MUCOPOLYSACCHARIDES PRODUCED BY A STRAIN OF *CLOSTRIDIUM PERFRINGENS*

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ABSTRACT

IZUMI, KUNIHIKO (Kanazawa University, Kanazawa, Japan). Mucopolysaccharides produced by a strain of *Clostridium perfringens*. J. Bacteriol. **83**:956–959. 1962.—A new series of mucopolysaccharides was isolated from the culture medium of *Clostridium perfringens* and partially purified by the use of a column of anion-exchange resin. A large part of the substance was composed of neutral sugars, amino sugars, uronic acids, and oligopeptides, suggesting a structure analogous to that of bacterial cell walls. Acidic amino acids, especially aspartic acid, were the main constituents of the oligopeptides. The substance exhibited high viscosity when dissolved in water. The degree of viscosity in each fraction seemed to depend on the content of amino sugars and the chain length of the oligopeptides.

The structure of surface layers of bacterial cells has been the subject of many investigations in recent years (e.g., Salton, 1960). It has recently been found by Smith (1959) that continuous subculture of the smooth variants of *Clostridium perfringens* in meat broth often gives rise to mucoid variants which are covered with slime and resistant to the group phages. This phenomenon has been further confirmed in many strains of bacteria by one of our colleagues. Thus, it appears that sooner or later, in the course of repeated subculture, bacteria acquire an ability to produce a large amount of viscous substance and excrete it into the medium. Since the mechanism of excretion of the substance seems to be closely associated with variation in surface structure of bacteria, chemical investigation of the viscous substance was undertaken. The results have shown it to be a new series of mucopolysaccharides.

MATERIALS AND METHODS

Organism, growth media, and cultural conditions. The organism used was isolated from human feces after heating at 100 C for 1 hr. It was identified as a strain of *C. perfringens* after growth on a modified Nagler's egg yolk-agar medium (Willis and Hobbs, 1958) at the laboratory of bacteriology, Kanazawa University. This strain, which was designated WK-44, could produce the mucopolysaccharides without repetition of subculture. The cells were grown at 37 C in a screw-capped bottle containing a medium composed of peptone, beef extract, and glucose (each 1%) at pH 7.4. Cells grown previously for 20 hr in 20 ml of medium were used directly to inoculate 2 liters of the growth medium. After 20 hr of growth, the culture medium became highly viscous, owing to the appearance of the mucopolysaccharides produced by the cells.

Analytical methods. Neutral sugars were quantitatively determined by the orcinol-H_2SO_4 method of Hewitt (1937). Amino sugars were determined according to the Svennerholm (1956) modification of the Elson-Morgan reaction, after hydrolysis in 2 N HCl at 100 C for 16 hr, using glucosamine as the standard. Methyl pentoses were determined according to the method of Dische and Shettles (1948), using fucose as the standard. Uronic acids were determined by the carbazole-H_2SO_4 method of Gurin and Hood (1939). A suitable correction was made by the analysis of a mixture of hexose and glucuronic acid.

For quantitative determination of amino acids, the dinitrophenyl (DNP) method was applied. After hydrolysis in 2 N HCl at 100 C for 44 hr, the solution was neutralized with NaOH, and dinitrophenylation was carried out according to the procedure of Schroeder and LeGette (1953). DNP amino acids were estimated by two-dimensional paper chromatography, using tertiary amyl alcohol saturated with 0.05 M phthalate buffer (pH 6.0) and 1.5 M phosphate buffer (pH 6.0).

For the identification of the amino acids and the sugars, paper chromatography was carried out

after hydrolysis in 2 N HCl at 100 C for several hours, using *n*-butanol–pyridine–water (6:4:3) or phenol–water (4:1) in an atmosphere of ammonia. Sugars and amino acids were detected on the paper by aniline hydrogen phthalate and ninhydrin, respectively. Amino acids were also identified as their phenyl thiohydantoin derivatives, according to the method of Blombäck and Yamashina (1958).

Free amino groups were determined by the ninhydrin method of Troll and Cannan (1953). Specific viscosity was measured with an Ostwald viscometer in 0.4% solution in water at 12 C.

RESULTS

Isolation of the mucopolysaccharides from C. perfringens culture medium. The organism was grown for 20 hr at 37 C as described previously. Two liters of culture solution were diluted with an equal volume of 0.15 M NaCl and centrifuged for 60 min at 4,000 × *g*. The viscous supernatant fluid was adjusted to pH 6.0 with 1 N NaOH. The addition of 0.02 volume of 1 M zinc acetate and 1 M barium acetate to the supernatant, and allowing it to stand overnight, yielded a precipitate which included nonviscous peptide-like substances derived from the peptone used in the culture medium. These were removed by centrifugation. One and one-half volumes of ethanol were added to the resulting supernatant fluid and allowed to stand overnight at 0 C. A very fibrous precipitate formed, which was centrifuged, washed with ethanol, and immediately dissolved in about 2 liters of water. The yellowish turbid solution was then passed through a Dowex 50X 2 (H^+ form, 200–400 mesh) column (2.5 by 28 cm) at a flow rate of 50 ml/hr. The colorless viscous effluent was concentrated in vacuo and 1.5 volumes of ethanol were added to it. About 470 mg of a white fibrous precipitate (fraction I) appeared, which produced a very viscous solution when dissolved in water.

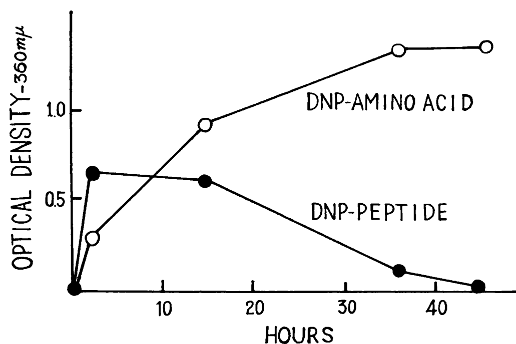

FIG. 1. *Liberation of amino acids and peptides from the mucopolysaccharides produced by Clostridium perfringens. A 1-mg amount of the sample (fraction I) was heated at 100 C in 2 N HCl for various times. After dinitrophenylation, DNP amino acids, mainly aspartic acid, in the ethyl acetate phase were estimated after chromatographic development and subsequent elution with 3.5 ml of 1% $NaHCO_3$ from the paper. The aqueous phase, containing DNP peptides, was estimated after adjusting the volume to 3.5 ml. The estimations were made by measuring the optical density at 360 mμ.*

Further fractionation of the mucopolysaccharides by means of a Dowex 1 column. About 400 mg of fraction I were dissolved in 100 ml of water, and applied to a Dowex 1X 8 (formate form, 100–200 mesh) column (2.5 by 40 cm). Initially, elution with water was done at a flow rate of 20 ml/hr. The effluent was concentrated, and a white nonfibrous precipitate (fraction IIa) was obtained by the addition of 4 volumes of ethanol. Fractions IIb and IIc, both fibrous precipitates, were obtained by elution from the column with 0.1 M and 1.0 M sodium formate, respectively, and by subsequent concentration and addition of 4 volumes of ethanol. Recovery, as estimated by neutral sugar analysis, was about 75%. The solution of each fraction in water showed marked differences in viscosity: fraction IIa, 0.2; IIb, 2.0; and IIc, 25.

Sugar composition of the mucopolysaccharides. The sugar composition of each fraction is shown in Table 1. Sugars detected by paper chromatography in the acid hydrolyzate of fraction I, which contained 56% of the total sugars, were glucose, galactose, mannose, rhamnose, glucosamine, and galactosamine. This pattern of sugar composition, especially the presence of glucose and rhamnose, is quite similar to that of cell walls of gram-positive bacteria, and differs clearly from that of animal mucopolysaccharides usually found (e.g., Bettelheim-Jevons, 1958). No fraction contained sialic acid. Fraction IIa, IIb, and IIc exhibited the same pattern of sugars qualitatively, but quantitatively they showed clear differences in content of total sugars, and particularly in content of hexose and uronic acid. The lowest amino sugar content was found in fraction IIa, which showed the least affinity towards the resin.

Amino acid composition of the mucopolysaccharides. Since, in the presence of sugars, amino

TABLE 1. *Sugar and amino acid composition of the mucopolysaccharides produced by Clostridium perfringens*

Component	Fractions			
	I	IIa	IIb	IIc
	%	%	%	%
Hexose	30	76	49	18
Methyl pentose	6.2	11	11	5.3
Amino sugar	16	1.2	3.7	12
Uronic acid	4.2	16	11	2.6
Amino acid	6.1	0.3	1.6	7.5

acids have been known to be markedly decomposed by acid hydrolysis under conditions usually employed for peptide cleavage, the liberation of amino acids from the mucopolysaccharides (fraction I) was followed in relatively mild conditions, i.e., in 2 N HCl at 100 C. Along with the measurement of DNP amino acids extracted with ethyl acetate and developed on the paper, the yellow material remaining in the aqueous phase after extraction was also measured at 360 mμ. This was ascribed to such substances as DNP peptides. The rates of liberation of amino acids and peptides, which were measured as their DNP derivatives, are shown in Fig. 1.

The intact mucopolysaccharides, without acid treatment, were not dinitrophenylated, as indicated by practically zero values at zero time (Fig. 1), in spite of their positive reactions for ninhydrin. It seems that dinitrophenylation was completely prevented by the presence of a large amount of sugars. After hydrolysis for less than 2 hr, however, the dinitrophenylation took place readily. The peptides released from the mucopolysaccharides increased at an early stage of the hydrolysis, but after about 10 hr began to decrease gradually.

After hydrolysis for 44 hr, maximal yields of amino acids were attained, and the aqueous phase no longer contained peptides. Based on these results, it was concluded that the amino acids found in the 44-hr hydrolyzate represented the whole amount of amino acids contained in the mucopolysaccharides.

The total amino acid content of each fraction is shown in Table 1; increasing amounts of amino acid are in parallel with other properties, such as increasing viscosity and increasing content of amino sugars. Mean peptide chain lengths were calculated from the content of amino acids and free amino groups. The free amino group content of each fraction was as follows (μmole/mg): fraction IIa, 0.024; IIb, 0.027; IIc, 0.057. The mean peptide chain lengths were calculated to be: fraction IIa, 1; IIb, 4 to 5; IIc, 10 to 11. Thus, in fraction IIa it appears that one residue of amino acid is linked to the sugar. Since the free amino group content does not differ as markedly as do the contents of other components, the amino acid content reflects the length of the peptide chain of each fraction.

Among the amino acids detected, acidic amino acids, especially aspartic acid, accounted for about 80% of the total; others detected were serine, threonine, glycine, alanine, and proline.

DISCUSSION

Chemical features of the viscous substance produced by *C. perfringens* have been characterized. A large part of the substance was composed of neutral sugars, amino sugars, uronic acids, and oligopeptides with various chain lengths. Thus, the substance seems to belong to the so-called mucopolysaccharides, though, based on the analytical values, there are differences in sugar and amino acid compositions among the fractions. The correlation could be rather clearly demonstrated between the content of amino sugars, the length of peptides, and the viscosity of each fraction, suggesting that amino sugars and peptides may contribute to the high viscosity.

However, the presence of some components other than sugars and amino acids in the mucopolysaccharides can not lightly be dismissed. The absence of phosphorus and also of ultraviolet absorption in the range of 230 to 300 mμ indicated that nucleic acid derivatives or substances like teichoic acids (Armstrong, Baddiley, and Buchanan, 1960) were not present. No nitrogen other than that accounted for in amino sugars and amino acids was found by the usual analysis. Further characterization of the residual components and elucidation of the biological significance of the substance must await further study.

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