HAF, Hepatoma Aggregation Factor Produced by *Streptomyces* sp. Strain No. A-6143

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We searched for a new cell aggregation factor for hepatoma AH109A cells, and found one we called HAF in the culture filtrate of *Streptomyces* sp. strain No. A-6143 isolated from a soil sample. HAF was purified by salting-out with ammonium sulfate, DEAE-cellulose column chromatography, gel filtration on Sephadex G-100, and hydroxylapatite column chromatography. HAF was a glycoprotein which had a molecular weight of about 73,000. HAF was stable from pH 6 to 8 at 37°C and up to 40°C at pH 8.0, and the aggregation activity of HAF was maximum around pH 8 at 30°C. The activity was not influenced by some saccharides, but it was inhibited by EDTA and EGTA; moreover HAF activity was restored by the addition of calcium ions. HAF aggregated hepatoma AH136B and COS-7 cells as well as hepatoma AH109A cells, but it was inert to other cancer cells and human erythrocytes. These properties proved that HAF is completely different from other aggregation factors for cancer cells so far reported.

Intracellular adhesion is of fundamental importance in the physiology of multicellular organisms. Several attempts to clarify the mechanism of intracellular adhesion have proved that it is a highly complex phenomenon in which many factors operate, *i.e.*, adhesive substances, divalent cations, and so on.

Extracellular factors having cell adhesion activity have been obtained from normal and malignant cells.^{1–4)} Moreover, a number of cell aggregation substances are obtained from various microorganisms. Among them, some factors,^{5–9)} such as mouse tumor cell agglutinin⁶⁾ and canacelunin⁷⁾ which aggregate cancer cells, are produced by streptomycetes, and do not aggregate erythrocytes. And also, we reported that SAF,^{10,11)} 3315-AF,¹²⁾ and 3315-AF2¹³⁾ produced by streptomycetes did not aggregate erythrocytes, and that these factors aggregated microorganisms and cancer cells differentially.

To obtain a new factor aggregating hepatoma AH109A cells, we screened various microorganisms, and a factor was found in the

culture filtrate of *Streptomyces* sp. strain No. A-6143, which was isolated from a soil sample. By comparing biochemical and physicochemical characteristics of the factor with those of other known aggregation substances, we found that this is a new aggregation factor. Hereafter, this factor will be referred to as HAF. In this paper, the purification procedure and some properties of HAF are described.

Materials and Methods

Organisms and materials. Hepatoma AH109A¹⁴⁾ cells have been maintained in our laboratory by routine 6-day-interval passage of 2×10^6 cells inoculated intraperitoneally into 70–90 g male rats of the Donryu strain. Leukemia P388 cells (mouse DBA, *i.p.*, 5 days culture), sarcoma 180 cells (mouse ddY, *i.p.*, 7 days culture) and hepatoma AH136B (Donryu rat, *i.p.*, 10 days culture) are all cells maintained in our laboratory. After removal of cells from the animals by injector, each cell type except for hepatoma AH136B forming cell islands *in vivo* was washed several times with Hanks' balanced salt solution (HBSS). To obtain dissociated cells of hepatoma AH136B, AH136B cells were suspended in Ca, Mg-free HBSS, and trypsin was added to the suspension to a final concen-

tration of 25 mg/ml, followed by shaking at 37°C for 15 min. The cells suspended in Rabinowitz' balanced salt solution containing 0.45 mM EDTA were dissociated by gentle pipetting for 15 min at 37°C and eluted on a nylon wool column. The dissociated cells of hepatoma AH136B thus obtained were washed with Ca, Mg-free HBSS.

Neutrophils, eosinophils, and macrophages of guinea pig, and lymphocytes and L-cells of mouse were kindly provided by Dr. Tominaga of the College of Medicinal Sciences, Kumamoto University. Human cells such as HL-60, U-937, and THP-1 were purchased. Human erythrocytes were prepared from commercially obtained blood. The reagents used were guaranteed grades commercially available.

Screening test for microorganisms that produced aggregation factor. The strains of *Streptomyces* sp. isolated from soils were inoculated in S medium consisting of 2% glucose, 3% starch, 1% corn steep liquor, 1% soybean flour, 0.5% peptone, 0.3% NaCl, and 0.5% CaCO₃ (pH 7.0), and cultivated at 28°C for 3 to 5 days on a rotary shaker (180 rpm, 5 cm radius). After cultivation, the mycelia were removed by centrifugation and the culture filtrates were assayed for aggregation activity by the microtiter method using cell suspension of hepatoma AH109A.

Assay of aggregation activity. Aggregation activity was observed directly with a light microscope. The cell suspension was diluted with HBSS to obtain a cell count of 1×10^7 cells/ml. Forty μ l of a serial dilution of aggregation factor in HBSS was prepared in flat wells of a microtiter plate. Then 10 μ l of the above cell suspension was added to each well and mixed well. The mixture was left to stand for 60 min at 28°C and then 250 μ l of HBSS was added to each mixture. The mixture was examined with a microscope for the occurrence of aggregation relative to the control con-

taining no factor. The activity was expressed as the reciprocal of concentration of aggregation factor with positive aggregation.

Disc electrophoresis. Polyacrylamide gel disc electrophoresis was done by the method of Davis¹⁵⁾ at pH 8.0. A sample was put on the top of the stacking gel (7.5% acrylamide) and run at the constant current of 3 mA per gel column for 60 min at 5°C. After electrophoresis, the gel was stained with 0.2% Coomassie brilliant blue R-250 in methanol-acetic acid-water (5:1:5, v/v), and destained with 7.5% acetic acid in 5% methanol. Fuchsin-sulfite staining of the gels was done using the periodic acid-Schiff's base technique of Zacharius *et al.*,¹⁶⁾ which was originally used for the detection of glycoprotein after gel electrophoresis.

Results and Discussion

Results of screening

Culture filtrates of streptomycetes were tested by assay method described in Materials and Methods. Among 200 strains of streptomycetes tested, 11 strains produced a kind of aggregation factor toward hepatoma AH109A cells in their culture filtrates. Nine strains among them also aggregated human erythrocytes. Only two strains aggregated hepatoma AH109A cells without aggregating erythrocytes. The strain No. A-6143 had higher aggregation activity than another one, and the activity was not observed in the mycelia of this strain. Therefore, further studies were

Fig. 1. Changes in Strain No. A-6143 Culture.

●, aggregation activity (unit/μl); ○, growth; △, pH.

made using *Streptomyces* sp. strain No. A-6143.

Conditions for producing aggregation factor by the strain No. A-6143 culture

The culture conditions were examined to obtain the highest production of aggregation factor, and optimal conditions are summarized in Table I. Under these culture conditions, changes in aggregation activity and pH in the culture filtrate, and growth of mycelia with cultivation time are shown in Fig. 1. Aggregation activity appeared in its death phase. The activity reached a maximum at 5 days of cultivation, and thereafter its activity gradually decreased as the mycelia began to decrease and the pH rose. The factor in the

culture filtrate which aggregates hepatoma AH109A cells was designated as HAF, as described in the introduction.

Production and purification of HAF

Strain No. A-6143 was cultivated for 5 days, and the mycelia were removed by centrifugation at $10,000 \times g$ for 20 min. HAF required calcium ions for its activity and stability, so we were careful to maintain the calcium ion concentration in all purification procedures.

At first, to the clear supernatant was added solid ammonium sulfate to give 80% saturation. After standing overnight at 5°C, the precipitate was collected by centrifugation, dissolved in a minimal volume of 0.02M Tris-HCl (pH 8.0) containing 5mM CaCl_2 and then dialyzed against the same buffer for 2 days to free it from ammonium sulfate. This dialysate was put on a DEAE-cellulose column (2.7×30 cm) that had been equilibrated with the above buffer. HAF was eluted with the same buffer containing a linear concentration gradient of NaCl from 0 to 0.5 M. The active fractions (tubes 45–55, active fraction I) were combined and two volumes of cold ethanol were added to the solution. The pre-

Table I. CULTURE CONDITIONS FOR THE PRODUCTION OF HAF

Strain used	: <i>Streptomyces</i> sp. strain No. A-6143
Culture medium	: S medium
Initial pH	: 7.0
Culture temp.	: 28°C
Culture volume	: 50 ml medium/200 ml flask
Agitation	: 180 rpm
Seed culture	: 2 days
Main culture	: 5 days (4% seed)

Fig. 2. Column Chromatography of HAF on Hydroxylapatite.

Column size, 1.8×13 cm; fraction size, 5 ml; flow rate, 10 ml/hr; eluant, phosphate buffer (pH 7.0) of linear concentration from 0.01M to 0.4M. ●, aggregation activity (unit/ μ l); ○, absorbance at 280 nm; ----, concentration of phosphate buffer (M).

precipitate was collected by centrifugation and dissolved in a minimal volume of 0.02 M Tris-HCl (pH 8.0) containing 0.1 M NaCl and 5 mM CaCl_2 , and then dialyzed against the same buffer at 4°C. The dialysate thus obtained was put on a Sephadex G-100 column (1.5×76 cm); and eluted with the above dialysing buffer. The active fractions (tubes 36–45, active fraction II) were combined and two volumes of cold ethanol were added. The resulting precipitate was dissolved in 0.01 M phosphate buffer (pH 7.0) and then dialyzed against the same buffer at 4°C. The dialysate was put on a hydroxylapatite column (1.8×13 cm) previously equilibrated with the same buffer. HAF was eluted with a linear gradient of the phosphate buffer (10–400 mM). The elution pattern is shown in Fig. 2. Two peaks of protein (OD at 280 nm) appeared, and the aggregation activity was detected in both fractions. From the result of polyacrylamide gel electrophoresis, the second peak eluted with 0.15 M phosphate buffer was contami-

nated with some proteins having no aggregation activity, but the first peak that passed through the column was shown as a single band in the gel. Therefore, the active fraction (tubes 5–9) were combined and dialyzed against water to remove any salt and then lyophilized.

Homogeneity of HAF was examined by polyacrylamide gel disc electrophoresis at pH 8.0. As shown in Fig. 3, HAF gave a single, clear band in the gel stained with Coomassie brilliant blue R-250 or periodic acid-Schiff's base, so HAF was regarded as a homogeneous glycoprotein.

Table II shows a summary of purification of HAF from the culture filtrate. The factor was purified 1990 times on the basis of protein (unit/OD at 280 nm) with a yield of 26% from the culture filtrate. Finally, about 800 μg of HAF was obtained from 710 ml of the culture filtrate, and $1.4 \times 10^{-3} \mu\text{g}$ of HAF gave 1 unit

Fig. 3. Polyacrylamide Gel Disc Electrophoresis of HAF.

The arrow indicates the position of the sample. A: Gel stained with Coomassie brilliant blue R-250. B: Gel stained with periodic acid-Schiff's base.

Fig. 4. Molecular Weight Measurement of HAF on Sephadex G-100 Column.

●, trypsin (MW: 24,000); ■, ovalbumin (MW: 46,000); ▲, bovine serum albumin (MW: 67,000); ○, HAF.

Table II. SUMMARY OF PURIFICATION STEPS OF HAF

Purification step	Volume (ml)	Total activity (unit $\times 10^{-3}$)	Yield (%)	Specific activity (unit/OD ₂₈₀)	Purification rate
Culture filtrate	710	2,272	100	215	1
Active fraction I	114	1,459	64	10,490	48
Active fraction II	35	224	10	20,000	93
Dialysate	7	364	16	40,950	190
Purified HAF	23	591	26	428,300	1,990

in the assay system as described in Materials and Methods.

Ultraviolet absorption spectrum of HAF

Lyophilized HAF was dissolved in deionized water (1 mg/ml) for ultraviolet absorption measurement, and recorded on a Hitachi U-2000 spectrophotometer. The spectrum of HAF showed a maximum at 278 nm and minimum at 245 nm. The spectrum was analogous to typical absorption spectra of proteins, indicating the presence of tyrosine and tryptophan in the HAF molecule. A 1% solution of HAF had an absorbance of 16.7 at 280 nm.

Molecular weight measurement of HAF

The molecular weight of HAF was mea-

sured by the method of Andrews¹⁷⁾ with a Sephadex G-100 column at 5°C in 0.02 M Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl and 5 mM CaCl₂. In Fig. 4, the ratio of elution volume to void volume was plotted against the logarithm of molecular weight for each reference protein. From the result, an approximate molecular weight of 73,000 was obtained for HAF.

Effects of pH and temperature on the aggregation activity of HAF

The aggregation activity was measured while changing the pH from 3 to 11.0. As shown in Fig. 5(A), the activity of HAF turned out to be pH-dependent. HAF showed weak aggregation activity at acidic pH, the activity increased as the pH rose, and the activity was

Fig. 5. Effects of pH and Temperature on the Aggregation Activity of HAF.

(A): The effects of pH on the activity were assayed at each pH at 28°C for 60 min. The activity at pH 8.0 was expressed as 100. (B): The effects of temperature on the activity were assayed at each temperature at pH 8.0 for 60 min. The activity at 28°C was expressed as 100.

Fig. 6. Effects of pH and Temperature on the Stability of HAF.

(A): After incubation at each pH at 37°C for 60 min, remaining activity was measured at pH 8.0. Relative activity was expressed at pH 8.0 as 100. (B): After incubation at each temperature at pH 8.0 for 60 min, remaining activity was measured at 28°C. Relative activity was expressed at 28°C as 100.

not shown at pH above 10. The optimal pH of HAF for aggregation toward hepatoma AH109A cells was 8 to 9.

The aggregation activity was examined at incubation temperatures from 0°C to 80°C. Figure 5(B) shows that the activity was temperature-dependent as with pH, and its activity was maximum at around 30°C.

Effects of pH and temperature on stability of HAF

The stability of HAF was tested in buffer solution of various pHs at 37°C for 60 min. Residual activity was measured at pH 8.0. As shown in Fig. 6(A), HAF was stable from pH 7 to 8, but its activity was about 50% of the initial activity.

After treatment at various temperatures for 60 min at pH 8.0, each remaining activity was assayed at 37°C. As shown in Fig. 6(B), HAF was stable up to 40°C, but gradual inactivation occurred above 40°C, and HAF was completely inactivated at 60°C.

Effects of various reagents on the aggregation activity of HAF

It is known that the productivity of L-fucose- or D-mannose-specific lectins was widely distributed in streptomycetes.^{18,19)} And also, we reported that the aggregation activities of SAF^{10,11)} and 3315-AF¹²⁾ were not inhibited by various saccharides, but both factors were inhibited by bovine serum albumin. The effects of various reagents on HAF activity were investigated by measuring the remaining aggregation activity toward hepatoma AH109A cells after incubation of HAF with each reagent at pH 8.0 and 37°C for 10 min. As shown in Table III, HAF was not affected by any saccharide or bovine serum albumin, but HAF was strongly inhibited by ethylenediaminetetraacetic acid (EDTA) and ethylene glycol bis(β -aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA).

Oishi *et al.*⁶⁾ reported that the aggregation at pH 6 of mouse tumor cell agglutinin from streptomycetes was strongly inhibited by EDTA and the inhibition was not restored by

Table III. EFFECTS OF VARIOUS REAGENTS ON THE AGGREGATION ACTIVITY OF HAF

Reagent	Concentration (mM)	Aggregation ^a activity (%)
D-Fucose	10	100
L-Fucose	10	100
D-Galactose	10	100
D-Glucose	10	100
D-Mannose	10	100
<i>N</i> -Acetylgalactosamine	10	100
<i>N</i> -Acetylglucosamine	10	100
Lactose	10	100
Hyaluronic acid	100 μ g ^b	100
Bovine serum albumin	100 μ g	100
Ovalbumin	100 μ g	100
EDTA	10	0
EGTA	10	0

^a The activity of HAF was measured with each reagent in the standard assay solution (50 μ l), and the activity without each reagent was expressed as 100.

^b Each 100 μ g/incubation mixture.

Table IV. REACTIVATION OF EDTA-TREATED HAF WITH CALCIUM IONS

The activity was shown as a relative value to that obtained from the buffer without EDTA.

	With EDTA	Without EDTA
CaCl ₂ not added	0	100
CaCl ₂ added	100	100

the addition of a sufficient amount of calcium and/or magnesium ions. Therefore, reactivation of EDTA-treated HAF with calcium ions was examined. After HAF was incubated with final 1 mM EDTA at 0°C for 10 min, CaCl₂ solution was added to make a final concentration of 1 mM in the mixture, and then the aggregation activity was assayed using Ca, Mg-free HBSS. As shown in Table IV, HAF completely lost its activity by incubation with EDTA, however this EDTA-inhibition disappeared upon the addition of calcium ions, thus differing from mouse tumor cell agglutinin. The fact also suggested that calcium atoms, which were essential for its activity, might be contained in the HAF molecule.

Before treatment with HAF

After treatment with HAF

Fig. 7. Hepatoma AH109A Cells before and after Treatment with HAF.

Table V. EFFECTS OF HAF ON VARIOUS ANIMAL CELLS

Animal cell	Amount required to cause aggregation (ng/ml of inc. mix.) ^a
Hepatoma AH109A (rat)	28
Hepatoma AH136B (rat)	28
COS-7 (monkey)	28
L-Cell (mouse)	> 7000
Leukemia P388 (mouse)	> 7000
HL-60 (human)	> 7000
U-937 (human)	> 7000
THP-1 (human)	> 7000
HeLa (human)	> 7000
Erythrocyte (human; A, B, O, AB-type)	
Lymphocyte (mouse)	> 7000
Macrophage (guinea pig)	> 7000
Neutrophil (guinea pig)	> 7000
Eosinophil (guinea pig)	> 7000

^a The aggregation was observed directly by microscope as described in the text.

Effects of HAF on various animal cells

The aggregation activity of HAF was tested toward various animal cells. The results of Table V shows the minimal amount of HAF causing cell aggregation. The dissociated cells of hepatoma AH136B and COS-7 cells were aggregated by HAF and the concentration of HAF causing aggregation was similar to that toward hepatoma AH109A cells, but HAF did not aggregate the other tumor cells, erythrocytes, and so on. Figure 7 shows hepatoma AH109A cells before and after treatment with HAF. HAF had rather high selectivity toward

a kind of cancer cells. While a change of cell-surface by HAF is not proven, this differential aggregation indicated that some specific interaction between the surface of the cells and HAF must be involved in the aggregation.

Numerous microorganisms produce cell aggregation substances which do not aggregate human erythrocytes. Among them, SAF,¹⁰⁻¹¹⁾ 3315AF,¹²⁾ mouse tumor cell agglutinin,⁶⁾ and canacelunin,⁷⁾ which aggregate cancer cells are produced by *Streptomyces* sp. as is HAF. HAF is distinguished from these four factors in the cell-specificity of aggregation activity. Wheat germ agglutinin from *Triticum vulgaris* aggregates hepatoma AH109A, but this lectin also aggregates human erythrocytes.²⁰⁾ Calcium ions are concerned with the aggregation activity of HAF. Sponge aggregation factor¹⁾ and cadherins²⁾ from animal cells require calcium ions for their activities. HAF was also distinguished from these factors by other properties. HAF was similar in its molecular weight and selectivity toward cancer cells to cell surface-associated adhesive factor,³⁾ which is a glycoprotein, from rat hepatoma AH136B cells. However, HAF differs from this factor since the aggregation activity of this factor is inhibited by D-mannose. Thus, all data reported above suggested that HAF is a new aggregation factor that aggregates cancer cells.

HAF has high selectivity toward hepatoma AH109A cells. It would be interesting to find the HAF binding site on the cell surface. The

receptor for HAF and the mode of action are now under investigation, and the results will be described in our next paper.

References

- 1) P. Henkart, S. Humphreys and T. Humphreys, *Biochemistry*, **12**, 3045 (1973).
- 2) Y. Shirayoshi, T. S. Okada and M. Takeichi, *Cell*, **35**, 631 (1983).
- 3) S. Kurano, M. Ishida and Y. Ishimaru, *J. Cell Science*, **66**, 367 (1984).
- 4) S. Benchimol, A. Fuks, S. Jothy, N. Beauchemin, K. Shirota and C. P. Stanners, *Cell*, **57**, 327 (1989).
- 5) M. Crandall and T. D. Brock, *Bacteriol. Review*, **32**, 139 (1968).
- 6) K. Oishi and K. Aida, *Agric. Biol. chem.*, **39**, 183 (1975).
- 7) T. Ikekawa, T. Asari, T. Manabe, M. Umeji, S. Yanoma and K. Oishi, *J. Antibiot.*, **33**, 776 (1980).
- 8) K. Sakka, T. Endo, M. Watanabe, S. Okuda and H. Takahashi, *Agric. Biol. Chem.*, **45**, 497 (1981).
- 9) K. Kodama and M. Murata, *Agric. Biol. Chem.*, **48**, 2423 (1984).
- 10) K. Suzuki, M. Kosai, K. Yokomizo, M. Uyeda and M. Shibata, *Agric. Biol. Chem.*, **51**, 3017 (1987).
- 11) K. Suzuki, M. Kosai, K. Yokomizo, M. Uyeda and M. Shibata, *Agric. Biol. Chem.*, **52**, 555 (1988).
- 12) K. Suzuki, N. Nakano, R. Tanaka, M. Uyeda and M. Shibata, *Agric. Biol. Chem.*, **52**, 2589 (1988).
- 13) M. Uyeda, K. Suzuki and M. Shibata, *Agric. Biol. Chem.*, **54**, 251 (1990).
- 14) S. Odashima, *J. Natn. cancer Inst. Monog.*, **16**, 51 (1964).
- 15) B. J. Davis, *Ann. N. Y. Acad. Sci.*, **121**, 404 (1964).
- 16) R. M. Zacharius, T. E. Zell, T. H. Morrison and J. J. Woodlock, *Anal. Biochem.*, **30**, 148 (1969).
- 17) P. Andrews, *Biochem. J.*, **96**, 595 (1965).
- 18) T. Kameyama, K. Oishi and K. Aida, *Biochim. Biophys. Acta*, **587**, 407 (1979).
- 19) I. Matsui, K. Oishi, K. Kanaya and N. Baba, *J. Biochem.*, **97**, 399 (1985).
- 20) D. F. Smith, G. Neri and E. F. Walborg, *Biochemistry*, **12**, 2111 (1973).