PRODUCTION OF STAPHYLOCOAGULASE IN A SPECIAL MEDIUM

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Occasional reports over the past several decades have indicated that coagulase production by Staphylococcus aureus (Micrococcus pyogenes var. aureus), in various media could be enhanced by the addition of whole blood or plasma (Fisher, 1936). More recently, attempts have been made to produce special media which would permit the elaboration of coagulase but little success has been achieved (Lominski et al., 1950). On the whole, only limited progress has been made toward the elucidation of the optimal conditions for coagulase production (Davies, 1951; Duthie, 1954); for example, Davies suggested that a factor contained in heart infusion and partially replaceable by peptone is necessary for maximum coagulase yields. The present report deals with the production of coagulase in a derivative of heart infusion broth and a characterization of its coagulase production capacity.

MATERIALS AND METHODS

Bacterial strain. A single strain of Staphylococcus aureus was used in this study. This potent coagulase producing organism was kindly supplied by Dr. Morris Tager of Emory University. The culture was maintained on heart infusion agar slants (Difco) at refrigerator temperatures. Preparations of staphylocoagulase were made periodically and checked against the standard coagulase described below. No quantitative change in coagulase production could be detected over a period of approximately one year.

Standard coagulase. A modification of the method of Gerheim et al. (1947) was used. An inoculum from a subculture of the stock strain, which had been grown on an agar slant for 24 hr at 37 C, was placed in 250 ml of sterile heart infusion broth (Difco) contained in a 1 L Erlenmeyer flask. This was incubated at 37 C for 24 hr. The bacteria were largely cleared by centrifugation at 4000 rpm for 10 min. To the cold supernatant were slowly added 3 volumes of cold 95 per cent ethanol with stirring. The mixture was maintained at 5 C for 2 hr and the resulting precipitate was packed by centrifugation in the cold. The precipitate was collected and dried with cold, absolute methyl alcohol and the light brown product was stored in an evacuated dessicator. No loss in potency could be detected over a period of 1 year. The material was completely soluble in 2 per cent solution with borate buffer; these solutions were stable at room temperatures for at least several days.

Borate buffer. H3BO3, 11.25 g; Na3B4O7·10H2O, 4.00 g; and NaCl, 2.25 g, were put into solution with distilled water and the volume was made up to 1 L. The pH of the buffer was 7.4.

Standard plasma. Human plasma was prepared from Kahn positive whole blood obtained from the Blood Bank Laboratories of the Detroit Receiving Hospital. These whole blood samples, which had been stored at refrigerator temperatures from 1 to 14 days, were allowed to stand for 24 hr at 5 C, the plasma carefully drawn off with suction and centrifuged at 2500 rpm for 20 min. The plasmas were then individually tested. Refractory plasmas were discarded and the reactive plasmas were pooled. Aliquots of 10 to 20 ml from the pool were distributed into test tubes and stored in the frozen state.

Assay of coagulase. This test was devised with the assumption that coagulase production could be indicated quantitatively by reacting serial dilutions of a coagulase preparation with the standard plasma and noting the greatest dilution which would clot the plasma. Coagulase preparations were serially diluted with borate buffer and 0.6 ml of the standard plasma was mixed with 0.4 ml portions of each of the dilutions in a series of 10 by 75 mm tubes. Merthiolate was added to the borate buffer so that a final concentration of 0.01 per cent was achieved. The tubes were plugged and placed in a 37 C water bath. Final readings were made at 24 hr. To indicate the
relative amounts of clot formed, 4+ was used to signify a firm invertible clot; ±, a trace of a clot; and 1+, 2+, and 3+, intermediates between the two.

Media. (a) Heart infusion broth (Difco) (b) Heart infusion broth dialyze: 25 g of heart infusion broth were put into solution with 1 L of demineralized water. An equal volume of 0.9 per cent saline was placed into dialysis bags, which consisted of 29/32 in cellulose casing (Visking), and statically dialyzed against the broth at 5 C for approximately 72 hr. The bags were rinsed carefully with distilled water and their contents, the broth dialyze, collected and sterilized by autoclaving for 20 min at 15 pounds pressure. (c) Chemically defined medium: Several modifications of the medium described by Gladstone (1937) were tested. One of these had the following composition: 0.05 g each L-arginine, DL-alanine, L-cysteine, DL-leucine, L-proline, DL-tryptophan, D-glutamic acid, DL-valine, DL-phenylalanine, DL-threonine, L-lysin and glycine; 0.1 g DL-methionine; 0.5 mg each nicotinic acid HCl and thiamin HCl; 4.0 g glucose; 0.5 g each MgSO4·7H2O and FeSO4; 1.0 g NaCl; 0.4 g KH2PO4 and 4.0 g K2HPO4. These were dissolved in 490 ml demineralized water. L-Aspartic acid, L-tyrosine and L-cystine, 0.05 g each, were dissolved in 10 ml of 0.2 N NaOH which was brought to boiling to effect solution. The two solutions were combined and the pH adjusted to 7.4 with 1 N HCl. The medium was then sterilized by Seitz filtration. (d) Pancreatic digest of casein (Difco Vitamin-Free Casitone) and casein hydrolyzate (Difco Vitamin-Free Casamino Acids): These materials were made up respectively as 1.0 per cent and 1.5 per cent solutions in demineralized water. To each 100 ml of the above solutions were added glucose, 2 g; K2HPO4, 1 g; KH2PO4, 0.1 g; MgSO4·7H2O, 0.1 g; 0.01 g each L-cysteine, L-tyrosine and glycine; 0.5 mg each thiamin HCl and niacin. These media were sterilized by autoclaving for 20 min at 15 pounds pressure.

RESULTS

Coagulate production in various media. While chemically defined and other special media either inhibited or permitted the production of coagulate to lie fallow in some fashion, it was found that the dialyze of heart infusion broth sustained the production of high titers of coagulate.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Biure Reaction†</th>
<th>Coagulate Titer‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart infusion broth</td>
<td>Positive</td>
<td>1:1024</td>
</tr>
<tr>
<td>Heart infusion broth dialyze</td>
<td>Positive</td>
<td>1:512</td>
</tr>
<tr>
<td>Pancreatic digest of casein</td>
<td>Trace</td>
<td>1:32</td>
</tr>
<tr>
<td>Casein hydrolyzate</td>
<td>Negative</td>
<td>1:16</td>
</tr>
<tr>
<td>Chemically defined medium</td>
<td>Negative</td>
<td>1:8</td>
</tr>
</tbody>
</table>

* Inoculated with 24 hr subcultures of stock strain and incubated at 37 C for 24 hr.
† Pancreatic digest of casein, casein hydrolyzate and chemically defined medium were biuret tested before being supplemented with glucose, vitamins and salts.
‡ Assays were performed on the supernatants following centrifugation at 4000 rpm for 20 min. (table 1). Although growths in each of the media listed in table 1 were similarly luxurious, considerable disparity can be noted in coagulate titers. It is interesting that in the case of the chemically defined medium, where coagulate titers were low, the number of viable bacteria consistently exceeded that in the heart infusion broth. Modifications of the chemically defined medium, for example, with twice the amino acid concentrations, failed to increase the coagulate yields.

A correlation was noted between the ability to give the biuret reaction and the capacity to support coagulate production. In dealing with the media listed in table 1 and modifications of these media, the clearly biuret-positive demonstrated much higher coagulate titers, usually in the range of 1:256 to 1:1024, when compared to the biuret-negative media, the titers of which never exceeded 1:16.

Concentration of coagulate from broth dialyze. The broth dialyze in addition to supporting the production of coagulate, also made convenient its concentration. Since this special medium is entirely dialyzable and since coagulate is retained by a dialysis membrane, a simple expedient is offered for the separation of the medium from the coagulate. A satisfactory method for the concentration of coagulate involved dialysis, to rid the centrifuged medium of its dialyzable constituents, and lyophilization, to effect a concentration of the remainder. After
inoculation and incubation, the medium was centrifuged at 4000 rpm for 20 min, and the supernatant was dialyzed against demineralized water to a resistance of more than 20,000 ohms. The dialysis was accomplished at 5 C in about 72 hr. The resulting solution was then dried from the frozen state. A 2 per cent solution of the resulting material in borate buffer, when assayed against a reactive plasma, yielded an invertible clot in 20 to 30 sec. In contrast, a 2 per cent solution of the standard coagulase required approximately 20 to 30 min before an invertible clot formed.

Characterization of the capacity of broth dialyzate to support coagulase production. A series of 10 individually prepared samples of broth dialyzate were studied with respect to some of their properties. Average values for the clear, amber colored samples were as follows: Nitrogen content, 0.84 mg per ml; pH, 7.2; specific gravity, 1.026; conductivity, 86 ohms. Each of the samples yielded a positive Bial reaction; in this connection, when the chemically defined medium was supplemented with the pentoses ribose or arabinose, in concentrations of 0.5 g per cent, no change could be detected in the coagulase yields. With respect to the protein constituents of broth dialyzate, positive Millon and glyoxylic acid reactions were obtained suggesting the presence of tyrosine and tryptophan. With respect to molecular size, careful dialysis techniques insure that the largest particles are limited by the dialysis membrane and that the broth dialyzate represents an ultrafiltrate of heart infusion broth. This observation, together with the fact that broth dialyzate yields a positive biuret reaction, suggests that a part of the fraction related to protein may consist of peptides. Preliminary experiments with paper chromatography using a water-butanol-acetic acid solvent system were carried out to establish the presence or absence of peptides. By this technique, the ninhydrin-reacting substances in broth dialyzate consisted of several amino acids and at least four peptides. From this it seems likely that the biuret forming substances of broth dialyzate are peptides.

Samples of broth dialyzate were subjected to a number of conditions in order to characterize further their capacity to support coagulase production. In each case, the sterile, treated broth dialyzate was inoculated with a 24 hr subculture of the stock strain, incubated for 24 hr at 37 C, centrifuged at 4000 rpm for 20 min, and the clear supernatant assayed as previously described.

This capacity to support coagulase production showed stability over a wide pH range. When broth dialyzate was incubated at a pH from 1.0 to 11.5 for 24 hr at 37 C and readjusted to about pH 7.2 no alterations could be detected. This capacity was stable over a wide range of temperatures. When stored at refrigerator temperatures for periods of up to 3 months, no loss in coagulase production capacity was noted. Neither refluxing at 100 C for 24 hr nor autoclaving at 20 pounds pressure and 121 C for 1 hr had any effect. The capacity was not altered by passage through a Seitz filter. Repeated extractions with diethyl ether failed to materially alter broth dialyzate with respect to its coagulase production capacity.

This capacity, however, appeared to be altered by hydrolysis with H2SO4. In the process of removing the H2SO4 a considerable amount of BaSO4 is formed. In an attempt to minimize the possibility that adsorption on BaSO4 might have modified the broth dialyzate, relatively large amounts of dry heart infusion broth were used as starting materials. Aliquots of heart infusion broth (14 g) were added to 60 ml quantities of 6 N H2SO4 and refluxed at about 100 C for periods of 2 to 108 hr. Solid Ba(OH)2-8H2O was added with stirring to each of the hydrolyzates in a cold, running water bath until it was alkaline to pH indicator paper. The resultant material was centrifuged at 3000 rpm for 20 min. The supernatant was decanted and CO2 gas was bubbled through the solution to react with the excess barium. The material was then heated to boiling until no further precipitate formed and, when the pH of the mixture was approximately 9, it was considered that the CO2 had been driven off completely. The solution was filtered, the filtrate made up to 100 ml with demineralized water, and

<table>
<thead>
<tr>
<th>Time of Hydrolysis in 6 N H2SO4</th>
<th>Biuret Reaction</th>
<th>Coagulase Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>Positive</td>
<td>1:128</td>
</tr>
<tr>
<td>2</td>
<td>Positive</td>
<td>1:32</td>
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<td>Trace</td>
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<tr>
<td>48</td>
<td>Trace</td>
<td>1:2</td>
</tr>
<tr>
<td>72</td>
<td>Trace</td>
<td>1:1</td>
</tr>
<tr>
<td>108</td>
<td>Negative</td>
<td>1:1</td>
</tr>
</tbody>
</table>

TABLE 2
Coagulase production in hydrolyzed heart infusion broth

[The table is not fully visible, but the header and some entries are given.]

With the decrease in pH, the coagulase titers became less sensitive to changes in the conditions of the broth dialyzates, with the exception of the effect of pH 5.2

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the pH readjusted to approximately 7. At this stage the solution was biuret tested. The filtrate was reconstituted with respect to glucose (1.0 g per cent) niacin and thiamin HCl (0.5 mg per cent, each) and sterilized by autoclaving. The present study confirmed and extended the observation that synthetic and other special media present environments which are suboptimal for coagulase production. One explanation is that these media are deficient in materials necessary for coagulase synthesis or release. A special medium, namely the dialyze of heart infusion broth, was described in which staphylococci are capable of producing high titers of coagulase. It appears from this that materials contained in heart infusion broth which support the production of coagulase are dialyzable. Since the constituents of the chemically defined medium do not substitute for them, it seems likely that the capacity to support coagulase production would reside in those features which make the two media dissimilar.

It is known that the broth dialyzate possesses peptides which are demonstrable in chromatographic procedures. Since broth dialyzate differs in this respect from the protein-free, chemically defined medium, it is possible that these peptides may account for its coagulase production capacity. Visualized as being consistent with this view is the observation that acid hydrolysis modifies the capacity of broth dialyzate to sustain coagulase production. As the medium became less biuret reactive, its coagulase production capacity decreased. Further, when subjected to a variety of extreme conditions, a stability was displayed which could be the property of a peptide.

The fact that broth dialyzate is initially completely dialyzable makes it convenient to concentrate coagulase activity. Extensive inroads have been already made toward the purification of coagulase (Tager, 1948). It is suggested that one might achieve convenient purification from the potent concentrates which can be obtained from broth dialyzate since much of the material which constituted the bulk of crude preparations is eliminated in the preparation of the medium.

ACKNOWLEDGMENT
Grateful acknowledgment is made to Professor Walter H. Seegers for his kind support and to Dr. Wells Shulls for the performance of the paper chromatography.

SUMMARY
A medium, the dialyzate of heart infusion broth, which supports the production of coagulase from *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) was described. Utilizing broth dialyzate, the preparation of potent coagulase concentrates was made convenient.

The capacity of broth dialyzate to sustain coagulase production was characterized. The possibility that peptide structures, contained in broth dialyzate, are responsible for this capacity has been discussed.

REFERENCES


