 disappeared on heating the non-dialyzable extract of amaranth seeds at 80°C for 20 min.

Discussion

The present study revealed that the non-dialyzable extract of amaranth seeds had IPS activity on HF10B4 hybridoma cells. The IPS activity of the non-dialyzable extract remained even after heating treatment at 40, 60 or 80°C for 20 min. It was also found that a certain amount of the non-dialyzable extract increased the number of viable cells of HF10B4, suggesting that the extract had an enhancing effect on the proliferation of HF10B4 cells. The active fraction with IPS activity was found to exist in the fraction with a molecular weight of over 30000. The SDS-PAGE analysis of the IPS active fraction showed clear bands at molecular weights of about 12000, 27000, 32000 and 41000. CALDERON DE LA BARCA and VARQUEZ-MORENO6) demonstrated that about 2% of the protein of Amaranthus cruentus was lectin, the molecular weight of which was 32500. The active fraction strongly agglutinated HF10B4 cells. This suggested that a lectin-like substance existed in the
Fig. 6 Agglutinating effect of the fraction chromatographed on a column of Sephacryl S-100HR toward HF10B4 cells

Experimental conditions were the same as in Fig. 5.
(1) No treatment as control
(2) The cells cultured with the fraction of molecular weight of 30,000
(3) The cells cultured with the fraction of molecular weight of 30,000

Fig. 7 DEAE ion-exchange Sepharose chromatography of the fraction with a molecular weight of over 30,000 and effect of the separated fraction on IgM production of HF10B4 cells

The fraction with a molecular weight of over 30,000 was applied to a DEAE-Sepharose FF column (2.6 cm i.d. × 45 cm) equilibrated with 0.1 M phosphate buffer (pH 6.8) and the adsorbed substances were eluted with phosphate buffer containing a continuous gradient of NaCl (0~1.0 M) at a flow rate of 120 ml/h at 5°C. Each fraction was dialyzed and then added to the culture medium of HF 10B4 cells (2 × 10⁵ cells/ml). The cells were cultured for 3 h and the amount of IgM was determined by the ELISA method.

active fraction. The band with a molecular weight of 32,000 in the faction seemed to be a lectin–like substance because the band was removed by fetuin affinity chromatography, in which fetuin easily bound to the lectin–like substance (data not shown). However, the band with a molecular weight of 32,000 disappeared on heating the non-dialyzable extract of amaranth seeds at 80°C for 20 min. It was also found that the band did not exist in
the adsorbed fractions from DEAE ion-exchange chromatography which had IPS activity (data not shown). Nevertheless, the supernatant of the heat-treated extract and the adsorbed fractions of DEAE ion-exchange chromatography retained the IPS activity. Therefore, the IPS active principle of the amaranth seed extract is suggested not to be the lectin-like substance with a molecular weight of 32,000. In addition, the results of chromatography and SDS-PAGE shown in this paper suggest that the protein portion of the IPS active substances could consist of some monomers with a molecular weight of approximately 10,000~12,000. Further purification of these active substances and elucidation of their structure are in progress.

References


(Received Jul. 27, 1994)
対する増殖促進活性は，0.007 mg/ml から 0.01 mg/ml の添加濃度において，若干ではあるが示された。アラネクス種子抽出物の IgM 産生促進（IPS）活性は，40，60，80℃ で 20 分間の加熱処理後においても安定であった。Sephacryl S-100 HR を用いたゲル通過により，IPS 活性は分子量 3 万以下の画分に存在していることが確認された。DEAE-Sepharose イオン交換クロマトでは，IPS 活性は吸着部および非吸着部の両方に存在していた。