PHYSICAL DIFFERENCES OF VIRUS-ASSOCIATED DEPOLYMERASES

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Eklund and Wyss (1962) reported that azotobacter bacteriophages contained a polysaccharide depolymerase which was necessary for the successful initial attack on the encapsulated host cell. Their evidence explained the slow initiation of azotophage infection reported by Wyss and Nimeck (1961) and suggested that cell invasion occurred only after release of the entrapped phage by the enzyme action on the polysaccharide. Shortly after invasion, the infected cell begins to produce the enzyme and by the time of cell lysis, approximately 1000 extra molecules of enzyme are produced for each molecule incorporated into the new phage progeny. Preliminary studies with crude lysates indicated that infection of the azotobacter strain with other phage strains yielded enzymes different in heat stability suggesting that the phage does not activate a genetic determinant in the bacterium, but rather that the coded information leading to enzyme production is present in the phage. To prove more conclusively that the phage does not just activate some preexisting protein in the host, differences were sought in the depolymerase enzyme formed in one strain of azotobacter cells with two serologically different azotophages, and differentiation of the two enzymes by physical methods.

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Materials and Methods

Azotobacter vinelandii, strain O (A. agilis), and azotophages A-22 and A-31 (serologically unrelated phages) were propagated using the usual techniques (Duff and Wyss, 1961).

The liberation of reducing groups (aldehyde groups) was estimated by Nelson's colorimeter modification of the Somogyi method (Dische, 1962). A standard curve was prepared with certified reagent grade glucose.

Protein measurements were made at 700 mμ with the Folin phenol reagent using the reagents of Lowry, et al. (1951).

For the production and partial purification of the two enzymes, ten liters of sterile nitrogen-free sucrose broth were inoculated with 60 ml of a Klett 40 (2.0×10^7 cells/ml) culture of A. vinelandii, strain O, and incubated at 33 C with vigorous aeration. When the cell concentration reached approximately 2×10^7 cells/ml, a phage inoculum was added to obtain a 1:1 ratio of cells to plaque-forming-units and allowed to incubate for an additional 12 hours.

After numerous trials, a procedure was developed which retained essentially all of the enzyme activity while eliminating 98% of the protein in the lysate. This involved concentration of the phage lysate by flash evaporation, clarification at 78,000 x g, dialysis for 8 hours against phosphate buffer, and filtration through membrane filters ranging from 450 mμ to 10 mμ to free the preparation from phages as well as bacteria. The enzyme activity was in that protein precipitated between 50-75% saturation with ammonium sulfate. The precipitate was dissolved in 0.05 M phosphate buffer, pH 6.5, and the ammonium sulfate removed by dialysis. The final crude enzyme preparation was filter-sterilized and stored at 4 C. Enzymes from phages A-22 and A-31 were prepared

in identical fashion and used in this partially purified form for the comparative experimental work.

The extracellular capsular polysaccharide from A. vinelandii, strain O, was obtained and purified for use as the enzyme substrate by the procedure reported by Cohen and Johnstone (1964).

The liberation of reducing groups by enzymic activity on the polysaccharide substrate was followed at 33 C. To 2 ml of 0.3% polysaccharide substrate, dissolved in 0.05 M sodium phosphate, pH 6.5, 0.5 ml of a specified dilution of enzyme was added and allowed to react for a period of 2 hours before a 1.0 ml sample of the reaction mixture was assayed for reducing groups (Dische, 1962). A sonicate of uninfected cells contained no enzymic activity.

A unit of enzyme was defined as that dilution of enzyme required for the liberation of 0.2 μM of glucose equivalents from the polysaccharide substrate under the specified conditions. The pH curves were identical for the A-22 and A-31 enzymes showing a broad optimum for activity spreading from pH 6.2 to 7.5.

Protein samples of 20 μg from each of the enzyme preparations were applied to 1 x 8.5-inch pieces of cellulose acetate that had been soaked overnight in the barbitone buffer used in electrophoresis. After 3.5 hours in 0.07 M barbitone buffer, pH 8.5 at 2.5 mA, the membranes were removed, blotted to remove excess buffer, and air-dried at room temperature. A strip was cut out and stained with azocarmine G (Smith, 1960). Corresponding areas were cut out of the nonstained cellulose acetate membrane, cut up in little pieces, and eluted in 0.05 M phosphate buffer, pH 6.5, at 4 C overnight followed by shaking for 1 hour at 33 C. Each protein-containing area of the paper was tested for enzymic activity.

Results

Differences in temperature stability. To determine the stability of the two enzymes at different temperatures, 1.0 ml of an equal amount of protein (4 µg/ml) from each enzyme preparation in 0.05 M sodium phosphate, pH 7.5, was placed in a tube and incubated in water baths set at 33, 45, 50, and 55 C for 1 hour. After this period, 0.5 ml of each enzyme was removed, immediately cooled in water, and assayed for the ability to release reducing groups from the polysaccharide substrate at 33 C.

Using the 33 C sample as the control, the percentages of inactivation of the preparation were determined by measuring the decrease in the reducing groups liberated. Fig. 1 shows enzyme A-31 to be much more heat labile at 50 and 55 C than enzyme A-22. There was 45 and 95% inactivation of the A-31 enzyme at 50 and 55 C, respectively, in contrast to 20 and 40% inactivation at corresponding temperatures for the A-22 enzyme. The large degree

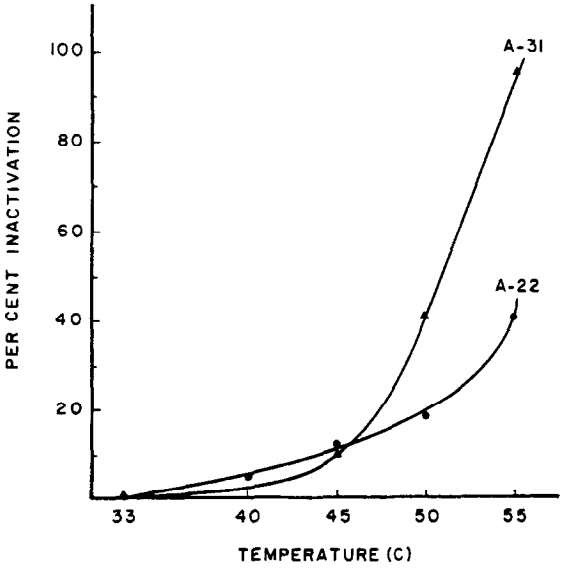

Fig. 1. Effect of temperature on the stability of depolymerases A-22 and A-31.

of difference in the denaturation of the enzymes provides strong evidence that the two proteins are different.

Differences in stability at pH 9.0. A sample of each of the enzymes in 0.05 M phosphate buffer, pH 6.5, was dialyzed against deionized water for 8 hours to remove the phosphate and diluted in 0.05 M sodium barbital buffer, pH 9.0, to give an equal protein concentration. The residual activity of the two enzymes in this buffer at pH 9.0 was determined after an incubation period of 48 hours at 33 C and compared with activity of each enzyme at 0 time. The results in Fig. 2 show that like with heat resistance, A-22 enzyme is more stable at 33 C at this adverse pH than the A-31 enzyme under the identical conditions.

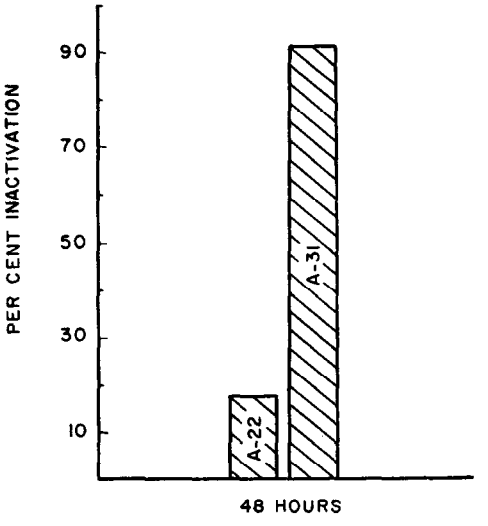

Fig. 2. Residual activity of depolymerases A-22 and A-31 after incubation in 0.05 M sodium barbital buffer, pH 9.0, at 33 C for 48 hours.

Differences in electrophoretic mobility of the enzymes. Upon the subjection of the two depolymerases to electrophoresis, two red protein-staining bands were detected for both enzyme samples on each cellulose acetate strip and each protein-containing

area of the paper was tested for enzymic activity. In both preparations the enzymic activity occurred only in the more rapidly moving band. Better band separation was obtained with the A-22 enzyme than with the A-31 enzyme but both bands moved more slowly in the former preparations (Fig. 3).

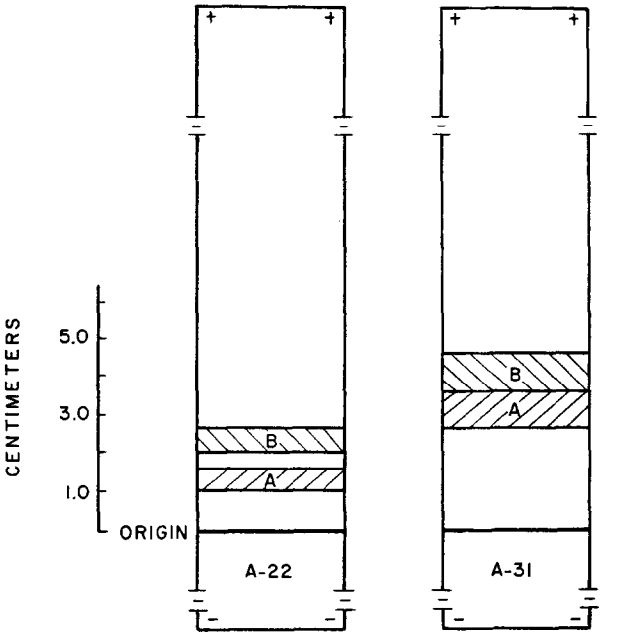

Fig. 3. Electrophoretic mobility of protein components in preparations purified from lysates of A. vinelandii with phages A-22 and A-31. Enzyme activity was found in band B.

Discussion

A method for the measurement of enzyme activity that was more sensitive than the viscosity measurements used by Eklund and Wyss (1962) was used and proved successful to detect physical differences in two polysaccharide depolymerases. The question proposed in this study concerned the location of the genetic information for an enzyme that was formed following the infection of a bacterium by a phage. If two phages produce different enzymes in the same host, the phage genome is the indicated site.

Physical characteristics such as those reported in this study have been used for differentiating proteins; for example, Johnson and Denniston (1964) depended on starch gel electrophoresis for differentiation of two genetic variations of alcohol dehydrogenase in Drosophila melanogaster and Han (1965) similarly proved that a bacteriolytic factor isolated from the allantoic fluid of chick embryo infected with influenza virus was different from egg lysozyme. In our opinion, the physical differences reported here supports the suggestion of Eklund and Wyss (1962) that the production of the polysaccharide depolymerase is under the control of the phage genome and not genetic material of the host.

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