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Production and Characteristics of Hemolysins of *Escherichia coli*

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ABSTRACT

SNYDER, IRVIN S. (University of Iowa, Iowa City), AND NANCY A. KOCH. Production and characteristics of hemolysins of *Escherichia coli*. J. Bacteriol. **91**:763–767. 1966.—Filterable and nonfilterable hemolysins were produced by *Escherichia coli* in beef-heart infusion, casein hydrolysate, and chemically defined media. Differences were found among the three media in the time of appearance and in the relationship between production of these two hemolysins. The nonfilterable hemolysin produced in the three media, as well as the filterable hemolysin produced in the beef-heart infusion medium, were destroyed in 1 hr at 56 C. The filterable hemolysin produced in the casein hydrolysate and chemically defined media was unaffected by exposure to 56 C for 1 hr. Formalin inactivated the hemolysins produced in all three media. The optimal *p*H for activity of the nonfilterable hemolysin varied with time of production, whereas the optimal *p*H for the filterable hemolysin was constant. Certain carbohydrates were required for the production of filterable hemolysin by *E. coli* grown in casein hydrolysate and chemically defined media.

Kayser (5) reported the existence of a heat-resistant, filterable hemolysin in cultures of *Escherichia coli*, but the occurrence of a filterable hemolysin was not confirmed by other investigators (1, 8, 9). Widholm (11) also reported the inability to separate the hemolysin from the bacterial cells by filtration and showed that the nonfilterable hemolysin was heat-labile. However, Smith (10) and Lovell and Rees (6) reported the production of a filterable hemolysin by *E. coli* grown in an alkaline meat-extract broth.

The purpose of this paper is to report the production of filterable and nonfilterable hemolysins in various media and to discuss some differences between the two hemolysins produced in different media.

MATERIALS AND METHODS

Preparation of media. Three types of media were used: beef-heart infusion medium, chemically defined medium, and casein hydrolysate medium. The beef-heart infusion medium was prepared from Beef Heart for Infusion as described by the manufacturer (Difco). The *p*H of the medium was adjusted to 8.0 before boiling, and the final *p*H after autoclaving was 7.5. The chemically defined medium was composed of the following (grams per liter): K_2HPO_4, 2.3; KH_2PO_4, 0.78; $(NH_4)_2SO_4$, 1.0; $MgSO_4$, 0.1; $Na_3C_6H_5O_7$, 0.6. Carbohydrates were sterilized by filtration through 0.45-μ (pore size) filters (Millipore Filter Corp., Bedford, Mass.), and were added to the chemically defined medium. Unless specified otherwise, glucose was used to give a final concentration of 0.2%. The final *p*H of the medium was 7.4. Flasks containing 100 ml of the medium were sterilized by autoclaving at 121 C for 10 min. The casein hydrolysate medium was prepared as follows. Enzymatic hydrolysate of casein (General Biochemicals Inc., Chagrin Falls, Ohio) was filtered through 0.45-μ Millipore filters and then added to the autoclaved chemically defined medium to give a final concentration of 10% casein hydrolysate. The casein hydrolysate was adjusted to *p*H 7.4 before filtration.

Preparation of inoculum. A hemolytic strain of *E. coli* type O6 (Iowa Stock Culture no. 447) was used. Several transfers of the organism were made through the chemically defined medium. A standard inoculum was made by diluting a 10-hr shake culture with chemically defined medium to give an optical density, as determined by use of a Spectronic-20 colorimeter, of 0.325 at 625 mμ. Samples of this suspension were frozen at −20 C. A 1-ml amount was used to inoculate 100 ml of medium for hemolysin production. All cultures were grown on a shaker at 37 C.

Assay of hemolysin. Hemolysin was titrated by preparing serial twofold dilutions of the hemolysin in 1 ml of saline containing 0.01 M $CaCl_2$. A 1-ml amount of a 1% suspension of three times washed sheep erythrocytes was added, and contents of the tubes were incubated for 1 hr at 37 C. The titer of hemolysin was the

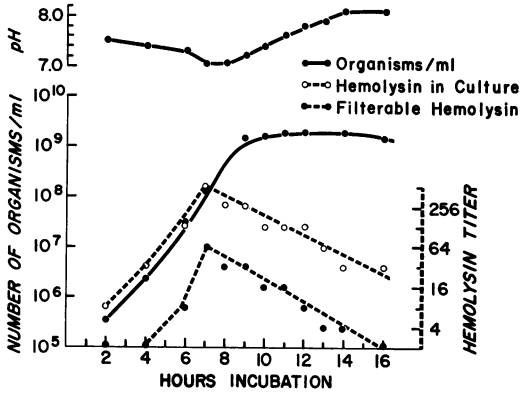

FIG. 1. *Hemolysin production in heart-infusion medium.*

highest dilution of hemolysin which produced complete lysis of the cells. Titers of nonfilterable and filterable hemolysins were obtained by use of unfiltered and Millipore-filtered cultures, respectively.

Stability of hemolysin. For studies on the heat stability of the hemolysins, samples of filtered and unfiltered cultures were placed in rubber-stoppered vaccine bottles and submerged in a 56 C water bath for 1 hr. The bottles were removed, rapidly cooled by immersion in ice, and immediately titrated.

The effect of formalin on the hemolysin was determined by addition of formalin to give a final concentration of 1%. Because of the temperature lability of hemolysin produced in heart-infusion medium, the hemolysin-formalin mixture was kept at 4 C for 1 hr and then titrated.

Control samples for both of the above studies were placed at 4 C during the period of treatment.

Studies on pH. For determination of optimal *p*H for hemolytic activity, the hemolysins were diluted in isotonic 0.2 M tris(hydroxymethyl)aminomethane (Tris)-phthalate-buffered saline at *p*H values of 8.0, 7.5, 7.0, 6.5, 6.0, and 5.5. Thrice washed sheep erythrocytes (1%) were also suspended in the buffer at the various *p*H values. Samples of the culture were removed at hourly intervals, and the hemolytic activity of the unfiltered and filtered cultures was determined at these *p*H values.

RESULTS

Production of hemolysins in different media. The production of filterable and nonfilterable hemolysins in various media and the relationships among bacterial numbers, *p*H, and hemolysin production were investigated. Beef-heart infusion, casein hydrolysate, and a chemically defined medium were used. Figure 1 shows these characteristics when beef-heart infusion medium was used. Bacterial numbers were determined by making viable counts of the suspension in nutrient agar. Determination of *p*H was made by use of a Beckman Zeromatic *p*H meter. Production of the nonfilterable hemolysins paralleled that of filterable

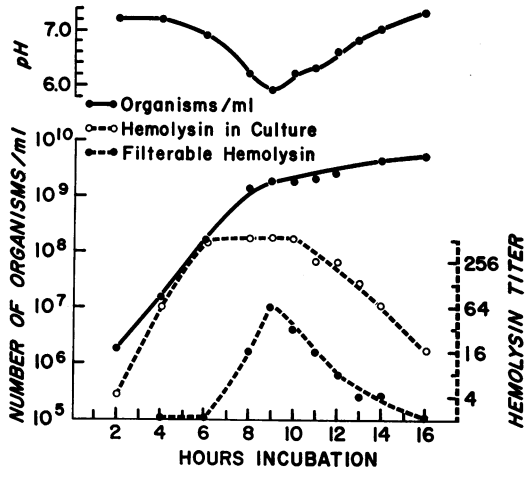

FIG. 2. *Hemolysin production in casein hydrolysate medium.*

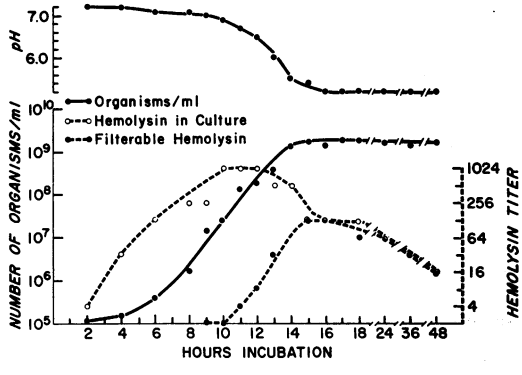

FIG. 3. *Hemolysin production in chemically defined medium.*

hemolysins, with maximal production of the hemolysins occurring 7 hr after inoculation of the beef-heart medium. Growth of the organisms in the casein hydrolysate medium (Fig. 2) and in the chemically defined medium (Fig. 3) shows that maximal filterability of the hemolysin was not obtained until several hours after maximal amounts of nonfilterable hemolysin were produced. Irrespective of the medium employed, the hemolysins were produced during the logarithmic phase of growth with maximal amounts of filterable hemolysin being produced when the *p*H of the medium was at or near its minimal value.

Influence of pH on the filterability of hemolysin. Because of the association of low *p*H with filterability of the hemolysin, cultures of *E. coli* were adjusted to various *p*H values after growth. Filterable hemolysin was not obtained.

Additional studies in which the organisms were grown in a fermentor (New Brunswick Scientific

Co., New Brunswick, N.J.) at constant *p*H values of 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 showed that maintenance of a constant extracellular *p*H does not affect the production of filterable hemolysin.

Effect of carbohydrates on the production of hemolysins. Various carbohydrates at a concentration of 0.2% were added to the casein hydrolysate medium and to the chemically defined medium. Filterable hemolysin was produced with glucose, lactose, and mannitol as the carbon source in the chemically-defined medium, but not with galactose mannose, maltose, or sorbitol. Nonfilterable hemolysin was produced with all the carbohydrates. Incorporation of carbohydrates into media, which permitted hemolysin production, resulted in better growth [optical density (OD), 0.98] and a lower final *p*H (5.4) than did addition of carbohydrates, which caused production only of nonfilterable hemolysin (OD, 0.64; *p*H, 6.5). Addition of sorbitol produced growth and final *p*H equivalent to those obtained with glucose (OD, 1.0; *p*H, 5.4), without production of filterable hemolysin. Increasing the concentration of sorbitol to 1.0% yielded filterable hemolysin without an increase in growth or a lower *p*H. Galactose at a 1% concentration produced filterable hemolysin as well as better growth (OD, 0.95) and a lower final *p*H (5.0). Mannose and maltose at a 1% concentration did not yield filterable hemolysin. With mannose, the amount of growth was 0.62 OD units and the final *p*H was 5.7; whereas, for maltose, the growth was equivalent to 0.38 OD units and the final *p*H was 6.5.

Addition of glucose, galactose, mannose, lactose, maltose, mannitol, and sorbitol at 0.2% concentration resulted in production of filterable and nonfilterable hemolysins in the casein hydrolysate medium. The final *p*H after growth ranged from 5.6 to 6.2. Rhamnose at a 0.2% concentration supported production of the nonfilterable, but not the filterable, hemolysin. With this carbohydrate the final *p*H was 6.7. The amount of growth in casein hydrolysate medium containing the various carbohydrates was essentially the same. Nonfilterable hemolysin was also produced in the casein hydrolysate medium lacking carbohydrates.

Effects of physical and chemical agents on the stability of hemolysins. Stability of the hemolysins at 56 C for 1 hr depends upon the medium in which the hemolysin is produced. The effect of heat on the hemolysins produced in beef-heart medium (Table 1) shows that both the filterable and nonfilterable hemolysins were labile. Table 2 gives the results obtained with the hemolysins produced in a chemically defined medium. These results are similar to the data for hemolysins produced in casein hydrolysate medium. These values show that the nonfilterable hemolysin was destroyed by heating, whereas filterable hemolysin was not affected by heat. It would appear that the change from heat lability to stability of hemolysins produced in chemically defined medium was not an abrupt change which occurs at the time the hemolysin became filterable. Some of the hemolysin became stable before it was filterable, with complete stability to heat occurring after filterable hemolysin appeared.

TABLE 1. *Effect of heat (56 C for 1 hr) on hemolysin stability of Escherichia coli in heart-infusion medium*

Age of culture	Titer of hemolysin			
	Unfiltered	Heated	Filtered	Heated
hr				
4	16	0	4	0
5	32	0	8	0
6	128	0	32	0
7	128	0	32	0
8	512	0	128	0
9	256	0	128	0
10	256	0	64	0

TABLE 2. *Effect of heat (56 C for 1 hr) on hemolysin stability of Escherichia coli in chemically defined medium*

Age of culture	Titer of hemolysin			
	Unfiltered	Heated	Filtered	Heated
hr				
8	64	0	0	0
9	64	0	0	0
10	128	4	0	0
11	256	8	0	0
12	256	8	8	4
13	512	16	32	8
14	512*	32	128	32
15	256	64	64	64
33	64	64	64	64

* Or greater than 512.

Data in Table 3 show the effect of formalin on the activity of the hemolysins. The results shown here are those obtained with hemolysin produced in chemically defined medium and are identical with those obtained with hemolysin produced in the beef-heart infusion medium and in the casein hydrolysate medium. Formalin destroyed the nonfilterable hemolysin, but it did not affect the filterable hemolysin. Again, part of the nonfilterable hemolysin produced in the chemically defined medium became stable shortly before the appearance of filterable hemolysin.

TABLE 3. *Effect of 1% formalin on stability of hemolysin of Escherichia coli in chemically defined medium*

Age of culture	Titer of hemolysin			
	Unfiltered	Formalin	Filtered	Formalin
hr				
8	64	0	0	0
9	64	0	0	0
10	128	8	0	0
11	256	16	0	0
12	512*	64	16	8
13	512*	64	32	16
14	128	128	128	64
15	256	128	128	64
33	64	32	32	32

* Or greater.

Effect of pH on activity of the hemolysins. The effect of *p*H on the hemolytic activity of the filterable and nonfilterable hemolysins produced in various media was determined by dilution in Tris-phthalate buffer at various *p*H values. The results shown in Table 4 are representative of the hemolysins produced in the heart infusion, casein hydrolysate, and chemically defined media. The filterable hemolysin was active over a broad *p*H range, whereas the optimal *p*H for activity of the nonfilterable hemolysin was dependent upon the age of the culture at time of assay. Nonfilterable hemolysin produced during the early period of production had a *p*H optimum of 6.0 or less (1-, 2-, and 3-hr cultures), whereas hemolysin produced later (4- and 5-hr cultures) exhibited optimal hemolytic activity over a wide *p*H range.

DISCUSSION

Widholm (11) reported the production of a nonfilterable hemolysin by *E. coli* in a chemically defined medium, but he was unable to demonstrate the filterable type. Others (6, 10) have reported a filterable hemolysin in alkaline meat-extract broth, but they have not presented data showing a relationship between these hemolysins.

The results presented in this paper show that hemolysin(s) can be produced in a beef-infusion medium, a casein hydrolysate medium, and in a chemically defined medium. Certain differences in the production of the hemolysin(s) are apparent. Both the filterable and nonfilterable hemolysins are produced concurrently by *E. coli* grown in heart-infusion broth. Filterable hemolysins first appeared in the casein hydrolysate medium and the chemically defined medium at the time the titer of nonfilterable hemolysin was at a maximum. The highest titer of filterable hemolysin is found several hours after maximal production of nonfilterable hemolysin. Irrespective of the medium employed, filterable hemolysin is found at the time the *p*H of the medium is at or near its minimum. These observations may indicate that nonfilterable hemolysin is released from the cell at a lowered *p*H or that it is released by some other mechanism which evolves coincidentally with this lowered *p*H. Gale (3) reported increased production or decarboxylases by microorganisms growing at low *p*H values. However, suspension of *E. coli* in medium of a *p*H value equivalent to that obtained during production of filterable hemolysin did not result in filterable hemolysin.

Pivnick et al. (7) found increased toxinogenesis of *Clostridium perfringens* by growth at a controlled *p*H. Growth of *E. coli* in a defined medium at various constant *p*H values neither inhibited nor increased the production of hemolysin. Therefore, extracellular *p*H does not affect hemolysin production or allow release of hemolysin from the bacterial cell.

Certain differences were observed in the lability of the filterable hemolysin at 56 C. The filterable hemolysin produced in both the casein hydrolysate medium and the chemically defined medium

TABLE 4. *Effect of pH on hemolysin titer of Escherichia coli in heart infusion medium**

Age of culture	*p*H											
	8.0		7.5		7.0		6.5		6.0		5.5	
	UF	F	UF	F	UF	F	UF	F	UF	F	UF	F
hr												
1	4	0	4	0	4	0	8	0	16	0	16	0
2	16	4	16	4	16	4	32	4	32	4	32	4
3	32	8	32	8	32	8	64	8	128	8	64	8
4	128	8	128	16	128	16	128	16	128	16	128	8
5	512	16	512	16	512	16	512	16	512	16	512	16

* UF = unfiltered; F = filtered.

was resistant to heating at 56 C for 1 hr, whereas the hemolysin produced in the beef-heart medium was labile at the same temperature. The nonfilterable hemolysin produced in all three media was labile to heating at 56 C for 1 hr. This probably indicates that hemolytic activity of the nonfilterable hemolysin is closely associated with cell viability. If the filterable hemolysin represents a release of nonfilterable hemolysin, then some change, which is expressed by the change from heat lability to heat stability, must occur in its chemical structure. The data obtained with the hemolysins produced in the chemically defined medium show that some of the nonfilterable hemolysin becomes heat-stable before the appearance of heat-stable filterable hemolysin. The change in optimal *p*H for hemolytic activity during production also indicates a change in molecular structure of the hemolysin during its production. These studies show that maximal filterable hemolysin is produced during the logarithmic-growth phase by *E. coli* grown in heart-infusion medium. In the casein hydrolysate and chemically defined medium, filterable hemolysin appears near the end of the logarithmic-growth phase with maximal filterable hemolysin appearing during the stationary phase. If cell disintegration or increased permeability during the stationary-growth phase was responsible for release of the hemolysin, filterable hemolysin would also appear in the casein hydrolysate medium lacking carbohydrate.

Bernheimer (2) has shown that the carbohydrate substrate affects the yield of streptolysin S. Haque and Baldwin (4) reported that low yields of staphylococcus β-hemolysin were obtained by addition of fermentable sugar to the basal medium. We found that the carbohydrate substrate did affect the yield of the *E. coli* hemolysin. Growth of *E. coli* in casein hydrolysate medium lacking carbohydrate results in the production of nonfilterable, but not filterable, hemolysin, whereas growth in the casein hydrolysate medium containing carbohydrate permitted production of both filterable and nonfilterable hemolysins. In chemically defined medium, carbohydrate is necessary for growth and therefore nonfilterable hemolysin, but only certain carbohydrates will allow production of filterable hemolysin.

These results indicate that carbohydrate is not required for the production of nonfilterable hemolysin, but that it is necessary for either release of the nonfilterable hemolysin or synthesis of a new hemolysin which is filterable.

ACKNOWLEDGMENT

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