

Immunostimulatory *in Vitro* and *in Vivo* Effects of a Water-Soluble Extract from Kale

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The water-soluble fraction of kale (*Brassica oleracea* L. var. *acephala* DC.) had immunoglobulin (Ig) production stimulating activity in human hybridoma HB4C5 cells and human peripheral blood lymphocytes. The biochemical and physical properties of the main active substance in kale were found to be a heat-stable protein with a molecular weight higher than 50 kDa. The Ig production-stimulating factors were assumed to act on the translational and/or secreting processes of Igs. This Ig production-stimulating effect was also observed in lymphocytes from the mesenteric lymph node and Peyer's patches of mice that had been administered with the kale extract for 14 d. The partially purified kale extract was analyzed by LC-ESI-MS/MS, the result indicating ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) as an active substance. Rubisco from spinach indeed exhibited Ig production-stimulating activity in HB4C5 cells. These findings provide another beneficial aspect of kale as a health-promoting foodstuff.

Key words: *Brassica oleracea*; immunoglobulin; immunostimulating factor; kale; ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco)

Brassica vegetables are some of the most consumed in the world, among which cabbage, broccoli, cauliflower, leaf mustard and kale are classified into *Brassica oleracea* species. These plants have high nutritional value due to their richness of such phytochemicals as vitamins, minerals, dietary fibers, glucosinolates, polyphenols, and phenolic acids. Since some vitamins, flavonoids and phenolic acids are able to act as scavengers of reactive oxygen species due to their antioxidative activities, these compounds are thought to be health-promoting substances,¹⁾ leading to the antioxidative capacity of *Brassica* vegetables having been well studied.²⁻⁴⁾ Moreover, epidemiological studies have revealed the potential of *Brassica* vegetables to reduce the risk of cardiovascular diseases and some types of cancer.⁵⁾

Kale is a leafy vegetable belonging to *Brassica* plants. The total polyphenol content in edible parts of kale has been reported to be higher than that of other *Brassica* vegetables such as califlower and broccoli,⁶⁾ and kale has therefore gained increased attention as a source of beneficial effects related to these substances. Kale juice has gained increasing attention as one of the popular health-promoting foods in Japan. In contrast to the antioxidative activity derived from secondary metabolites in this plant, there have been few studies on such other biological effects as the immunoregulatory activities derived from substances in kale. The present study was undertaken to examine the other potential effects of kale on human health other than its antioxidative effect. We performed extensive screening in terms of the immunological effects of a kale extract by using cultured cells *in vitro*. We demonstrate here that immunoglobulin (Ig) production-stimulating factors were present in the water-soluble fraction from a kale extract which enhanced the Ig production of hybridoma cells and lymphocytes. We also investigated the immunostimulatory effect of kale *in vivo* by using mice and identified an Ig production-stimulating factor (IPSF). These data provide valuable information to confirm another beneficial function of kale.

Materials and Methods

Reagents. Trypsin was obtained from Difco (Detroit, MI, USA). Insulin, transferrin, ethanolamine, sodium selenite, penicillin, streptomycin, the RPMI-1640 medium, and ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) were purchased from Sigma (St. Louis, MO, USA). The ERDF medium was purchased from Kyokuto Pharmaceutical (Tokyo, Japan), and fetal bovine serum (FBS) was purchased from SAFC Biosciences (Lenexa, KS, USA). Coomassie brilliant blue (CBB) R-250 was purchased from ICN Biomedicals (Aurora, OH, USA), and the molecular weight marker was purchased from Apro Science (Tokushima, Japan).

Preparation of the kale extract. Two grams of dried protophyll of kale (*Brassica oleracea* L. var. *acephala* DC.) was milled and soaked

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Abbreviations: ActD, actinomycin D; BSA, bovine serum albumin; CBB, Coomassie brilliant blue; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GALT, gut-associated lymphoid tissues; IFN, interferon; Ig, immunoglobulin; IL, interleukin; IPSF, immunoglobulin production-stimulating factor; ITES, insulin, transferrin, ethanolamine, and sodium selenite; MLN, mesenteric lymph node; PBL, peripheral blood lymphocyte; PBS, phosphate-buffered saline; PP, Peyer's patches; rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; TNF, tumor necrosis factor

in 40 mL of phosphate-buffered saline (PBS, pH 7.4) at 4 °C, before centrifuging the solution at $15,000 \times g$ for 10 min at 4 °C. The supernatant was used as the kale extract. The protein concentration was quantified with a DC protein assay (Bio-Rad, Hercules, CA, USA), using bovine serum albumin (BSA) as a standard.

Cells and cell culture. Human-human hybridoma HB4C5 cells producing lung cancer-specific monoclonal IgM were used for the assay to assess the Ig production-stimulating activity. The HB4C5 cell line was a fusion product of human B lymphocytes from a lung cancer patient and the human fusion partner, NAT-30.⁷⁾ HB4C5 cells were cultured in an ERDF medium supplemented with 100 units/mL of penicillin, 10 µg/mL of streptomycin, 10 µg/mL of insulin, 20 µg/mL of transferrin, 20 µM ethanolamine, and 25 nM sodium selenite (ITES-ERDF) at 37 °C under 5% CO₂ in humidified air.⁸⁾

Human peripheral blood lymphocytes (PBLs) were obtained from peripheral blood of a healthy female donor as described in a previous report.⁹⁾ Briefly, human peripheral blood diluted with an equal volume of PBS was centrifuged at $800 \times g$ for 20 min on a lymphocyte separation medium (Nycomed Pharma, Oslo, Norway). After washing the cells twice with the ERDF medium, PBLs were cultured at 1×10^6 cells/mL in the ERDF medium supplemented with 10% FBS and a varying concentration of the kale extract for 3 d. The supernatant was then used for enzyme-linked immunosorbent assays (ELISA).

Assay of the human Ig production-stimulating activity. The Ig production-stimulating activity was examined by measuring the amount of secreted Igs by ELISA as mentioned in a previous report.¹⁰⁾ Briefly, a goat anti-human IgM or IgG antibody solution at 1 µg/mL (Cappel, Durham, NC, USA) in a 50 mM sodium carbonate-bicarbonate buffer (pH 9.6) was added to a 96-well microplate (Nunc, Roskilde, Denmark) at 100 µL/well, and incubated for 2 h at 37 °C. After washing the wells three times with 0.05% Tween 20-PBS (PBS-T), each well was blocked with PBS containing 1% (w/v) BSA for 2 h at 37 °C. Following the blocking reaction, each well was treated with 50 µL of the culture supernatant for 1 h at 37 °C. After washing with PBS-T, each well was then treated for 1 h at 37 °C with 100 µL of the horseradish peroxidase (HRP)-conjugated anti-human IgM or IgG antibody (Biosource International, Camarillo, CA, USA) diluted at 1:1000 in PBS containing 1% BSA. After washing again, 0.6 mg/mL of 2,2'-azino-bis(ethylbenzothiazoline-6-sulfonic acid) (ABTS) dissolved in a 0.03% H₂O₂-0.05 M citrate buffer (pH 4.0) was added to each well at 100 µL, and the absorbance at 415 nm was measured after adding 100 µL of 1.5% oxalic acid to terminate the coloring reaction. The Ig production assays were conducted in triplicate.

Assay of the cytokine production-stimulating activity of human PBL. Human PBLs were inoculated at 1×10^6 cells/mL in the ERDF medium containing 10% FBS and a varying concentration of the kale extract. After cultivating in a CO₂ incubator at 37 °C for 3 d, the tumor necrosis factor (TNF)-α, interleukin (IL)-4, and IL-5 concentrations in the culture medium were measured by ELISA kits (Biosource International). Cytokine production assays were conducted in triplicate.

Real-time RT-PCR. HB4C5 cells were cultured at around 5×10^4 cells/mL in an ITES-ERDF medium containing the kale extract for 6 h. The cells were then harvested, and total RNA was extracted by using Sepasol RNA I Super G (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. First-strand cDNAs were synthesized from 1 µg of total RNA from the cells by using an oligo-dT primer and MMLV reverse transcriptase. A 20-µL real-time PCR mixture consisted of 10 µL of 2 × Fast SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA), 2 µL of a forward primer, 2 µL of a reverse primer, 2 µL of a cDNA sample, and 4 µL of water. The thermal cycling conditions were as follows: 2 min at 95 °C, 40 cycles of 15 s each at 95 °C and 15 s at 60 °C. The PCR products were measured by the StepOne real-time PCR system with StepOne software v2.0 (Applied Biosystems). The primer pairs used here for amplification were as follows: constant region of human IgM, 5'-CTCCCAAAGTGAGCGTCTTC-3' and 5'-CAGCCAGGACACCT-GAATCT-3'; and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-GCACCGTCAAGGCTGAGAAC-3' and 5'-TGGTG-AAGACGCCAGTGA-3'. Data are shown as the number-fold differ-

ence in IgM expression normalized to the GAPDH housekeeping gene as an endogenous reference.

Inhibition of transcription. The effect of the kale extract on the transcription activity of HB4C5 cells was investigated. The transcription activity of HB4C5 cells was suppressed by actinomycin D (Act D) that is an irreversible inhibitor of RNA synthesis.¹¹⁾ HB4C5 cells were inoculated at 1.0×10^5 cells/mL in the ITES-ERDF medium and treated with 2 µg/mL of Act D for 2 h in a CO₂-incubator at 37 °C. Following this treatment, a varying concentration of the kale extract was added, and the mixture cultured at 37 °C. The supernatant was used for ELISA after 4 h of cultivation to determine the IgM amount.

Oral administration. BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). The mice were housed in a room maintained at 24 °C on a 12 h light/dark cycle in a specific pathogen-free facility, and provided with tap water and the diet *ad libitum*. All animal experiments described here were carried out in accordance with the protocol approved by the Ehime University Animal Care and Use Committee and were performed in accordance with applicable guidelines and regulations. The kale extract was administered to 8-week-old female BALB/c mice at 2 mg of protein/kg of body weight per day for 14 d. The mice were sacrificed on the 15th day, and the mesenteric lymph node (MLN) and Peyer's patches (PP) were excised. Lymphocytes from these tissues were collected and washed with PBS. The cells were then cultured for 48 h in an RPMI-1640 medium containing 100 units/mL of penicillin, 10 µg/mL of streptomycin, 5% FBS, and 5 µg/mL of concanavalin A.

Assay of murine the Ig production-stimulating activity. The Ig production-stimulating activity was examined by measuring the amount of secreted Igs by ELISA as described in a previous report.¹²⁾ Briefly, the rabbit anti-mouse IgA, IgM or IgG antibody (Zymed Laboratories, Carlsbad, CA, USA) was diluted at 1:1000 in a 50 mM sodium carbonate-bicarbonate buffer (pH 9.6) and added to a 96-well plate at 100 µL/well, and the mixture incubated for 2 h at 37 °C. After washing three times with PBS-T, each well was blocked with 1% BSA/PBS for 2 h at 37 °C. Each well was then treated with 50 µL of the culture supernatant for 1 h at 37 °C. After washing three times again with PBS-T, each well was then treated for 1 h at 37 °C with 100 µL of the HRP-conjugated goat anti-human IgA, HRP-conjugated goat anti-human IgM or HRP-conjugated goat anti-human IgG antibody (Zymed Laboratories) diluted at 1:1000 in 1% BSA/PBS. A 100-µL amount of 0.6 mg/mL of ABTS dissolved in a 0.03% H₂O₂-0.05 M citrate buffer (pH 4.0) was then added to each well, and the absorbance at 415 nm was measured after adding 100 µL of 1.5% oxalic acid. Assays were conducted in triplicate.

Partial purification and SDS-PAGE. The kale extract was loaded into a Toyopearl SuperQ-650M anion-exchange chromatographic column (1.6 × 10 cm, Tosoh, Tokyo, Japan) that had been equilibrated with a 10 mM sodium phosphate buffer (pH 7.4) and then washed with the same buffer until the OD value at 280 nm became stable. Proteins were eluted at a 2 mL/min flow rate with a linear gradient of 0–1 M NaCl containing the 10 mM sodium phosphate buffer. The eluted fractions were used for an IgM production-stimulating assay and for SDS-PAGE on a 15% polyacrylamide slab gel as described by Laemmli.¹³⁾ Proteins on the gel were stained with CBB R-250.

Mass spectrometric analysis. The proteins were cut from the SDS-polyacrylamide gel and in-gel digested by trypsin at 35 °C for 20 h by using XL-TrypKit (Apro Science). A liquid chromatographic-electrospray ionization tandem mass spectrometric (LC-ESI-MS/MS) analysis of each digested sample was carried out essentially as described by Batycka *et al.*¹⁴⁾ Peptide mixtures were separated by using a microflow-HPLC system (Ultimate; Switchos; Famos; LC Packings, Amsterdam, The Netherlands). A sample volume of 20 µL was loaded by the autosampler into a home-made 2-cm fused silica pre-column (75 µm i.d.; 375 µm o.d.; Reprosil C18-AQ, 3 µm (Ammerbuch-Entringen, DE, USA)). The peptides were sequentially eluted by using a flow rate of 250 µL/min and a linear gradient from solution A (5% acetonitrile and 0.1% formic acid) to 50% of solution B

(95% acetonitrile and 0.08% formic acid) in 35 min. The peptides were eluted directly into a HCTplus high-capacity ion trap (Bruker-Daltonik, Germany). The capillary voltage was 1.5 kV and a dry gas flow rate of 5 L/min was used at a temperature of 320 °C. The scan range used was 350–1250 *m/z*. Proteins were identified by searching in the National Center for Biotechnology Information non-redundant (NCBI nr) database with the Mascot MS/MS Ion Search program (<http://www.matrixscience.com>). The following parameters were adopted for the database search: a peptide mass tolerance of ± 1.0 Da, fragment mass tolerance of ± 0.6 Da, and 2 missed cleavages. The score for the result of $(-10 \text{ Log}(P))$ had to be over the significance threshold level ($p < 0.05$) for positive identification. Replicated measurements confirmed the identity of these protein hits.

Statistical analysis. Each result is expressed as the mean \pm standard deviation (SD), and Tukey's test was used to assess the statistical significance of the difference. Each value of * $p < 0.01$, ** $p < 0.005$, or *** $p < 0.001$ is considered to be statistically significant.

Results and Discussion

Immunostimulating effect of the kale extract on HB4C5 hybridoma cells

In order to demonstrate the immunostimulatory effect of the water-soluble extract of kale, we determined the Ig production-stimulating activity by measuring the monoclonal IgM concentration produced by human-human hybridoma HB4C5 cells. HB4C5 cells were cultured for 6 h at 5×10^4 cells/mL in an ITES-ERDF medium supplemented with various concentrations of the kale extract in a 96-well culture plate, and the amount of IgM secreted into the culture medium in each well was measured by ELISA. As shown in Fig. 1, the IgM production by the HB4C5 cells was stimulated by the kale extract in a dose-dependent manner. The stimulating effect on IgM production reached approximately a 5-fold increase as compared to the control (PBS) at 40 $\mu\text{g/mL}$ of protein concentration. The IgM productivity was lower at the highest protein concentration, caused by cytotoxicity of the kale extract at this high concentration.

The heat stability of IPSFs in the kale extract was then examined. The kale extract was heated at 100 °C for 30 min and immediately cooled on ice. The sample was

then used for an assay of the IgM production-stimulating activity. The heated kale extract showed slightly higher Ig production-stimulating activity than the non-heated kale extract (Fig. 2A), suggesting that IPSFs in the kale extract were stable at high temperature and rather enhanced the activity. To further examine the properties of IPSFs in kale, the kale extract was treated with 150 $\mu\text{g/mL}$ of trypsin, one of the proteases, at 37 °C for 15 min. The treatment was terminated by heating at 100 °C for 2 min, and the sample was immediately cooled on ice. As shown in Fig. 2B, the Ig production-stimulating activity of the kale extract was completely lost by the trypsin digestion, suggesting that the active substances in the kale extract were proteins with heat stability. We had previously found lysozyme to be one of the strong Ig production-stimulating proteins, and the activity was increased by heating up to a 30-fold increase compared with the intact protein.¹⁵⁾ The Ig production-stimulating effect of these proteins might therefore have been related to their distinctive confor-

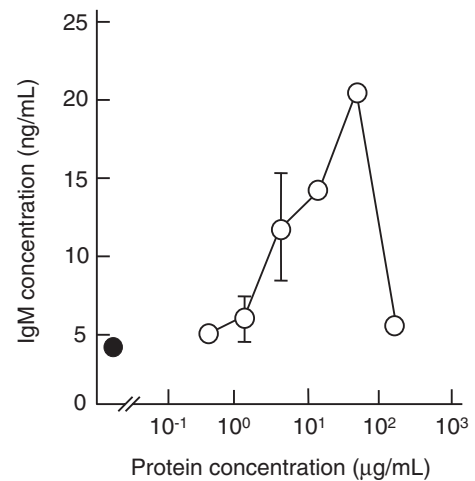


Fig. 1. Effect of the Kale Extract on IgM Production by HB4C5 Cells.

The control (●) was supplemented with PBS instead of the kale extract. Each result is presented as the mean \pm standard deviation of three independent measurements.

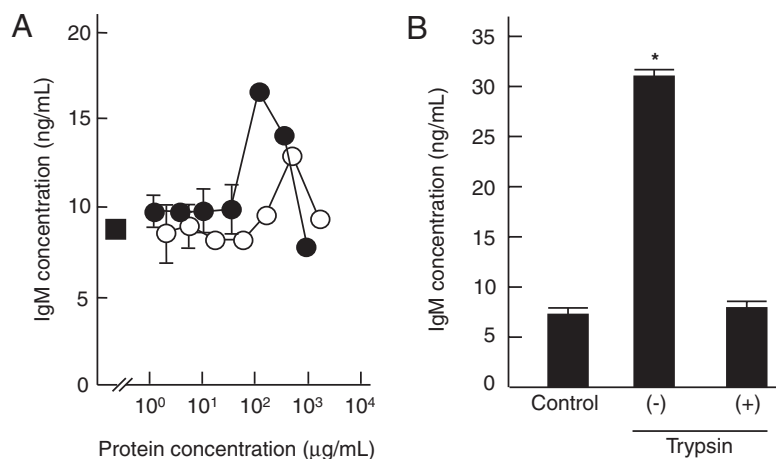


Fig. 2. Effect of Heating and Trypsin Digestion on the Ig Production-Stimulation Activity of the Kale Extract.

Each result is presented as the mean \pm standard deviation of three independent measurements. A, Heating effect on the IgM production-stimulating activity of the kale extract. Filled and unfilled circles respectively represent heated and non-heated kale samples. Control (■) represents the addition of PBS instead of the kale extract. B, Effect of trypsin digestion on the kale extract. Control represents the addition of PBS instead of the kale extract. * $p < 0.01$.

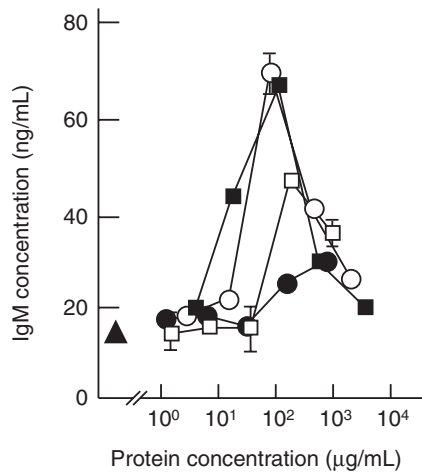


Fig. 3. IgM Productivity of the Fractionated Kale Extract by Ultra-filtration.

Filled and unfilled squares, and filled and unfilled circles respectively represent the HMW fraction (≥ 50 kDa), MMW fraction (10–50 kDa), LMW fraction (≤ 10 kDa), and the extract before fractionation. Control (\blacktriangle) was supplemented with PBS instead of the kale extract. Each result is presented as the mean \pm standard deviation of three independent measurements.

mations that prevented denaturation under the heated condition.

The active proteins in the kale extract were fractionated into three fractions by ultrafiltration into the LMW fraction (≤ 10 kDa), MMW fraction (10–50 kDa), and HMW fraction (≥ 50 kDa) to roughly examine their molecular masses. When the separated fractions were examined for the IgM productivity of HB4C5 cells, the HMW fraction exhibited greater specific activity than the MMW and LMW fractions, although relatively high activity was also shown by the MMW fraction (Fig. 3). These results therefore indicated that the molecular weight of the main IPSF in the kale extract was higher than 50 kDa and that another minor IPSF was present with a molecular weight between 10 and 50 kDa.

Effect of the kale extract at the transcriptional level

The effect of the kale extract at the transcriptional level was examined by RT-PCR. The HB4C5 cells were cultured with the kale extract for 6 h, and then the culture medium and cells were respectively subjected to ELISA to determine the IgM productivity, and to real-time RT-PCR to determine the mRNA amount encoding IgM protein in the HB4C5 cells. Figure 4A shows that the mRNA amount was not increased by treating with the kale extract, although the IgM productivity of HB4C5 cells was stimulated (Fig. 4B), indicating that the kale extract did not affect the transcriptional level to increase the IgM productivity. To confirm that the kale extract did not affect transcription, the HB4C5 cells were treated with 2 μ g/mL of Act D for 2 h to suppress mRNA production, and the effect of the kale extract on IgM production was then examined. The results showed that the IgM productivity was respectively accelerated by around 7-fold and 5-fold in the non-treated (Fig. 5A) and Act D-treated (Fig. 5B) HB4C5 cells. Although the relative productivity was slightly lower in the Act D-treated cells than in the non-treated cells, IPSFs in the kale extract seem not to have influenced transcription for IgM stimulation, because the IgM productivity was still

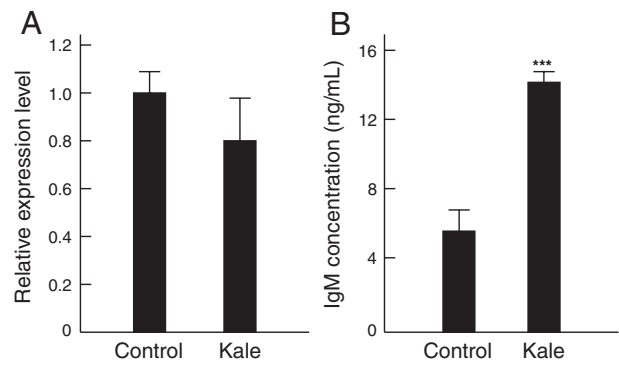


Fig. 4. Effect of the Kale Extract on the mRNA Transcriptional Level.

The control was supplemented with PBS instead of the kale extract. A, Production levels of IgM mRNAs; B, Production levels of IgM proteins. Each result is presented as the mean \pm standard deviation of three independent measurements. *** $p < 0.001$ vs. control.

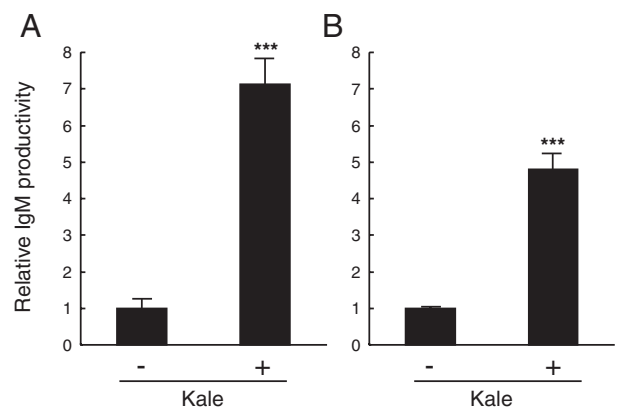


Fig. 5. Effect of the Kale Extract on IgM Production in Transcription-Suppressed Cells.

The IgM productivity is presented as the relative value against the control in each of the Act D-treated and non-treated HB4C5 cells. Each result is presented as the mean \pm standard deviation of three independent measurements. A, Non-treated HB4C5 cells; B, HB4C5 cells treated with 2 μ g/mL of Act D for 2 h. *** $p < 0.001$ vs. without the kale extract.

stimulated in the transcription-suppressed cells. It is therefore suggested that the kale extract affected the HB4C5 cells in the post-transcriptional stages such as the translational and/or protein-secreting processes to enhance the IgM production. These results are consistent with other IPSFs;^{16–19)} for example, lysozyme is one of the strong IPSFs and accelerated IgM production in both Act D-treated and non-treated HB4C5 cells.²⁰⁾ The stimulatory effect of the kale extract on the Act D-treated HB4C5 cells was lower than that on the intact HB4C5 cells. This difference may have arisen from the cytotoxic activity of Act D against HB4C5 cells.

Effect of the kale extract on Ig and cytokine production by human PBLs

The Ig production-stimulating activity of the kale extract on lymphocytes other than HB4C5 cells was examined. Human PBLs were cultured for 3 d at 1×10^6 cells/mL in the ERDF medium supplemented with 10% FBS and the kale extract, and the culture medium was subjected to ELISA. The IgM production by human PBL was facilitated 1.8-fold by treatment with the kale extract (Table 1). The IgG production was

Table 1. Effect of Kale Extracts on IgM and IgG Productions by Human PBLs

Ig class	Control	Total extract	High MW extract
IgM	10.2 ± 0.5	18.3 ± 1.0	20.9 ± 2.0
IgG	70.3 ± 0.9	81.7 ± 0.8	89.8 ± 0.2

Each result is presented as the mean ± standard deviation of three independent measurements.

Table 2. Effect of Kale Extracts on Cytokine Productions by Human PBLs

Cytokine	Control	Total extract	High MW extract
IL-4	2.3 ± 0.2	2.7 ± 0.1	2.1 ± 0.3
IL-5	9.6 ± 1.8	10.5 ± 3.1	15.7 ± 2.5
TNF- α	35.6 ± 0.1	73.4 ± 1.0	35.1 ± 3.1

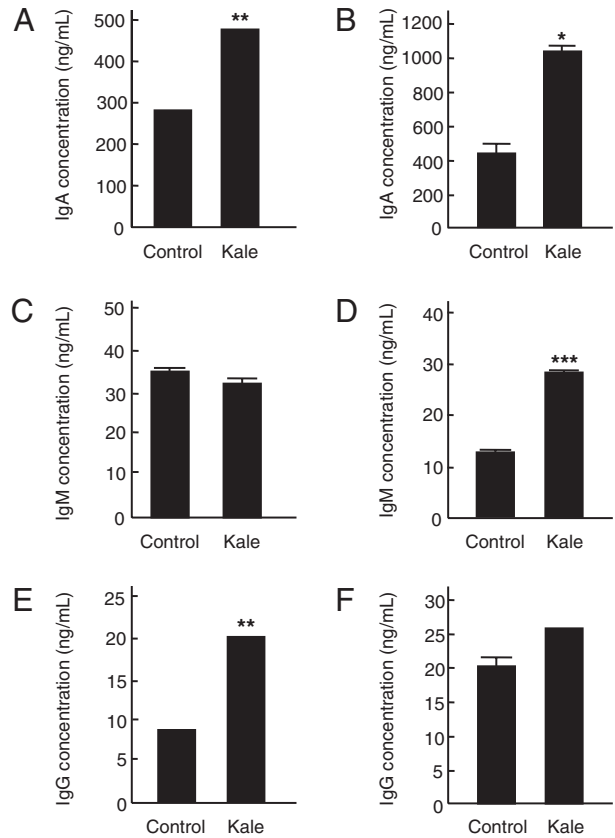
Each result is presented as the mean ± standard deviation of three independent measurements.

also enhanced by around 1.2-fold. These results suggest that IPSFs in the kale extract affected not only a specified hybridoma cell line, but also unspecified human Ig producers. Increased production of both IgM and IgG was shown when the HMW fraction of the kale extract was assayed (Table 1). This result supports the molecular weight of the main IPSFs in the kale extract being higher than 50 kDa as already shown in Fig. 3.

The effect of the kale extract on the production of typical cytokines in PBLs that affect Ig production was also examined. As shown in Table 2, the kale extract stimulated the production of TNF- α by around 2-fold. Interestingly, the HMW kale extract did not enhance the TNF- α production by PBLs, suggesting that the molecular weight of TNF- α stimulators in the kale extract was less than 50 kDa and that the active substances were different from the TNF- α stimulators in the kale extract. On the other hand, the IL-5 production was increased by treating with the HMW fraction of the kale extract, suggesting that the molecular weight of the IL-5 stimulators in the kale extract was more than 50 kDa and that the IL-5 and TNF- α inducers were different from each other. The kale extract did not stimulate the production of IL-4, although IL-4 is involved in B cell stimulation.²¹⁾

Effect of the kale extract on Ig production in mice

The kale extract was administered to 8-week-old female BALB/c mice for 14 d at 2 mg/kg/d. PP and MLN, two important intestinal lymphoid structures, were then excised, and the effect of the kale extract on Ig production in the gut-associated lymphoid tissues (GALT) in the mice was investigated *ex vivo*. The kale extract stimulated the IgA production by lymphocytes 1.7-fold in MLN (Fig. 6A) and 2.4-fold in PP (Fig. 6B). The result shown in Table 2 shows that the HMW kale extract had an IL-5-stimulating effect. Since IL-5 enhances the IgA secretion by B cells,²²⁾ increased IgA production by lymphocytes in these tissues is assumed to have been due to a continuous intake of IL-5 inducers in the kale extract. The production of IgM by lymphocytes was around 2-fold increased in PP (Fig. 6D), although not in MLN (Fig. 6C). On the other hand, the kale extract enhanced the IgG production by

**Fig. 6.** Effect of the Kale Extract on Ig Production in Mice.

Each result is presented as the mean ± standard deviation of three independent measurements. * $p < 0.01$, ** $p < 0.005$, and *** $p < 0.001$ vs. control. A, IgA production by lymphocytes in MLN; B, IgA production by lymphocytes in PP; C, IgM production by lymphocytes in MLN; D, IgM production by lymphocytes in PP; E, IgG production by lymphocytes in MLN; F, IgG production by lymphocytes in PP.

lymphocytes in MLN (Fig. 6E), but not in PP (Fig. 6F). These results indicate that the kale extract stimulated the Ig production in GALT, although the effects on Ig production were different among tissues.

Identification of IPSFs in the kale extract

The kale extract was fractionated by anion-exchange chromatography (Fig. 7A), and each fraction was assessed in terms of its IgM production-stimulating activity by using HB4C5 cells (Fig. 7B) and SDS-PAGE (Fig. 7C). A strong, common major band on the CBB-stained gel, whose molecular weight was estimated to be around 53 kDa from a molecular weight marker, was gained from fractions 1 and 2 including IPSFs. The band signal of F4 on CBB-stained gel was weak compared with the UV absorbance of the fraction. It is supposed that F4 mainly contained peptides that were not apparent on the gel. This result is consistent with that of the HMW fraction exhibiting the highest IgM production-stimulating activity in HB4C5 cells. This 53-kDa protein was then in-gel digested and analyzed by LC-ESI-MS/MS. The result of NCBI database searching showed that the 53-kDa protein was similar to the rubisco large subunit. In particular, the Mascot score between *Brassica oleracea* rubisco (52.9 kDa) and the 53-kDa protein was very high (626), and an amino acid sequence coverage between fragments of the 53-kDa

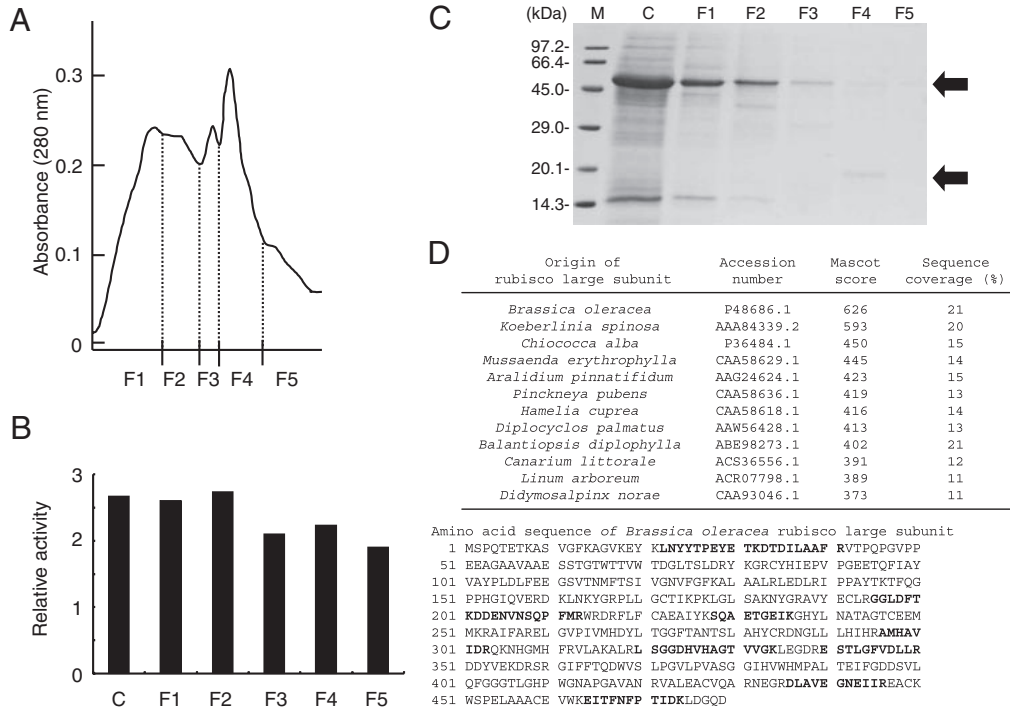


Fig. 7. Identification of the Ig Production-Stimulating Protein in Kale.

A, Partial purification of the kale extract by anion-exchange chromatography. The eluate was collected into five fractions (F1 to F5). B, IgM production-stimulating activity of the crude kale extract and partially purified kale extracts F1 to F5 as shown in A. C represents the crude kale extract. C, SDS-PAGE run on a 15% polyacrylamide slab gel using the crude kale extract and partially purified kale extracts F1 to F5 as shown in A. C and M respectively represent the crude kale extract and a molecular weight marker. Then arrow indicates the 53-kDa protein band. D, Result of the NCBI database search for the 53-kDa protein by the Mascot MS/MS Ion Search program. Bold characters indicate peptide fragments of the 53-kDa protein identified by the LC-ESI-MS/MS analysis shown in the amino acid sequence of the *Brassica oleracea* rubisco large subunit.

protein and *Brassica oleracea* rubisco was 21% (Fig. 7D). In addition, the 53-kDa protein was similar to the rubisco large subunit with other organisms. Rubisco is well known as the key photosynthetic enzyme responsible for CO₂ fixation. An IgM production-stimulating assay was then conducted using commercially available rubisco from spinach, and the activity was compared with that of the kale extract. The result shows that spinach rubisco had Ig production-stimulating activity in a dose-dependent manner and higher specific activity than that of the kale extract (Fig. 8). Although rubisco exhibited Ig production-stimulating activity with statistical difference when compared with the control production level, the Ig production-stimulating effect was not so strong. It is therefore considered that this weak activity might have been derived from the species difference of rubisco proteins or that there was another strong IPSF in the HMW kale extract other than rubisco. In addition, another active substance was observed in F4 obtained by anion-exchange chromatography. The molecular weight of the active protein was estimated to be 18.1 kDa. It is supposed that the reason for the high activity of the kale extract at the highest concentration may have arisen from a synergetic action between the rubisco large subunit and this 18.1-kDa protein. Identification of this protein is still under investigation.

In conclusion, we have demonstrated the immunostimulating effect of the water-soluble extract from kale by using human IgM-producing hybridoma cells, human PBLs, and mice. The data obtained here describe the immuno-stimulating effect of kale not only *in vitro*, but

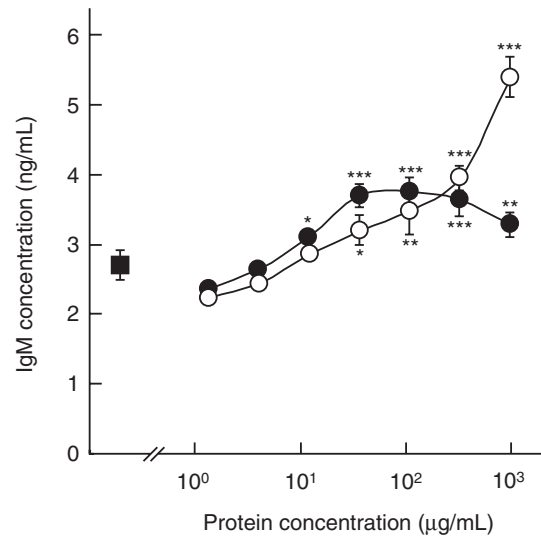


Fig. 8. Effect of Rubisco on the IgM Production of HB4C5 Cells.

Unfilled and filled circles respectively represent the kale extract and rubisco from spinach. Control (■) was supplemented with PBS instead of the kale extract. Each result is presented as the mean ± standard deviation of three independent measurements. **p* < 0.01, ***p* < 0.005, or ****p* < 0.001 vs. control.

also *in vivo*. We identified rubisco as one of the IPSFs in the kale extract and confirmed its immuno-stimulating effect by using human IgM-producing hybridoma cells. The kale extract obviously stimulated IgA production in mice. Since IgA is the predominant Ig in normal intestinal mucosa, accounting for 70–90% of all Igs in GALT and thus plays a pivotal role in host defense,²³⁾

the intake of kale might provide a beneficial effect on humans to enhance the defense against such pathogens as viruses, bacteria, and toxins. The immuno-stimulating effect will provide an additional advantage of kale, as well as its antioxidative capacity and other effects. We have also reported rubisco to have Ig production-stimulating activity. Further investigations on the molecular mechanism for rubisco and other substances in the kale extract are now underway.

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