Note

3315-AF2, a Cell Aggregation Factor Produced by *Streptomyces* sp. Strain No. A-3315

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Some cell aggregation substances produced by microorganisms do not aggregate human erythrocytes, but do aggregate microorganisms and animal cells differentially.^{1–3)}

We have previously reported that *Serratia* aggregation factor (SAF), produced by *Streptomyces murinus* strain No. A-2805, is a glycoprotein and that the factor was able to aggregate some bacterial cells, and sarcoma 180 and HeLa cells in the presence of calcium or manganese ions.^{4–5)} Recently, we found that *Streptomyces* sp. strain No. A-3315 produced two kinds of aggregation factors, designated as 3315-AF and 3315-AF2, in the culture filtrate. 3315-AF was purified and some of its properties were described in the previous paper.⁶⁾ We now report the purification of 3315-AF2 and some differences between SAF and 3315-AF.

The aggregation activity toward bacterial cells was measured as the degree of the decrease in the turbidity of a cell suspension. One unit of aggregation activity was defined as the amount of factor causing a decrease in the optical density at 660 nm of 0.2 per 120 min. The reaction mixture and conditions were described in our previous paper.⁴⁾

Streptomyces sp. strain No. A-3315 was cultivated for 14 days under the same conditions as described in the preceding paper⁶⁾ and the mycelia were removed by centrifugation. To the culture filtrate was added active carbon powder, followed by stirring 30 min. After the carbon had been removed by filtration, an equal volume of cold ethanol was added to the filtrate. The precipitate was dispersed in deionized water, and applied on a Sepharose 2B column and eluted with a NH_4OH solution (pH 8). The active fraction was extracted with ether at pH 4, and then the extract was applied on a silica gel column and eluted with the solvent pair of *n*-hexane and ether. The active fractions (crude solution) containing 3315-AF and 3315-AF2 were combined, and then the solution was applied on a Sephadex LH-20 column equilibrated with 2-propanol. Two peaks of aggregation activity appeared, the first

fraction being 3315-AF, which was reported in the previous paper.⁶⁾ To obtain 3315-AF2, the fractions comprising the second peak were combined, and then silica gel column chromatography and gel filtration on Sephadex LH-20 were repeated under the respective conditions. 3315-AF2 was purified about 21-fold on the basis of the OD at 275 nm, with a yield of 1.3% from the crude solution. Finally, we obtained about 1.4 mg of 3315-AF2 from one liter of culture filtrate, the aggregation activity of the factor toward *Serratia* being 68 units per mg.

3315-AF2 was a colorless and oily liquid. It was soluble in ethanol, 2-propanol and *n*-hexane, but insoluble in water. It showed a positive H_2SO_4 reaction, and negative Dittmer, ninhydrin, FeCl_3 and SbCl_5 reactions on TLC. 3315-AF2 gave a single spot, R_f 0.49, with *n*-hexane–ether (4:1) on TLC on a Merck Silica gel 60 F_{254} plate, 0.89 with 2-propanol–acetonitrile (1:1) on a Merck RP-8 F_{254} plate and 0.94 with benzene–ether (4:1) on a silica gel (type G) containing 20% AgNO_3 plate. The UV spectrum in 2-propanol showed a maximum at 275 nm (E value, 34.4), and the IR spectrum showed peaks at 2964, 2936, 2868, 1734, 1124 and 1074 cm^{-1} . The ^1H and ^{13}C NMR signals are shown in Table I. From these results, the structure of 3315-AF2 was determined to be bis(2-ethylhexyl) phthalate. Garner and Watson⁷⁾ reported that this compound was obtained from a reaction mixture of 2-octanol, phthalic anhydride and xylene by means of steam distillation. However, it is interesting that this compound was obtained from the culture filtrate of a streptomycetes strain.

In Table II, some properties of 3315-AF2 are sum-

Table I. NMR DATA FOR 3315-AF2

Position	^{13}C NMR	^1H NMR
1	11.0*(q)	0.90*(t)
2	23.0 (t)	1.26–1.48
3	23.8 (t)	1.26–1.48
4	29.0 (t)	1.26–1.48
5	38.8 (d)	1.56–1.72
6	68.2 (t)	4.22 (m)
7	167.8 (s)	
8	14.1 (q)	0.92 (t)
9	30.4 (t)	1.26–1.48
10	132.5 (s)	
11	130.9 (d)	7.69 (m)
12	128.8 (d)	7.54 (m)

* ppm

3315-AF2, bis(2-ethylhexyl) phthalate.

Table II. COMPARISON OF 3315-AF2 WITH 3315-AF AND SAF

	3315-AF2	3315-AF	SAF
Producer	<i>Streptomyces</i> sp.	No. A-3315	<i>St. murinus</i> No. A-2805
Localization	Extracellular	Extracellular	Intracellular
Structure	Phthalate	Triglyceride	Glycoprotein
Activity (unit/mg)	68.0	76.0	15.0
Optimum pH	7.0	8.0	8.0
Optimum temp. (°C)	28.0	27.0	30.0
Activator	Ca, Co, Ba	Ca, Co, Ba	Ca, Mn (essential)
Inhibitor; ID ₅₀ (μg)			
BSA	5	24	26
Hyaluronic acid	13	24	> 100
Laminarin	> 100	> 100	56
Aggregation	<i>S. marcescens</i> , <i>S. cerevisiae</i> , <i>C. neoformans</i> ,	<i>P. vulgaris</i> <i>C. albicans</i> HeLa	<i>S. marcescens</i> <i>E. coli</i> , <i>M. luteus</i> <i>B. subtilis</i> , <i>S. aureus</i> HeLa, Sarcoma 180

marized, and compared with those of 3315-AF and SAF. 3315-AF2 is similar to 3315-AF in its properties, but is distinguishable from SAF. The activity of 3315-AF2 turned out to be pH- and temperature-dependent, the optimum pH and temperature being 7.0 and 28°C, respectively. 3315-AF2 and 3315-AF aggregated *Serratia* in the absence of metallic ions, and the activity was enhanced by the addition of CaCl₂, CoCl₂ and BaCl₂. On the other hand, SAF required metallic ions for aggregation. The aggregation activities of 3315-AF2 and 3315-AF were not inhibited by mono- or disaccharides, but both factors were inhibited by hyaluronic acid and bovine serum albumin. The degree of this inhibition against 3315-AF2 was stronger than that against 3315-AF. 3315-AF2 aggregated *Proteus vulgaris* and HeLa cells as well as *Serratia marcescens*, and showed weak activity toward *Saccharomyces cerevisiae*, *Candida albicans* and *Cryptococcus neoformans*, but could not aggregate *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus subtilis* or the animal cells such as human erythrocytes (A, B, O and AB-type), leukemia P388 cells, lymphocytes, macrophages, sarcoma 180 cells or hepatoma AH109A

cells (data not shown).

3315-AF2 turned out to be a kind of phthalate derivative and is thought to undergo some interaction with the hydrophobic sites on the cell membranes from its lipophilic nature.

References

- 1) K. Oishi and K. Aida, *Agric. Biol. Chem.*, **39**, 183 (1975).
- 2) T. Ikekawa, T. Asari, T. Manabe, M. Umeji, S. Yanoma and K. Oishi, *J. Antibiot.*, **33**, 776 (1980).
- 3) K. Sakka, T. Endo, M. Watanabe, S. Okuda and H. Takahashi, *Agric. Biol. Chem.*, **46**, 497 (1981).
- 4) K. Suzuki, M. Kosai, M. Uyeda and M. Shibata, *Agric. Biol. Chem.*, **51**, 3017 (1987).
- 5) K. Suzuki, M. Kosai, K. Yokomizo, M. Uyeda and M. Shibata, *Agric. Biol. Chem.*, **52**, 555 (1988).
- 6) K. Suzuki, N. Nakano, R. Tanaka, M. Uyeda and M. Shibata, *Agric. Biol. Chem.*, **52**, 2589 (1988).
- 7) P. J. Garner and G. Watson, U.S. Patent 2,508,911 (May 23, 1950).