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Stimulation of cell growth in the U-937 human myeloid cell line by honey royal jelly protein

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Abstract

Royal jelly was fractionated by ion-exchange chromatography and a protein (DIII protein) that had growth stimulating activity to the U-937 human myeloid cell line was obtained. The molecular weight of the DIII protein was 58 kDa on SDS-PAGE. The growth stimulating activity of the DIII protein was shown to be relatively heat and pH stable.

Introduction

Royal jelly has been reported to be a mixture of secretions of the hypopharyngeal gland and mandibular glands of young honey bees (*Apis mellifera*) (Yatsunami et al., 1987). Royal jelly is known to be a special exclusive food of the queen honey bee. Its chemical composition was reported by Echigo et al. (1986). The major components of honey royal jelly are carboxylic acids including 10-hydroxy-2-decenoic acid (Yatsunami and Echigo, 1984), sugar and proteins. Some reports described that royal jelly contained an antibacterial protein (Royalisin, Yatsunami and Echigo, 1985; Fujiwara et al., 1990), and stimulating factor(s) on the development of genital organs in male mice (Kato et al., 1988).

A royal jelly extract (ethanol extract) is widely used for non-alcoholic beverages. In the process of ethanol extraction of royal jelly, we obtained precipitated protein residue as a by-product. We postulated whether royal jelly protein contained cell growth factor(s) which could be useful for animal cell culture.

The object of this report is to elucidate the cell growth stimulating effect of royal jelly protein. We assessed the growth stimulating effect in the U-937 human myeloid cell line. The purification and some properties of a growth stimulating protein (DIII protein) from honey royal jelly are reported.

Materials and methods

Fractionation of royal jelly

Royal jelly from honey bees (*Apis mellifera*) was obtained from API Co. Ltd. (Gifu, Japan). Royal jelly was mixed with the same volume of deionized water and dialyzed against 50 mM sodium phosphate buffer (pH 6.2). The solution retained in a dialysis tube (molecular weight cut off of 10 kDa) was centrifuged at 20 000 × *g* for 20 min at 4 °C; the supernatant was centrifuged again at 110 000 × *g* for 60 min at 4 °C. The supernatant from the second centrifugation was used as crude royal jelly protein.

Crude royal jelly protein was charged on a DEAE-Toyopearl 650M column (1.6 × 25 cm) equilibrated with 50 mM sodium phosphate buffer (pH 6.2) and materials adsorbed were eluted with a linear gradient of

0.3 M sodium chloride. Eluted proteins were dialyzed against water and lyophylized.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The DIII fraction from DEAE-Toyopearl column chromatography was analyzed by SDS-PAGE under non-reduced conditions using a 10–20% gradient gel (Daiichi Kagaku, Japan) (Laemmli, 1970). The proteins were stained with the Coomassie brilliant blue dye. Carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa) and phosphorylase b (94 kDa) were used as standard marker proteins.

Cell culture

Human myeloid cell lines U-937 (JCRB9021) and THP-1 (JCRB0112) were obtained from the Japanese Cancer Research Resources Bank (JCRB). A phorbolester-induced macrophage-like cell line UM was established from U-937 (Kong et al., 1990), and a human-human hybridoma HB4C5 secreting IgM-antibody to human lung cancer cells was obtained from Kyushu University (Murakami et al., 1985). The cells were maintained by culturing in RPMI1640 medium (Nissui, Japan) supplemented with 10% fetal calf serum (FCS, GIBCO, USA) or an ERDF medium (Kyokuto Co., Japan) supplemented with 5% FCS (Kobori et al., 1995).

For the assay of growth stimulation of cells, 1 ml of cell suspensions (1×10^4 cells ml^{-1}) each was plated in 24 well microculture plates (Falcon, USA), and 100 μl of serially diluted samples (dissolved in phosphate buffered saline, filter sterilized) was added. After several days of culture, 110 μl of cell suspensions were harvested and cell proliferations were measured by MTT assay as described by Mossman (1983) using a 96 well plate reader. Results were expressed as the mean value for triplicate assays.

Heat treatment of an active fraction

The DIII protein was dissolved in phosphate buffered saline and heated at 20–100 °C for 30 min. Insoluble materials were removed by centrifugation at 10 000 × *g* for 10 min and the remaining growth stimulating activity was measured.

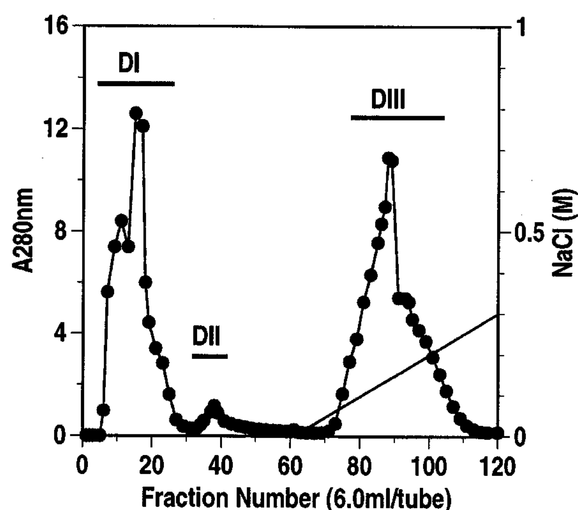

Figure 1. Elution profile of water soluble proteins from royal jelly on a DEAE-Toyopearl 650M column. Crude royal jelly proteins were charged on a DEAE-Toyopearl 650M column (1.6 × 25 cm) equilibrated with 50 mM sodium phosphate buffer (pH 6.2) and materials were eluted with a linear gradient of 0.3 M sodium chloride.

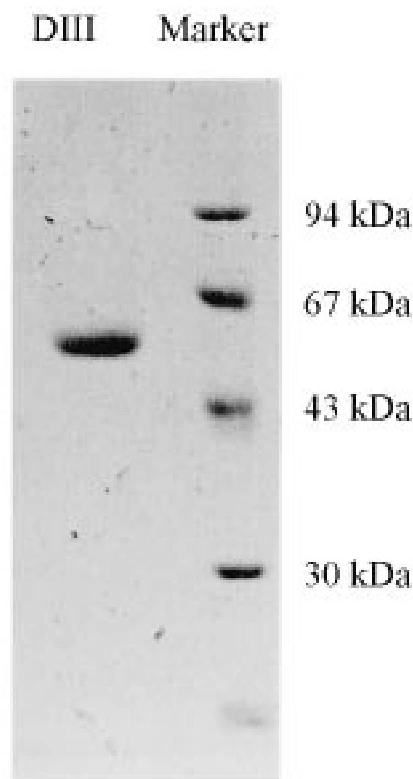

Figure 2. SDS-PAGE of royal jelly DIII protein. The DIII fraction from DEAE-Toyopearl column chromatography was analyzed by SDS-PAGE. Carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa) and phosphorylase b (94 kDa) were used as standard marker proteins.

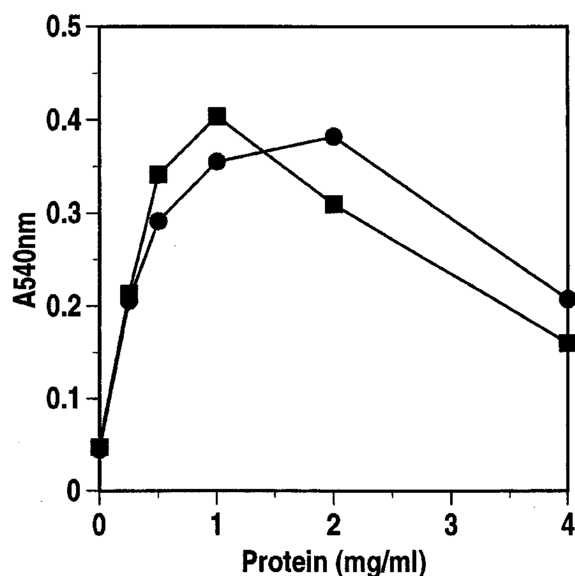

Figure 3. Dose response curves of crude proteins and the DIII protein from royal jelly on the proliferation of U-937 cells in an ERDF medium. U-937 cells were cultured in the presence of royal jelly crude proteins (●) or the DIII protein (■) for 4 days. Cell proliferation was determined by MTT assay.

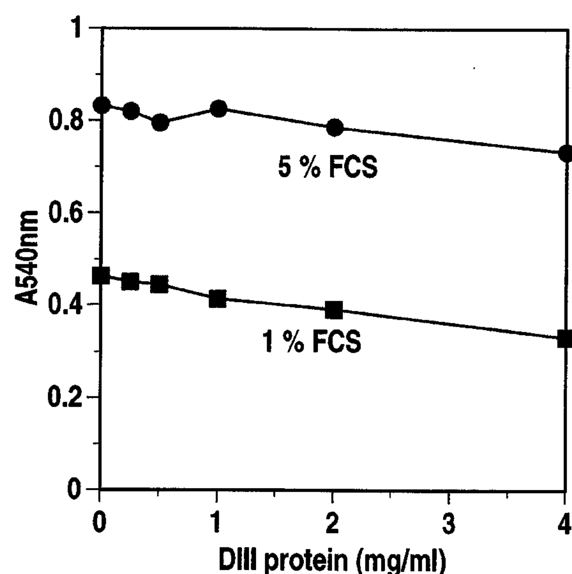

Figure 4. Dose response curves of the DIII protein on the proliferation of U-937 cells in an ERDF medium supplemented with FCS. U-937 cells were cultured in the presence of the DIII protein in an ERDF medium with 1% (■) or 5% (●) FCS for 4 days.

Acid and alkaline treatment of an active fraction

The active fraction was dissolved in distilled water (20 mg ml^{-1}), mixed with the same volume of buffer (50 mM KCl-HCl pH 2.0, 50 mM sodium acetate pH 4.0, 50 mM sodium phosphate pH 6.0, 50 mM Tris-HCl pH 8.0 and 50 mM Glycine-NaOH pH 10.0) and incubated at room temperature for 24 h. The samples were dialyzed against water, and growth stimulating activity was measured.

Measurement of the induction of NBT reducing activity

A nitro Blue Tetrazolium (NBT) assay to produce active oxygen from U-937 cells was done according to a previous report (Kobori et al., 1995). U-937 cells cultured with royal jelly samples for 3 or 4 days in an RPMI1640 medium supplemented with 10% FCS were incubated with royal jelly samples and 2 μM TPA (12-*o*-tetradecanoylphorbol 13-acetate) at 37 °C for 1 h. The number of cells stained blue was counted under light microscopy.

Results and discussion

Fractionation of royal jelly

Royal jelly proteins were fractionated by DEAE-Toyopearl column chromatography. As shown in Figure 1, we obtained three protein fractions (DI, DII, and DIII). Among these, the DIII fraction showed growth stimulating activity to the U-937 human myeloid cell line in a serum-free condition.

Figure 2 shows the result of SDS-PAGE analysis. The DIII fraction had a single protein band. The molecular weight of the DIII protein was calculated to be 58 kDa. The DIII protein seemed to be one of the major proteins of royal jelly. Otani et al. (1985) reported an analysis of royal jelly proteins by SDS-PAGE and showed that a major protein of royal jelly had a molecular weight of 45 and 54 kDa. The 54 kDa protein in their report might be the DIII protein we obtained.

Growth stimulation activity of the DIII protein

Growth stimulating activity for U-937 cells in a protein-free ERDF medium of the DIII protein was measured by MTT assay. Figure 3 shows growth stimulating activities of crude royal jelly proteins and the DIII protein. The addition of crude proteins and the DIII protein stimulated the growth of U-937 cells significantly. The optimum concentrations were 2.0 mg

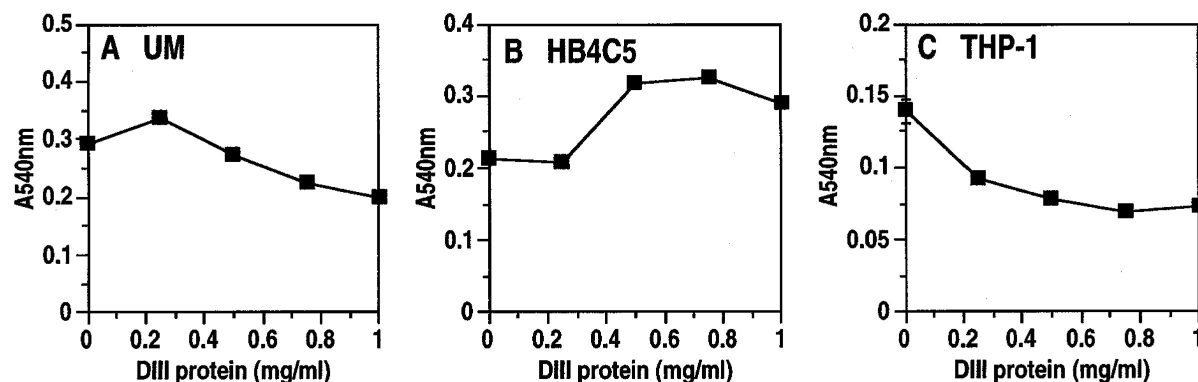

Figure 5. Dose response curves of the DIII protein from royal jelly on the proliferation of three cell lines in an ERDF medium. UM cells (A), HB4C5 cells (B), and THP-1 cells (C) were cultured in the presence of the DIII protein for 4 days.

ml^{-1} for crude proteins and 1.0 mg ml^{-1} for the DIII protein in a serum-free condition.

The effects of the DIII protein on the growth of U-937 cells in the presence of FCS were examined. As shown in Figure 4, adding the DIII protein suppressed cell growth slightly. The growth of U-937 cells at a DIII protein concentration of 4 mg ml^{-1} in the presence of 1% FCS was calculated to be 72% of the control. We thus postulated that the DIII protein has growth stimulation effects when it is added to serum-free media.

Figure 5 shows the effects of the DIII protein on the growth of macrophage-like UM cells (Figure 5A), human-human hybridoma HB4C5 cells (Figure 5B) and human myeloid THP-1 cells (Figure 5C). The DIII protein suppressed the growth of UM cells and THP-1 cells at a concentration of 1 mg ml^{-1}. The growth of HB4C5 cells increased slightly at a concentration of 0.5 mg ml^{-1} of the DIII protein.

Stability of the DIII activity

The DIII protein was heat treated and the growth stimulating activity was measured. As shown in Figure 6, heat treatment at 60 °C did not cause a significant decrease in the activity. Treatment at 80 °C decreased growth stimulating activity by 28% and treatment at 100 °C decreased 76% of the growth stimulating activity.

Results of acid and alkaline treatments are shown in Figure 7. The DIII protein seemed to be strongly acid stable even at a pH 2.0, but seemed alkaline labile.

Table 1. Effects of crude proteins and the DIII protein from royal jelly on the NBT reducing activity of U-937 cells

Sample	Incubation (days)	NBT positive cells (%)
None	3	0.8
	4	0
Crude proteins	3	0.7
	4	1.9
DIII protein	3	1.1
	4	0.6

U-937 cells were cultured with 1 mg ml^{-1} of royal jelly crude proteins or the DIII protein for 3 or 4 days. The number of NBT positive cells was counted under microscopy.

NBT assay of U-937 cells cultured with the DIII protein

The U-937 monocytic cell line can be differentiated into monocyte/macrophage by chemicals such as TPA, 1-a,25-dihydroxyvitamin D3 and all-*trans* retinoic acid. We examined the differentiation inducing effect of the DIII protein by measuring NBT-reducing activities of differentiated U-937 cells. As shown in Table I, the addition of the DIII protein did not increase the number of NBT-positive cells. We concluded that the DIII protein could stimulate the growth of U-937 cells but could not stimulate differentiation.

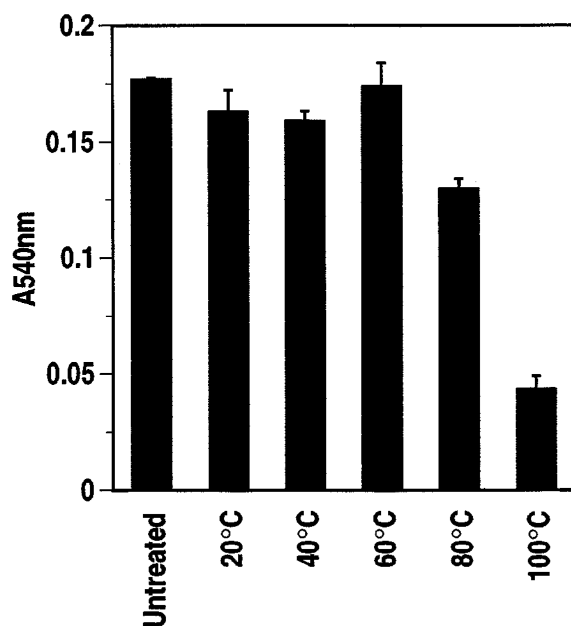

Figure 6. Heat stability of the DIII protein on the proliferation of U-937 cells. The cells were cultured for 4 days in the presence of 1 mg ml^{-1} DIII protein heated for 30 min at each temperature.

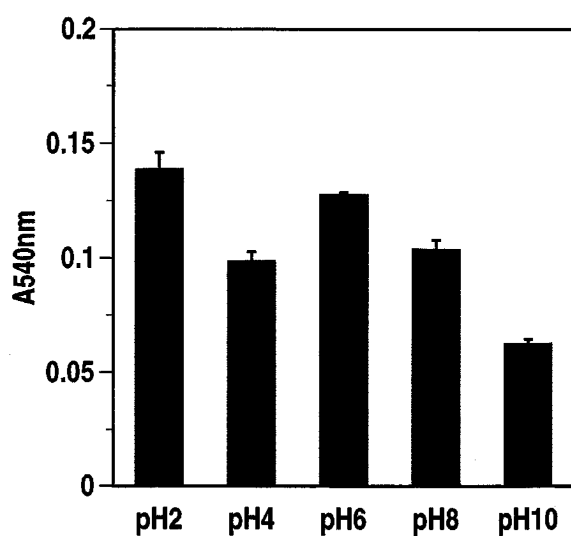

Figure 7. PH stability of the DIII protein on the proliferation of U-937 cells. The cells were cultured for 4 days in the presence of 1 mg ml^{-1} DIII protein treated with each PH for 24 h at room temperature.

Conclusions

In this paper we showed the growth stimulating activity of the royal jelly DIII protein. The DIII protein was very easy to prepare from honey royal jelly and it had growth stimulating effect to the U-937 human myeloid cell line. From these results, we concluded that the DIII protein is useful as a serum-free supplement in U-937 cell cultures.

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