BBA 75490

SOLUBILIZATION OF THE FACTOR REQUIRED FOR POLYMERIZATION OF FLAGELLIN INTO P-FILAMENT

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(Received April 14th, 1970)

SUMMARY

1. A membrane fraction derived from deflagellated cells of a Salmonella strain initiates polymerization of flagellin into "P-filaments" which differ in many respects from flagellar filaments[1].

2. When the membrane fraction was treated with 1% (w/v) Tween-20, it was partially solubilized, giving a fraction which was effective for initiation of polymerization.

3. The soluble fraction contained protein, (poly-)saccharide and organic phosphorus presumably originating from phospholipid. The weight ratio between protein and saccharide was about 1:1.5 and that between organic phosphorus and saccharide was about 1:10.

4. These components appeared to be tightly associated into a complex, which had an $s^{\circ}_{20,w}$ value of 4.2.

5. On the addition of the soluble fraction practically 100% of the total flagellin was polymerized into P-filaments. When P-filaments were washed with distilled water, they contained only trace amounts of saccharide and organic phosphorus.

INTRODUCTION

HOTANI *et al.*[1] have reported that a membrane fraction, derived from an autolysate of deflagellated cells of a Salmonella strain, initiates polymerization of flagellin into "P-filaments" which largely differ in morphological and physicochemical properties from flagellar filaments. However, the preparation of the membrane fraction used in that study was contaminated with proteolytic enzymes, by which a part of the mixed flagellin was digested before polymerization. Indeed, only less than 50% of the total flagellin could be recovered in the form of P-filament.

Subsequently to the above study, we attempted to solubilize the membrane fraction without loss of "activity" for the initiation of polymerization. When the membrane fraction was treated with detergent, it was partly solubilized into a fairly homogeneous small component, and on the addition of this component practically 100% of the flagellin was polymerized into P-filaments. Hereafter, the preparation of this component will be referred to as "the soluble fraction". This success made it possible to investigate the biochemical properties of P-filament.

HOTANI *et al.*[2] have shown that the primary structure of flagellin remains unchanged upon polymerization and that P-filament contains small amount of protein originating from the added soluble fraction.

In this paper we report the preparation of the soluble fraction and some of its properties. It contains protein, (poly-)saccharide and presumably phospholipid. These componens have not been separated successfully, and it remains unresolved whether the factor required for the initiation of polymerization is associated with one or two of the components or with a complex of them.

MATERIALS AND METHODS

Preparation of flagellin

Salmonella strain SJ25, which produces normal flagella with 1,2 antigen, was used. This was a gift from Dr. T. IINO[3]. Cultivation of the organisms and the isolation and partial purification of flagella were carried out as described by HOTANI *et al.*[2]. Partially purified flagella were suspended to give a concentration of about 10 mg/ml in a solvent containing 0.15 M NaCl, and were depolymerized into monomeric flagellin by heating at 65° for a few min. After clarification by centrifugation at 105000 × *g* for 1 h, the solution was mixed with concentrated $(NH_4)_2SO_4$ to a final concentration of 0.7 M and left standing at room temperature. In a few hours long flagellar filaments were reformed in the solution[4,5]. The filaments were sedimented by centrifugation at 78000 × *g* for 1 h and resuspended in 0.15 M NaCl to a final concentration of about 10 mg/ml. Flagellin solutions were obtained by heating purified flagella solutions at 65° for 3 min.

Preparation of membrane fraction

The membrane fraction was prepared by the procedure of HOTANI *et al.*[1] with slight modifications (see Fig. 1). Deflagellated cells were washed twice with large volumes of distilled water and incubated at a density of about 1 g wet wt. per ml

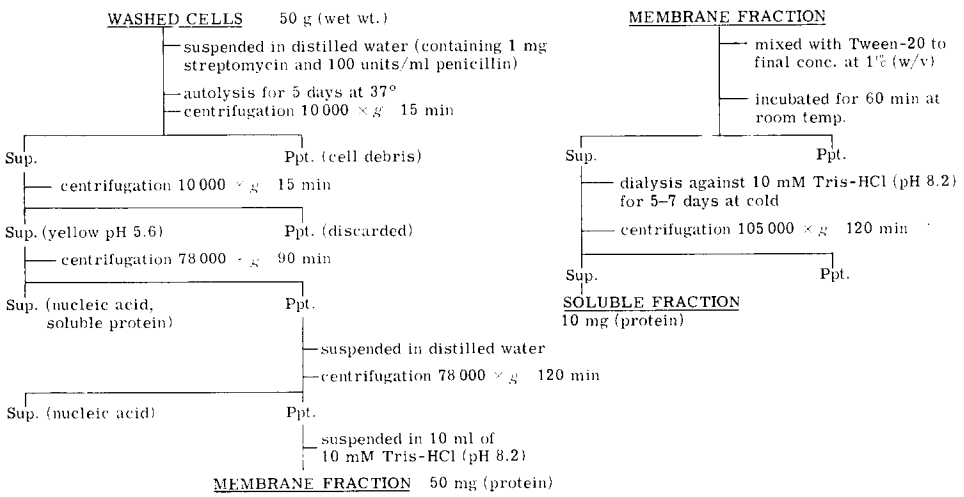

Fig. 1. Procedure for the preparation of soluble fraction.

at 37° for 5 days in the presence of 1 mg/ml streptomycin and 100 units/ml penicillin. In this period the cells underwent autolysis. The autolysate was centrifuged twice at 10000 × *g* for 15 min to remove heavy material. The supernatant was yellow and slightly opalescent; its pH was 5.3–5.6. This fluid was centrifuged at 78000 × *g* for 90 min; the precipitate was washed with a large volume of distilled water, centrifuged and resuspended in 10 ml of solvent containing 10 mM Tris–HCl buffer (pH 8.2). This suspension will be called "the membrane fraction". The procedure for obtaining the soluble fraction from this fraction will be described later.

Assay for activity

We assayed the activity by following the increase in viscosity associated with the formation of P-filaments. 0.1 ml of a given sample of membrane fraction or soluble fraction was added to 0.53 ml of a flagellin solution containing 5–10 mg/ml protein, 0.15 M NaCl and 50 mM Tris–HCl buffer (pH 8.2) or 10 mM phosphate buffer (pH 7.5); 0.5 ml of the mixture was used for measurement of viscosity. An Ostwald-type viscometer was used. The initial rate of increase in viscosity was taken as a measure for expressing the initial rate of formation of P-filaments[2].

Other methods

The sedimentation property of soluble fraction was examined using a Spinco Model-E ultracentrifuge.

P-filaments were observed in a JEM T7 electron microscope with negative staining.

The concentration of protein contained in flagellin solutions was determined by the biuret reaction[6] and that in the membrane or soluble fraction by the Lowry–Folin method[7], using bovine serum albumin as a standard.

The saccharide component was determined by the phenol–H_2SO_4 method[8].

DNA and RNA were determined by the diphenylamine reaction[9] and the orcinol reaction[10], respectively.

Organic phosphorus was determined by the method of LOWRY AND LOPEZ[11].

Detergents

Tween-20 (polyoxyethylene sorbitane monolaurate), sodium deoxycholate, sodium cholate, digitonin, and sodium dodecyl sulphate were used. The detergents were obtained commercially and used without purification.

RESULTS

Partial solubilization of membrane fraction with detergents

First, several kinds of detergent were compared with respect to usefulness for the present purpose. The experimental results obtained are given in Table I. The extent of overall solubilization of membrane fraction increased in the order sodium dodecyl sulphate >sodium deoxycholate >sodium cholate >digitonin >Tween-20 (Columns II and III), whilst the soluble fraction prepared with Tween-20 was highest in relative activity (Column V). The relative activity depended on the kind of detergent used. The reason for this remains uncertain. However, Tween-20 seems to be most useful for the present purpose. In a separate experiment, 0.1 ml of 0.01–10 %

TABLE I

SOLUBILIZATION OF MEMBRANE FRACTION WITH VARIOUS KINDS OF DETERGENT

Various kinds of detergent (Column I) were added to a final concentration of 1 % (w/v) to suspensions of membrane fraction. After incubation at room temperature for 1 h, each suspension was centrifuged at 105000 × *g* for 1 h to precipitate heavy material. As a semi-quantitative measure for expressing the amount of precipitated material, the diameter of pellet formed on the bottom of the centrifuge tube was measured (Column II). Supernatant fluids were dialysed against large volumes of solvent containing 10 mM Tris–HCl buffer (pH 8.2) for 10 h in the cold, and concentrations of protein contained in dialysates were determined (Column III). Before centrifugation each suspension contained 4.0 mg/ml protein. 0.1 ml of each dialysate was mixed with 0.53 ml of a flagellin solution containing 5.0 mg/ml protein, 0.15 M NaCl and 50 mM Tris–HCl buffer (pH 8.2). The mixture was incubated at 60° for initiation of polymerization. The initial rate of increase in viscosity associated with this process was measured (Column IV). Column V denotes the ratio IV/III.

(*I*) *Detergent*	(*II*) *Diameter of pellet (mm)*	(*III*) *Protein (mg/ml)*	(*IV*) *Polymerization rate (η_{spec}/min)*	(*V*) *Relative activity (η_{spec}/min per mg protein)*
Sodium dodecyl sulphate	9.5	3.1	0.018	0.006
Sodium deoxycholate	10	2.1	0.040	0.019
Sodium cholate	12	1.4	0.035	0.025
Digitonin	13	1.5	0.029	0.020
Tween-20	15	0.9	0.043	0.047
Without added detergent	16	—	—	—

(w/v) Tween-20 was added to 0.53 ml of a flagellin solution containing (5 mg/ml) protein, 0.15 M NaCl and 50 mM Tris–HCl buffer (pH 8.2), and the solution was incubated at 60° for 2 h. No P-filaments were formed. Taking into account these experimental results, we decided to use Tween-20 in the present study.

Effect of the concentration of Tween-20

Fig. 2 shows the effect of the concentration of Tween-20 on the extent of the extraction of protein and saccharide from the membrane fraction. The membrane fraction used in this experiment contained protein and saccharide at a weight ratio of 1:1.6. In the range between 2 and 10 % of Tween-20, the extent of extraction was approximately constant, and the extracted protein and saccharide amounted to about 15 and 16 %, respectively. This means that selective extraction of either protein or saccharide was unsuccessful, and each fraction contained protein and saccharide approximately at the same ratio as the membrane fraction.

Soluble fractions obtained in the above experiment were added to flagellin solutions at a constant volume ratio and, after incubation for a definite period, the amount of P-filament produced in each mixture was determined. The result is shown in Fig. 3. Under the given set of experimental conditions, about 80 % of the total flagellin was polymerized when soluble fraction was prepared with 1–2 % Tween-20 and, thereafter, the extent of polymerization decreased gradually with increasing concentration of detergent. The reason for this decrease may be that high concentrations of detergent inhibit polymerization. It seems that the optimal concentration of detergent lies between 1 and 2 %. From these experimental results, we decided to use 1 % of Tween-20 for the preparation of soluble fraction (see Fig. 1). If the concen-

tration of detergent were fixed at 1 %, the extent of extraction was little influenced by changing other conditions, namely, temperature (0–37°), salt concentration (0–0.15 M NaCl) and period of extraction (30 min to overnight).

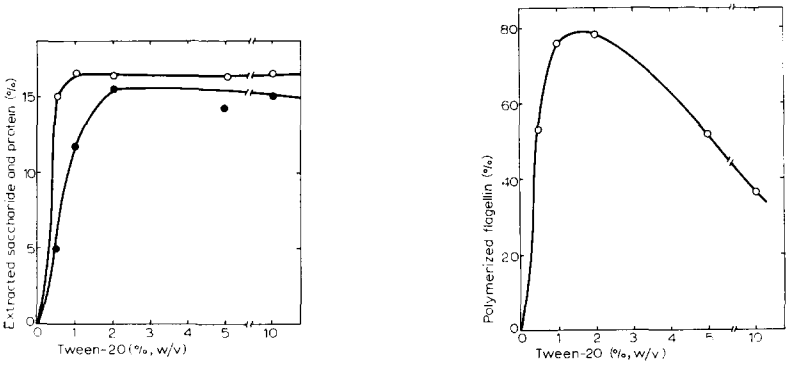

Fig. 2. Extraction of protein and saccharide from the membrane fraction with various concentrations of Tween-20 .The detergent was mixed with suspensions of membrane fraction at various concentrations, and mixtures were left standing at room temperature for 1 h. Final concentrations: 7.8 mg/ml protein, 11.6 mg/ml saccharide; 10 mM Tris–HCl buffer (pH 8.2); and various concentrations of Tween-20 .Thereafter, each mixture was centrifuged at 105000 × *g* for 2 h, and concentrations of protein (●) and saccharide (○) in the supernatant were determined by the Lowry–Folin method and the phenol–H_2SO_4 reaction, respectively. In each colorimetry, the presence of Tween-20 in sample solutions was taken into account. The ordinate denotes percentages of extracted protein and saccharide in total amounts of these materials added to each original suspension.

Fig. 3. Polymerization of flagellin incubated with extracts of the membrane fraction with various concentrations of Tween-20. Supernatant fluids obtained in the experiment of Fig. 2 were added to 5.3 vol. of flagellin solutions containing 0.15 M NaCl, 6.0 mg/ml protein, and 10 mM phosphate buffer (pH 7.5). The mixtures were incubated at 60° for 30 min and then diluted with 10 vol. of cold distilled water. P-filament contained in each diluted solution was precipitated by centrifugation at 105000 × *g* for 90 min and determined by the biuret reaction. Ordinate denotes percent of the precipitated protein in the total amount of flagellin used.

Sedimentation property of soluble fraction

Fig. 4 shows sedimentation patterns of a soluble fraction. Each diagram appears to consist of a single peak, although this does not necessarily mean that the soluble fraction was chemically homogeneous[12]. Fig. 5 shows the dependence of sedimentation velocity on concentration. This result gives an $s^{\circ}_{20,w}$ value of 4.2.

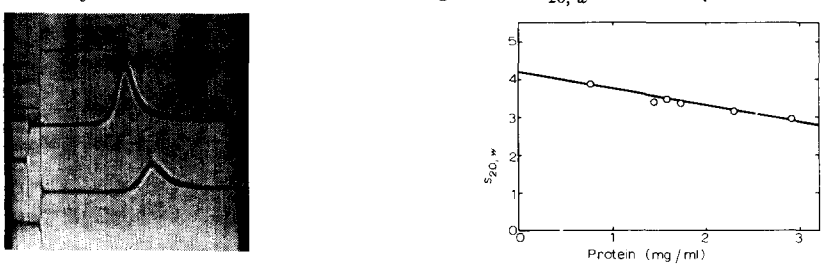

Fig. 4. Sedimentation diagrams of a preparation of soluble fraction. Centrifugation at 59780 rev./min at 20.8°. Protein concentrations: 2.9 (upper) and 1.8 mg/ml (lower). The photograph was taken 112 min after reaching the maximal speed at angle 75°.

Fig. 5. Sedimentation velocity of soluble fraction in the presence of 10 mM Tris–HCl buffer (pH 8.2). The abscissa denotes the concentration of soluble fraction expressed in terms of protein component.

Chemical composition of soluble fraction

It has been mentioned that the soluble fraction contains protein and (poly-) saccharide at a weight ratio of about 1:1.5. The soluble fraction was free from DNA and RNA, whilst the membrane fraction contained RNA as demonstrated by the orcinol reaction.

The soluble fraction contained organic phosphorus amounting to about 1/10 by weight of the saccharide component. Although the lipid content was not determined, it seems likely that a part of the organic phosphorus associates with lipid. It remains unresolved whether the factor essential for initiation of polymerization of flagellin is associated with one or two of these components or with a complex of them. Investigation of this problem is in progress.

Stability

The soluble fraction was stable to heat: its activity remained unchanged after boiling for 10 min. The soluble fraction precipitated when exposed to below pH 4, and the precipitate could be redissolved in 10 mM Tris–HCl buffer (pH 8.2) without loss of activity. Incubation of soluble fraction in 0.1 M NaOH at room temperature for 10 h led to complete irreversible loss of activity.

Formation of P-filaments

In the previous study[1], polymerization of flagellin, initiated by the addition of membrane fraction, took place in a peculiar manner and the associated increase

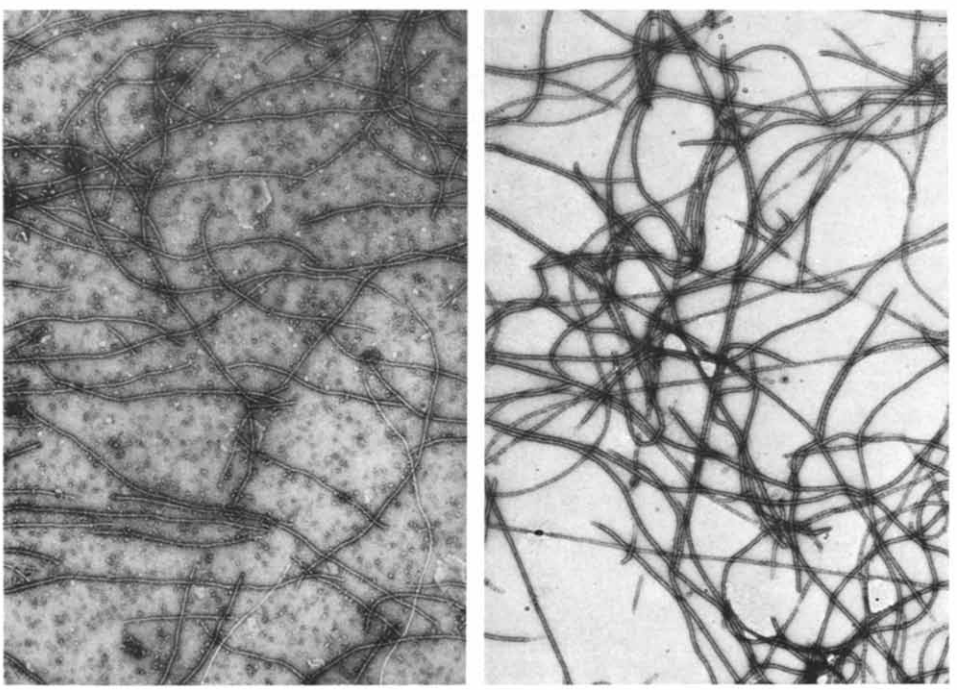

Fig. 6. Electron micrographs of P-filaments produced by the addition of membrane fraction (left) and of soluble fraction (right). Magnification × 15000. Negative staining with 1% uranyl acetate.

in viscosity involved a large lag period. By contrast, polymerization initiated by the addition of soluble fraction occurs in a simple manner[2].

Another important difference between the membrane fraction and the soluble fraction is in the final extent of polymerization. The difference became clear from the following experiment. Three independent preparations of membrane fraction or soluble fraction were added to flagellin solutions at a fixed protein ratio for initiation of polymerization: each mixture contained 10 mg flagellin per ml, 1.0 mg/ml protein from the added membrane or soluble fraction, 0.15 M NaCl and 50 mM Tris–HCl buffer (pH 8.2). After incubation at 60° for 1 h, each mixture was diluted with 10 vol. of distilled water, and P-filaments contained in the diluted solution were sedimented by centrifugation at 105000 × *g* for 2 h. P-filaments produced upon the addition of membrane fraction and soluble fraction amounted on average to 40 ± 7 and 91 ± 4 % of the total flagellin used, respectively.

In spite of these differences, filaments produced by the additon of membrane fraction or soluble fraction were indistinguishable in physicochemical properties as well as in morphology (Fig. 6).

Fig. 7 shows the temperature dependence of the rate of polymerization initiated by the addition of soluble fraction. Polymerization took place at physiological temperature, although the rate of polymerization at this temperature was a little less than that at the optimal temperature, 65°.

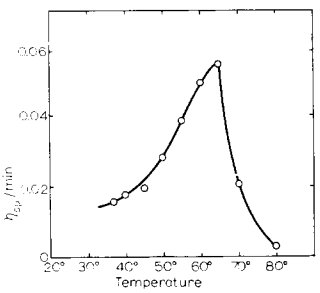

Fig. 7. Temperature dependence of the initial rate of polymerization initiated by the addition of soluble fraction. Polymerization was followed by viscosity measurement. Sample solutions contained 6.4 mg/ml flagellin, 0.15 M NaCl, 50 mM Tris–HCl buffer (pH 8.2) and a constant concentration of soluble fraction (1.0 mg/ml in protein). The ordinate denotes the increment of specific viscosity in the early period of polymerization.

DISCUSSION

When the membrane fraction was found to initiate polymerization of flagellin into P-filament, it was supposed that the relatively large structure of membraneous particulates might be an important factor for the initiation of polymerization. This possibility, however, has been eliminated in the present study. The factor associates with a protein–polysaccharide–lipid complex which has an $s^{\circ}_{20,w}$ value of 4.2.

Purification and characterization of the factor required for the initiation of polymerization has not been accomplished. The soluble fraction might perhaps be fractionated into components from which the essential one can be isolated. Nevertheless, the soluble fraction is useful for the investigation of P-filament, since this

fraction is free from proteolytic enzymes. HOTANI *et al.*[2] have shown, using the soluble fraction, that P-filaments contain little amount of the protein originating from the added soluble fraction and that the primary structure of flagellin remains unchanged after polymerization. In this connection, the following experiment is noteworthy. When P-filaments were washed with a large volume of distilled water, they contained only traces of saccharide and organic phosphorus, namely 0.4 and 0.05 % by wt. of the total amount of P-filament, respectively. Therefore, it is likely that components of the soluble fraction have incorporated into P-filaments as indispensable parts.

ACKNOWLEDGEMENTS

We wish to thank Mr. H. Hotani, Dr. S. Asakura and Prof. S. Suzuki for helpful discussions and technical advice. Determination of organic phoshporus was kindly performed by Miss K. Oshima in this institute.

REFERENCES

1 H. HOTANI, S. ASAKURA AND T. IINO, *Biochim. Biophys. Acta*, 194 (1969) 572.
2 H. HOTANI, T. OOI, H. KAGAWA, S. ASAKURA AND S. YAMAGUCHI, *Biochim. Biophys. Acta*, 214 (1970) 207.
3 T. IINO, *Genetics*, 46 (1961) 1465.
4 G. L. ADA, G. J. V. NOSEL AND A. ABBOT, *Nature*, 199 (1963) 1257.
5 K. WAKABAYASHI, H. HOTANI AND S. ASAKURA, *Biochim. Biophys. Acta*, 175 (1969) 195.
6 E. LAYNE, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 3, Academic Press, New York, 1957, p. 450.
7 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
8 J. E. HODGE AND B. T. FOFREITER, in R. L. WHISTLER AND M. L. WOLFROM, *Methods in Carbohydrate Chem.*, Vol. 1, Academic Press, New York, 1962, p. 388.
9 W. MEJBAUM, *Z. Physiol. Chem.*, 258 (1939) 117.
10 Z. DISCHE AND K. SCHWARZ, *Microchim. Acta*, 2 (1937) 13.
11 O. H. LOWRY AND J. A. LOPEZ, *J. Biol. Chem.*, 162 (1946) 421.
12 D. M. ENGELMAN AND H. J. MOROWITZ, *Biochim. Biophys. Acta*, 150 (1968) 376.