Purification of Growth-promoting Substances of Human Promyelocytic Leukemia Cell Line (HL 60) in Serum-free Medium from Melon Extracts

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The substances promoting the growth of a serum-free cultured human promyelocytic leukemia cell line (HL 60) were separated by ammonium sulfate precipitation from extract of melon (Amus).

A growth-promoting activity on HL 60 cells was found in the fraction precipitated with 70 % ammonium sulfate. By DEAE-Sephacel column chromatography of the fractions, two peaks exhibiting growth-promoting activity were detected. One of the growth-promoting substances was purified by DEAE-Sephacel chromatography and chromatofocusing chromatography, and found to have a molecular weight of 32 k dalton. The growth-promoting activity of melon extract was heat-stable, and the activity after heating for 10 min at 80°C remained. It was found that this growth-promoting substance was not associated with enzymes of fruits in terms of the protease activity.

Mammalian cell lines for studying the physiological effects of food components have been used¹⁾⁻⁷⁾.

The growth-promoting substances in food were discovered on cultured animal cells. However, few studies on the growth-promoting substances in plant origin food have been reported. Our previous study revealed that extracts of various kinds of fruits promoted the growth of a human-human hybridoma cell line, HB 4 C 5, and a human promyelocytic leukemia cell line, HL 60, cultured in serum-free medium with or without insulin (I), transferrin (T), ethanolamine (E), and selenite (S)⁸⁾. Among the fruits tested, the extracts from akebi, grapefruit, grape, plum (dry), mango and melon (Amus) exhibited the strong growth-promoting activity on HB 4 C 5 cells and the

extracts from akebi, kiwi fruit, pear, banana, grape, plum and some kinds of melon exhibited a strong activity on HL 60 cells in an ERDF medium. The present study aimed at purifying the substances promoting the growth of HL 60 cells from the extract of melon (Amus).

Materials & Methods

Cells and cell culture

The cell line used in this study was a serum-free cultured human promyelocytic leukemia cell line, HL 60⁹⁾. The cells were maintained in enriched RDF medium (ERDF, Kyokuto Pharmaceutical Kogyo Co., Tokyo, JAPAN) of ERDF medium supplemented with cultured additive RD-1 (Insulin ; 5 mg/l, Transferrin ; 10 mg/l, Ethanolamine ; 1.53 mg/l, Selenite ; 4.3 ng/l : Kyokuto Pharmaceutical Kogyo Co.).

The cells were cultured in a humidified atmosphere of 5% CO₂-95% air at 37°C.

Assay of growth-promoting activity

To the culture of HL 60 cells (2×10^4 cells/ml) in ERDF medium or ERDF medium supplemented with ITES, different amounts of the extracts of melon were added and each culture was incubated in a 5% CO₂-95% air atmosphere at 37°C for 3 day. In the control, phosphate-buffered saline solution was added instead of the melon extract. The cells were counted by a Sysmex F-500 cell counter (Toa Medical Electronics Co., Tokyo, JAPAN.), and the growth rate ratio calculated by comparison with the control.

Preparation of extracts from melon (Amus)

Melon (4 kg) purchased from a supermarket was washed with water, peeled and squeezed. The juice was centrifuged at 10 000 rpm for 10 min. The supernatant collected was dialyzed against distilled water for 24 h at 4°C and against 0.02 M phosphate buffer for 24 h at 4°C.

Fractionation of the supernatant

The supernatant was fractionated with 70% ammonium sulfate, allowed to stand for 18 h at 4°C. The precipitate was collected by centrifugation at 8 000 rpm for 20 min, dissolved in 50 ml of 0.02 M phosphate buffer (pH 6.5) and dialyzed against deionized water and the same buffer for 48 h at 4°C.

The active fraction obtained by ammonium sulfate precipitation was then applied to a DEAE-Sephacel gel column (2 × 50 cm) pre-equilibrated with 0.02 M phosphate buffer (pH 6.5). The column was washed with the same buffer and eluted with 0.02 M phosphate buffer with a linear gradient of 0~2 M KCl at the flow rate of 30 ml/h, by monitoring the absorbance at 280 nm. Five ml to every fractions was collected in a tube and dialyzed against the same buffer. The active fractions thus obtained were desalted and applied to a DEAE-Sephacel column (1 × 40 cm) equilibrated with 0.02 M phosphate buffer (pH 6.5). Elution was stepwisely performed with 0.2, 0.4, 0.5, 0.6 and 0.8 M KCl. The active fraction was then re-chromatographed on a DEAE-Sephacel column

(1 × 40 cm) with stepwise elution using 0.25, 0.30, 0.35 and 0.4 M KCl, and the fractions were collected, dialyzed, sterilized and assayed for growth-promoting activity.

The active fractions were subjected to chromatofocusing chromatography on a PBE-94 gel column (Pharmacia Fine Chem.) (1 × 15 cm) using Poly buffer (Pharmacia Fine Chem.) (pH 6.0 to 4.0). The fractions were dialyzed against the same buffer, concentrated with Mole Cut II (Millipore Co.) and sterilized, and the growth-promoting activity was assayed.

Electrophoresis

Electrophoresis was performed on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) by the method of WEBER and OSBORN¹⁰. Gels were stained with 0.25% Coomassie Brilliant Blue R-250.

Assay of protease activity

The protease activity in the extracts was measured using 1% casein in 0.05 M phosphate buffer (pH 6.5)¹¹. The protease fraction (100 µl) and the substrate (2 ml) were incubated at 40°C for 30 min, and then 10% trichloroacetic acid solution (2 ml) added to stop the reaction. The mixture was then filtered and the optical density of filtrate measured at 280 nm by a Hitachi spectrophotometer.

Results

Fractionation of the extract from melon by ammonium sulfate precipitation and DEAE-Sephacel chromatography

The extract of melon was fractionated by ammonium sulfate precipitation. The growth-promoting activity on HL 60 cells in ERDF medium with or without ITES was found in the precipitated fraction with 70% ammonium sulfate. A KCl linear gradient (0~2 M) chromatography on a DEAE-Sephacel column was performed with the sample dialyzed against phosphate buffer. And then the growth-promoting fractions were applied to DEAE-Sephacel column chromatography with a stepwise elution using 0.2, 0.4, 0.5, 0.6 and 0.8 M KCl.

Figure 1 shows the elution pattern (A) and the relative growth rate (B). The growth promoting activity of each peak (I~V) on HL

Fig. 1 DEAE-Sephacel chromatography (stepwise) (A) and growth-promoting activity of each fraction (B)

The extract of melon was first separated by ammonium sulfate precipitation and then fractionated by KCl linear gradient (0 ~ 2 M) on DEAE-Sephacel. The second peak was then applied on a DEAE-Sephacel column with stepwise elution 0.2, 0.4, 0.5, 0.6 and 0.8 M KCl. To the cultures of HL 60 cells (2×10^4 cells/ml) in ERDF medium or ERDF medium supplemented with ITES, different amounts of the fractions were added and each culture was incubated in a 5% CO₂-95% air atmosphere at 37°C for 3 days. In the control, phosphate-buffered saline solution was added.

Relative growth rate: Fraction I (●), Fraction II (○), Fraction III (■), Fraction IV (□), Fraction V (▲).

60 cells in ERDF medium with or without ITES showed that peaks I and II promoted the growth of cells. Eighty microliters of peak I increased cell numbers about 130%, as compared with the control. Peak II (80 μl) also promoted the growth of cells about 60%. Other peaks were low in growth-promoting

activity. In ERDF medium supplemented with ITES, the relative growth rate of fraction I (80 μl) on HL 60 cells was approximately 30%, while other peaks increased the growth of HL 60 by about 10~20%.

Partial purification of a growth-promoting substance

Both fractions I and II showed a higher growth-promoting activity on HL 60 cells in ERDF medium. Further purification of growth-promoting substances was done on these fractions. However, the activity on fraction I resulted in loss during the purification.

Therefore, the purification of growth-promoting substances from fraction II was attempted.

Fraction II was separated into four fractions (II-1, II-2, II-3 and II-4) by DEAE-Sephacel

chromatography with a stepwise elution using 0.25, 0.3, 0.35 and 0.4 M KCl (Fig. 2 A). The highest growth-promoting activity on HL 60 cells in ERDF medium with or without ITES was found in the fraction II-2.

Chromatofocusing chromatography of the II-2 fraction with a PBE-94 column produced two fractions of II-2-1 and II-2-2 (Fig. 3). The growth-promoting activity of these fractions on cells were shown Fig. 4. The relative growth rate on 40 μ l of II-2-2 fraction was only about 30%, however the increase in

Fig. 2 DEAE-Sephacel chromatography of fraction II (A) and growth-promoting activity of each fraction (B)

The fraction II was separated with a DEAE-Sephacel column equilibrated with 0.02 M phosphate buffer (pH 6.5). Elution was stepwisely performed using 0.25, 0.30 and 0.35 M KCl.

Measuring methods of relative growth rate were indicated in Fig. 1.

Relative growth rate: Fraction II-1 (●), Fraction II-2 (○), Fraction II-3 (▲), Fraction II-4 (■).

Fig. 3 Chromatofocusing chromatography of fraction II-2

The fraction II-2 was subjected to chromatofocusing chromatography on a PBE-94 gel column using Poly buffer (pH 6.0 to 4.0).

Absorbance at 280 nm (—), pH of eluting solution (●).

Fig. 4 Growth-promoting activity of the fractions obtained by chromatofocusing chromatography

Relative growth rate: Fraction II-2-1 (●), Fraction II-2-2 (○).

specific growth-promoting activity was not proportional to the degree of purification. SDS-PAGE analysis of peak II including peak II-2-1 and II-2-2 showed two bands. Peak II-2-2 having growth promoting activity on HL 60 cells had a molecular weight of about 32 k dalton as estimated from the elution profile.

Characterization of the partially purified growth-promoting substance

It was suggested that one of the growth-promoting substances in the extract of melon was a proteinous substance. It is probable that some of the proteinous components in fruits are proteolytic enzymes¹²⁾. Heat stability of the growth-promoting activity of the fraction obtained by 70% ammonium sulfate precipitation in ERDF medium was examined at various temperatures and the protease activity of fractions was also measured. As shown in Fig. 5, the fraction had the ability to promote the growth of HL 60 cells even after heating at 80°C for 10 min, although the relative growth rate at 70°C was slightly low. This indicated that the growth-promoting substances in the extract of melon were heat-stable. On the other hand, the protease activity decreased after heating at 80°C. These findings suggested that the growth-promoting substance in the extract of melon was not a protease.

Discussion

Our present study revealed that one of the growth-promoting substances in melon was a

Fig. 5 Heat-stability of growth-promoting activity and protease activity of the fraction obtained by 70% ammonium sulfate precipitation

The fractions obtained by 70% ammonium sulfate precipitation were heated at different temperatures for 10 min, and then the growth-promoting activity on HL 60 cells in ERDF medium and the protease activity were measured.

proteinous substance (non-protease substance) having 32 k dalton of molecular weight and that it was heat-stable. The reason why the growth promoting activity was reduced was unclear, although it was purified. It is probable that trace components responsible for the growth-promoting activity were removed during the purification procedures. A similar tendency was observed when growth-promoting substances were isolated from blue-green algae¹³⁾.

Further purification of other growth-promoting substances of the Amus melon extract and the study on growth-promoting action of the active principles on human-derived cell lines are currently being attempted.

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(Received May. 13, 1991)

メロン抽出液からの無血清培養 HL 60 に対する増殖促進物質の精製

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アムスメロンを試料として無血清培養ヒト細胞 (HL-60) に対して、増殖促進活性を示す物質の精製を試みた。HL-60 に対する増殖活性は、果汁の 70% 硫酸画分に移行することが確認された。DEAE-Sephacel によるカラムクロマトグラフィーにより 2 つの活性画分が得られ、その II-2 の画分をさらに DEAE-Sephacel, クロマトフォーカシングにより精製した。精製された 1 つの活性物質は、32 k ダルトンの分子量であった。このメロン抽出液中の増殖促進物質は、80℃ 10 min. の加熱においても活性が安定であった。同時に測定した酵素活性が同条件で完全に失活したことから、このタンパク性の物質は、タンパク分解酵素とは異なることが確認された。