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THE EFFECT OF SELECTED PROTEINS ON
THERMAL INACTIVATION OF STAPHYLOCOCCAL ENTEROTOXIN B

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Deborah Anne Lee

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K. E. Stevenson
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THE EFFECT OF SELECTED PROTEINS ON THERMAL INACTIVATION OF STAPHYLOCOCCAL ENTEROTOXIN B

By

Deborah Anne Lee

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ABSTRACT

THE EFFECT OF SELECTED PROTEINS ON
THERMAL INACTIVATION OF STAPHYLOCOCCAL ENTEROTOXIN B

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The purpose of this investigation was to determine the influence of selected proteins during thermal inactivation of staphylococcal enterotoxin B (SEB) at 110 C. Specifically, protein obtained from beef broth or chuck roast bouillon was used. Preliminary heat inactivation studies using the non-dialyzed protein or dialysis fractions i.e., the dialyzed and dialysate portions, were performed. Results suggested that a dialyzable factor(s) was responsible for retarding heat inactivation of SEB. Purification of the dialysis fractions by separation techniques such as ion exchange, gel filtration, and ultrafiltration was performed in order to determine the origin of the protective factor(s). Results suggested that several components which are similar in size and charge may be involved. In addition, the effect of pH, ionic strength, and denaturants on the non-dialyzed protein was studied during thermal inactivation of SEB. The protective factor(s) was effective over a broad pH range; ionic strength was also an important condition for protection.

DEDICATION

To the ones I love.

ACKNOWLEDGMENTS

I would like to express my appreciation to my adviser, Dr. Kenneth E. Stevenson for his guidance during the course of the investigation.

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INTRODUCTION

One of the major foodborne intoxicants is the thermally stable staphylococcal enterotoxin; it is responsible for numerous food poisoning outbreaks classified as staphylococcal food poisoning. Several investigations have been conducted on the thermal inactivation of staphylococcal enterotoxins. This research was involved with one aspect of heat inactivation of staphylococcal enterotoxins, the protective effect of protein on staphylococcal enterotoxins during heat inactivation. Specifically, a protein fraction from a commercial soup product and protein from laboratory-prepared chuck roast bouillon were studied. The objective of the research was to purify, isolate, and identify the component(s) active in the protection of staphylococcal enterotoxin B (SEB) during thermal inactivation. Although all the staphylococcal enterotoxins were found to possess greater thermal stability in the presence of the protein fraction from the commercial soup product, SEB was used in this study because of its commercial availability and the ability to prepare it in larger quantities than the other staphylococcal enterotoxins.

LITERATURE REVIEW

Staphylococcal Enterotoxins

Only six staphylococcal enterotoxins, types A, B, C, D, E, and F, have been identified (Fung, 1973; Niskanen, 1977; Arbuthnot, 1978). In general, these staphylococcal enterotoxins appear to be rather simple proteins. All appear to have similar structures; each is a single polypeptide chain containing a loop due to the formation of a disulphide bridge. The amino acid residues in the loop appear to be the same for the different toxins and are thought to be the site of toxicity; the composition of amino acids in the chain outside the loop varies for different serological types of staphylococcal enterotoxin, thus resulting in different isoelectric points (Bergdoll and Robbins, 1973; Bergdoll et al., 1974).

Enterotoxin A and B

Staphylococcal enterotoxins A and B have been more widely studied than the other enterotoxins. Type A is studied because it is most often isolated from foods involved in staphylococcal food poisoning outbreaks (Payne and Wood, 1974); type B because it is commercially available and is produced in larger amount than any of the other

enterotoxins.

Staphylococcal enterotoxins types A and B have similar structures and produce similar clinical symptoms; however, they have characteristics which distinguish them from each other very clearly. One difference is that SEA was bound by food more extensively than SEB (Bergdoll, 1970). Another difference is the growth phase during which the two enterotoxins are produced. SEA was produced mainly during the exponential phase, thus it is a primary metabolite, while SEB was produced during the late exponential or early stationary phase of growth, thus it is a secondary metabolite (Morse et al., 1969; Markus and Silverman, 1970; Carpenter and Silverman, 1976). SEA is more heat labile than SEB. After heating at 100 C for 1 min, SEA lost all biological activity (Chu et al., 1966); on the other hand, SEB retained greater than 50% of its biological activity after heating for 5 min at 100 C (Schantz et al., 1965). Finally, enterotoxins A and B differ in their antigenic properties more than any two other enterotoxins (Bergdoll and Robbins, 1973; Spero et al., 1978).

Staphylococcal enterotoxin B is the only enterotoxin for which the molecular weight is known exactly. SEB is a single polypeptide chain with 239 amino acid residues and a molecular weight 28,494 (Huang and Bergdoll, 1970). It contains two cysteine residues at positions 92 and 112 which join to form a single cystine residue, thus forming a loop

in the amino acid chain (Figure 1). SEB can be produced in higher amounts and is more heat stable than all of the other staphylococcal enterotoxins (Bergdoll, 1972).

Enterotoxin C

Two enterotoxin C's have been identified, C_1 and C_2. They were found to be immunologically identical (Metzger et al., 1975), however, they are distinct toxins based on isoelectric heterogeneity; C_1 with an isoelectric point of 8.6 and C_2 with an isoelectric point of 7.0 (Bergdoll, 1972; Stavric et al., 1975). SEC is antigenic in nature and has an electrophoretic behavior very similar to that of SEB (Borja and Bergdoll, 1967); partial cross reactions between these two have been noted (Johnson et al., 1972; Spero et al., 1978). However, SEC is less heat stable than SEB; when SEC was heated at 100 C for 1 min, only 20% of the original biological activity remained (Avena and Bergdoll, 1967).

Enterotoxin D

Staphylococcal enterotoxins A and D are produced most frequently by S. aureus strains of food poisoning origin. Casman et al. (1967) found that type A alone was produced by 50% of the food poisoning strains; in combination with type D, it was produced by an additional 25%; and type D alone was produced by 8% of the food poisoning strains in foods such as milk and frozen foods. Type D appears to be more resistant to heat than type A since it retained 15%

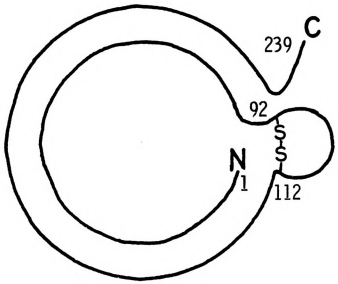


Figure 1. A schematic representation[a] of the structure of staphylococcal enterotoxin B.
[a]Warren et al. (1974a)

and 5% of its biological activity after heating at 100 C for 1 and 2 hrs, respectively (Chang and Bergdoll, 1979).

Enterotoxin E and F

Known facts concerning staphylococcal enterotoxins E and F are still limited. In a toxicity study with monkeys, 10-20 μg of SEE provoked vomiting in 60% of the test animals (Borja et al., 1972). This same study revealed other physiochemical properties of SEE: serological activity of SEE was reduced 95% after heating for 5 min at 100 C; and SEE was inactivated by extreme pH values of 2.0 or 12. Enterotoxins A and E have very similar amino acid compositions. Cross reactions have been observed between SEA and SEE; antiserum A was able to neutralize enterotoxin E when injected intravenously into monkeys (Bergdoll and Robbins, 1973).

Very little is known about staphylococcal enterotoxin F. SEF has been purified and a specific antiserum can be made. Factors influencing production of the toxin were reported by Thota et al. (1973), but more research concerning the characterization of enterotoxin F is needed.

Staphylococcal Food Poisoning

Staphylococcal food poisoning is strictly an intoxication which is caused by a water-soluble protein secreted by the microorganism, Staphylococcus aureus. The incidence of staphylococcal food poisoning is among the highest

reported for foodborne intoxications although exact numbers of cases are not known due to incomplete reporting of outbreaks. Some foods frequently involved in outbreaks are dairy products such as milk or cheese, custard- and cream-filled bakery goods, and cured meats (Minor and Marth, 1972b,c). These foods usually become involved in food poisoning outbreaks as a result of inadequate cooling and mishandling after being prepared.

All enterotoxins cause similar clinical symptoms of intoxication. Typically, symptoms such as nausea, vomiting, various degrees of abdominal cramp and often diarrhea will appear 1-6 hr after consumption of a contaminated food. Staphylococcal food poisoning is rarely fatal with recovery occurring usually within 24-72 hr. Although the exact mode of action of the toxin is unknown there is evidence that the toxin may act on sites in the abdominal viscera via sympathetic nerves and smooth muscles (Jeljaszewicz et al., 1978; Arbuthnot, 1978).

Due to the great variation in sensitivity which exists between individuals, it is difficult to determine the minimum dose of staphylococcal enterotoxin which would cause symptoms of food poisoning. Raj and Bergdoll (1969) found that 20-25 μg of pure SEB could produce clinical manifestations of staphylococcal food poisoning in man. In another study involving SEA, SEB, and SEC, a minimum dose of 10-13 μg for the development of symptoms was

observed (Gilbert et al., 1972). In the monkey, the only other primate order which is comparatively sensitive to enterotoxins per os, 5 μg of SEB was found to be sufficient for an emetic dose in 50% of the animals (Bergdoll, 1972); also, 5 μg of SEA produced symptoms of intoxication in monkeys (Ladany, 1973); SEC in the amount of 5-10 μg per monkey (2-3 kg) caused emesis (Avena and Bergdoll, 1967; Borja and Bergdoll, 1967). Casman and Bennett (1965) suggested that doses as low as 1-4 μg of enterotoxin were capable of causing food poisoning symptoms. Cheese containing 1 μg or less of SEA was consumed and caused staphylococcal food poisoning in humans (Bergdoll, 1970).

Thermal Stability of Staphylococcal Enterotoxins

There have been many investigations concerning factors such as temperature, pH, ionic strength, and type and concentration of medium which influence the growth of the microorganism, Staphylococcus aureus (Mah et al., 1967; Troller, 1971; Barber and Deibel, 1972; Tatini, 1973; Troller and Stinson, 1975; Tatini et al., 1976). Similar factors have been studied thoroughly with respect to production of staphylococcal enterotoxins (Genigeorgis and Saddler, 1966; Reiser and Weiss, 1969; Minor and Marth, 1972a; Jarvis et al., 1973; Miller and Fung, 1973; Tatini, 1973; Vanden Bosch et al., 1973; Keller et al., 1978). However, there is little information available on the influence of these factors on the thermal stability of

staphylococcal enterotoxins.

The thermal inactivation of staphylococcal enterotoxin is very complex. Denaturation of the enterotoxin is primarily due to the effects of heat. Neucere (1972) stated that heat might be expected to induce thermal effects on certain proteins at varying rates depending upon the temperature. After heating, proteins exist in their zwitterionic state (Haurowitz, 1963). This suggests that hydrogen bonds between peptide chains are cleaved by the thermal motion of peptide chains and hydrophobic bonds are disrupted. The changes result in conformations that make the protein less soluble, modify electric charge, and allow for formation of complex products.

Factors of the system being heated have a significant influence on the thermal inactivation of staphylococcal enterotoxins. Some of the important factors that should be considered are the following: type and concentration of enterotoxin, type of medium, pH, and ionic strength of the system.

Type and Concentration of Enterotoxin

The type and concentration of staphylococcal enterotoxin is an important consideration. Of the six staphylococcal enterotoxins identified, type B is the most heat stable. In addition, the slope of the thermal destruction curve (z value) for enterotoxin A or D was 27-28 C and 32 C for enterotoxin B regardless of the initial concentration of enterotoxin, heating medium, or assay system used to

detect the enterotoxin (Tatini, 1976).

Staphylococcal enterotoxins were more stable in concentrated protein solutions than in dilute solutions. Haurowitz (1963) suggested that concentrated protein solutions were more stable because peptide chains of closely folded native protein could not unfold unless water flowed into the space between the chains. Hilker et al. (1968) found that a larger initial concentration of crude SEA (90 µg/ml) had a much higher heat tolerance than a lower initial concentration (21 µg/ml) when heated in veronal buffer at pH 7.2. Heat inactivation of SEA was more effective at lower initial than at higher initial concentrations in both beef bouillon and phosphate buffer (Denny et al., 1971).

Crude preparations of SEB were found to be slightly more thermostable than purified preparations. Read and Bradshaw (1966) determined the D_{110} value to be 29.7 min for crude SEB and 23.5 min for purified SEB. Satterlee and Kraft (1969) found that 50 µg/ml of crude SEB was slightly more resistant to thermal inactivation than 50 µg/ml of partially purified SEB when heated in 0.013 M phosphate buffer containing 0.85% NaCl. However, Jamlang et al. (1971) found that at 70 C, an increase in concentration of SEB resulted in an increase in denaturation; this denaturation was explained by heat aggregation of molecules in their native state which could be reversed by heating at a

higher temperature so that renaturation occurred.

Type of Medium

The type of medium in which the toxin is being heated was found to have a significant effect on the thermal inactivation of staphylococcal enterotoxins. Tatini (1976) and Niskanen (1977) have made this generalization based upon numerous studies. Enterotoxin A was inactivated by less heat in a pH 7.2 phosphate buffer than in beef bouillon (Denny et al., 1971). The heating of enterotoxin B at 60, 80, and 100 C in the presence of two meat proteins, either myosin or met-myoglobin, resulted in a rapid loss of enterotoxins. Also, thermal loss of enterotoxin B in a ground round slurry was rapid when compared to inactivation in a phosphate saline buffer (Satterlee and Kraft, 1969). Reichert and Fung (1976) suspended SEB in various liquid food systems. After heating for 5 min at 100 C, they found 50% of the original toxin activity in the beef broth, brain heart infusion (BHI) broth, and protein hydrolysate medium (PHP) systems. Activity of SEB was determined to be very low in buttermilk, tomato soup, and milk after heating under the same conditions because of combined effects of low pH and/or heat treatment. Lee et al. (1977) determined that D-values were higher when partially purified SEB was heated in beef broth than when heated in BHI broth or veronal buffer. Finally, enterotoxin C had greater heat stability at 80 C in a casein hydrolysate solution than in

phosphate saline buffer (Fung et al., 1973).

Pure buffer solutions appeared to have less protective effect than food systems. Denny et al. (1971) found that the heat resistance of SEA in beef bouillon was greater by a factor of 3-5 times than when in 0.15 M phosphate buffer. Results presented by Reichert and Fung (1976) indicated that the rate of heat inactivation of 5 μg/ml SEB was faster in phosphate buffered saline than in BHI broth.

pH

The pH of the medium significantly influences the heat denaturation of staphylococcal enterotoxins. Denaturation by acid occurs via an impact on the total surface charge of the protein and on ionization of specific groups on particular amino acids. Addition of acid ionizes weakly acidic or basic groups which are in the interior, hydrophobic region of the protein molecule; subsequently these charged groups attract water molecules and form hydration shells which disrupt hydrophobic associations and cause unfolding (Kinsella, 1976). Bull and Breese (1973) correlated the effects of pH and heat. They stated that a non-buffered protein solution showed a significant and abrupt change in pH as the protein became heat denatured. This change in pH arose from normalization of pK values for the various ionizable groups in the protein.

In several studies with staphylococcal enterotoxin, inactivation was more rapid when the system heated had a pH

value in the acid range. For enterotoxin A, Denny et al. (1966) hypothesized that if acid denatures protein and enterotoxin is a simple protein, then the heat required for toxin inactivation at lower pH levels would be less. In a study by Humber et al. (1975) 5 µg/ml SEA in beef bouillon was inactivated faster at pH 5.3 than at pH 6.2. Tatini (1976) found that SEA in buffer was inactivated faster at a pH ≤5.5 than at pH ≥6.5; however, in contrast, SED in buffer was inactivated faster at the higher pH of 6.5 than at pH ≤5.5. For enterotoxin B, rapid denaturation occurred at a pH value less than 3.5 (Warren et al., 1974a). Jamlang et al. (1971) found that when the pH was changed from 6.4 to 4.5 or 7.5, no large change was seen in the inactivation curves of an initial concentration of 100 µg/ml SEB at 70 C if ionic strength was maintained at 0.10; however, under similar conditions at 100 C, SEB was more stable at pH 6.4 than at pH 4.5 or 7.5.

Ionic Strength

When sodium chloride was added to a test system to increase ionic strength, the resistance of enterotoxin to heat increased. This was demonstrated by Jamlang et al. (1971) who varied the ionic strength of phosphate (pH 6.4) or sodium acetate (pH 4.5) buffers by adding NaCl. When 100 µg/ml SEB was heated at 70 C there was a gradual increase in amount of remaining activity as ionic strength increased from 0.10 to 1.0 in the pH 6.4 buffer; however,

in the pH 4.5 buffer, the amount of activity decreased as ionic strength increased from 0.05 to 1.0. Stinson and Troller (1974) studied the inactivation of SEB under oil frying conditions. They observed that the lower water activity (a_w) of oil-fried foods exerted some protective effect on the toxin and NaCl afforded an additional protective effect.

Specific Components

The influence of protein on the thermal inactivation of staphylococcal enterotoxin has not been studied in detail. Sharma et al. (1978) discovered that addition of foreign proteins in dilution buffers prevented inactivation of the test protein in their system. In specific studies with staphylococcal enterotoxins in which SEA was heated in beef bouillon versus casamino acid medium (CAM), a slightly better inactivation occurred in CAM (Humber et al., 1975). Humber et al. (1975) postulated that this was because CAM lacked the larger molecular weight proteins or other materials that might bind or in some way protect the toxin molecule from heat inactivation. Satterlee and Kraft (1969) explained that the greater heat stability of crude SEB was due to the presence of other proteins which were abundant in the crude preparation. In the same study, there was rapid heat inactivation of SEB in meat protein solutions and in a meat slurry. Two possible reasons for the lack of protection by meat proteins were (1) some of

the enterotoxin bound to the meat proteins and was then undetectable by the gel diffusion assay and (2) the toxin that was not bound may have been inactivated rapidly by heat. Lee et al. (1977) found a low molecular weight protein fraction in a commercial soup product which increased the heat resistance of staphylococcal enterotoxins.

Concentrated solutions of glucose and other sugars are thought to protect protein during heat denaturation. Haurowitz (1963) stated that the action of these substances may be due to their adsorption to the protein, and to the formation of large hydrophobic complexes in which protein is coated, thus preventing formation of aggregates with other proteins. The thermal stabilization of proteins by sugar through hydrophobic interactions is also supported by Smith et al. (1978) and Oakenfull et al. (1978). Reducing sugars such as xylose, lactose, glucose, maltose, and fructose had a protective effect when 10 μg/ml SEA was heated at 60 C in 2.5% peptone medium (Chordash and Potter, 1976).

MATERIALS AND METHODS

Enterotoxin Production

Commercial Toxin

Staphylococcal enterotoxin B (SEB) was obtained in highly purified form as a lyophilized powder from Makor Chemicals Ltd. (Jerusalem, Israel). Data sheets concerning protein concentration (mg protein/mg solids) were supplied by Makor. The lyophilized SEB was stored dry at 4 C and was reconstituted in diluent consisting of a mixture of one part of brain heart infusion broth and nine parts of fluid base which contained 0.02 M phosphate buffer, 0.85% NaCl, and 0.01% thimersol prior to use.

Laboratory-prepared Toxin

Staphylococcus aureus strain 243 (ATCC 14458) was maintained on 3% NZ-amine-NAK:3% protein hydrolysate powder (3% NAK-PHP) agar slants. Prior to use for production of enterotoxin, the culture was transferred twice in 10 ml of 3% NAK-PHP broth and incubated at 37 C for 24 hr. One milliliter was inoculated into 100 ml of 3% NAK-PHP broth which was incubated at 37 C for 24 hr on a gyrotory shaker (New Brunswick Scientific Co.) operating at 150-160 rpm. Finally, 8 flasks containing 500 ml of 3% NAK-PHP broth were inoculated with 1% inoculum, and incubated at 37 C for

48 hr.

The supernatant was collected by centrifugation at 4 C in a Sorvall RC-2 centrifuge operating at 9500 rpm for 30 min.

The supernatant was concentrated by dialysis in Spectrapor 2 tubing (Fisher Scientific Co.) against 40% polyethylene glycol (Carbowax 20,000; Fisher Scientific Co.) for 2 days at 4 C. The crude SEB concentrate, an approximately 20-fold concentration of the supernatant, was obtained after a second dialysis against polyethylene glycol.

Partially purified SEB was obtained by gel filtration of the crude SEB on a Bio-Gel P-60 (Bio-Rad; Richmond, Ca.) column. Fractions were collected using a LKB Ultrorac, model 7000 fraction collector (LKB Produkter AB; Sweden). The SEB activity was assayed by the Casman and Bennett (1965) microslide technique. Fractions containing the highest activities were pooled and subsequently concentrated by dialysis. Ten milliliter aliquots of this partially purified SEB were stored at ⁻17 C.

Source of Protein

Beef Broth Bouillon

Protein from beef broth (bouillon), a product of Campbell's Soup Co., was obtained according to the method described by Lee (1974) and illustrated in Figure 2. Non-dialyzed (ND) and dialyzed (Dz) fractions of the protein

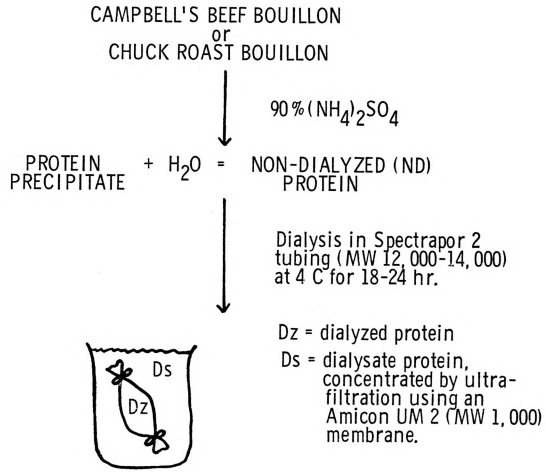


Figure 2. Flow Chart for Preparation of Protein from Bouillon.

were stored as liquids at 4 C. The dialysate (Ds) fraction was concentrated by ultrafiltration through a UM 2 membrane (Amicon) and by lyophilization using a manifold-style freeze dryer (Virtis; Ann Arbor, Mi.); the lyophilized Ds was subsequently stored at ⁻17 C.

Chuck Roast Bouillon

Bouillon was prepared from chuck roast (MSU Foodstores) according to the procedure described by Denny et al. (1971). Protein was obtained and separated into fractions and stored as described for the Campbell's soup protein.

Other Protein Sources

Other protein sources used were meat extract (L.J. Minor, Corp.; Cleveland, Ohio), soytone (Difco), Edi-Pro-N (Ralston Purina), and yeast extract (Difco).

Purification Techniques

Gel Filtration

Bio-Gel P-4: The chuck roast bouillon protein fractions (ND, Dz, Ds) were characterized by gel filtration on a Bio-Gel P-4 column (1.5 x 40 cm). The column was operated at a head pressure of 40 cm and flow rate of approximately 30 ml/hr. The eluant buffer was 0.1 M Tris-HCl, pH 7.5.

Bio-Gel P-10: The Campbell's soup dialysate (Ds) fraction was characterized by gel filtration on a Bio-Gel P-10 column (2.5 x 85 cm). The column operated at a head pressure of 85 cm and flow rate of 100-120 ml/hr. The eluant used was either water or 0.05 M phosphate buffer,

pH 7.4.

Bio-Gel P-60: SEB crude concentrate was partially purified by chromatography over a Bio-Gel P-60 column (4.5 x 52 cm). The column was operated at a head pressure of 26 cm and a flow rate of 30 ml/hr. The eluant used was 0.04 M veronal buffer, pH 7.4.

All gel filtration columns were conditioned according to the general instructions for column preparation of Bio-Gel P (Bio-Rad catalogue). For each column, void volume was determined by elution of Blue dextran 2000 (Pharmacia Fine Chemicals). All columns were operated at room temperature. Protein was detected in the effluent by measurement of absorbance at 280 nm by a UV monitor (Gilson; Middleton, Wi.); the chromatographic profiles were recorded (Sargent, Model SR).

Ion Exchange

Anion Exchange Cellulose: Cellex D (Bio-Rad): The dialysate from beef broth and the chuck roast bouillon protein fractions were characterized on a Cellex D column (1.2 x 20 cm). The column operated at 30 cm head pressure and 15-20 ml/hr flow rate. The eluant buffer was 0.01 M phosphate buffer, pH 7. For linear gradient elution, a 2-chambered mixing apparatus, one with 0.01 M phosphate buffer, pH 7 and the other with 0.01 M phosphate buffer containing 0.5 M NaCl, pH 7 in equal volume was employed; and for stepwise gradient elution, 0.01 M phosphate buffer, pH 7 containing the

desired NaCl concentration was used.

Cation Exchange Cellulose: Whatman CM 22 (W & R Balston, Ltd.):

The beef bouillon dialysate fraction which did not adsorb on Cellex D was characterized using a Whatman CM 22 column (1.2 x 13 cm). Head pressure over the column was 15 cm and flow rate of the eluant was 15-20 ml/hr. The composition of the eluant, 0.01 M phosphate buffer, was altered to vary the pH (Gormori, 1955).

The effluent in all ion exchange experiments was monitored by measurement of absorbance at 280 nm.

Electrophoresis

Isoelectric focusing: The procedure of Wrigley (1971) for gel electrofocusing was used. Ampholytes (LKB Produkter AB; Sweden) with the pH range 3.5-10 were used initially to obtain approximate locations of the isoelectric points of the proteins; subsequently ampholytes in the pH range of 3-6 were used. 7.5% total gels (7x75-mm tubes) were either polymerized chemically with persulfate or photopolymerized with riboflavin. A 100-200 µg sample was applied at the surface or throughout the gel. Isoelectric focusing at 2 mA/tube, up to a maximum of 400 V (Heathkit Power Supply, model IP-32) required 1.5-2 hr. The gels were stained by a Coomassie brilliant blue (Sigma; St. Louis, Mo.) stain according to the method of Malik and Berrie (1972). Duplicate unstained gels were scanned at 280 nm by a densitometer

(Gilford, model 2400-S; Oberlin, Ohio).

Disc Electrophoresis: Polyacrylamide disc gel electrophoresis was carried out in 7x75-mm tubes at room temperature according to the method of Davis (1964). A running buffer of pH 8.3 was employed with 7.5%, 12%, and 15% gels and the sample size applied ranged from 100-600 μg/gel. In addition, 7.5% and 10% disc gels containing 8M urea and 10% sodium dodecyl sulfate gels (Porzio and Pearson, 1977) were also employed. Gradient disc gels were made using a slight modification of the procedure described by Kasper (1978); a 5-25% gradient was pumped (Gilson Minipuls II peristalic pump; Middleton, Wi.) via a linear gradient mixing chamber into a vessel containing the electrophoretic tubes. Gels were stained with Coomassie brilliant blue (Malik and Berrie, 1972).

Ultrafiltration

An ultrafiltration cell (Amicon, model 52) operating at 60 psi nitrogen was used for concentration or fractionation purposes. The Amicon diaflo membranes UM 2 and UM 10 with exclusion at approximately MW1,000 and MW10,000 were used most frequently. They were washed with either dilute NaOH or 1-2 M NaCl and stored in 10% ethanol at 4 C. Upon reuse, they were rinsed several times in sterile deionized water.

Quantitation of Protein

Protein concentration (mg/ml) was determined by the Lowry method as described in Whitaker and Bernhard (1972). The protein standard was bovine serum albumin (Sigma) at concentrations of 0-125 µg/ml. Absorbance at 600 nm was measured on a Beckman DB-G spectrophotometer or a Spectronic 20 (Bausch & Lomb).

Thermal Inactivation of SEB in the Presence of Protein

Thermal inactivation treatments were made in a mini-steam retort in the Food Science Building at Michigan State University. All samples contained a total protein concentration of 7.7 mg/ml plus 55 µg/ml partially purified SEB. In order to conserve both sample and SEB, aliquots consisting of sample and SEB in a 1 ml total volume were sealed in small glass ampules. Each sample was prepared in quadruplet and was heated at 110 C (230 F) for various time intervals. Samples were cooled by three consecutive rinses in cold tap water.

Assay for Heat Inactivated SEB

The Casman and Bennet (1965) microslide technique, sensitive to 0.1-0.01 µg SEB/ml, was used. Details concerning preparation of media, reagents, and slides were described by Lee (1974) and in the 2nd supplement to the 12th edition of the Official Methods of Analysis of the AOAC (1976). In this assay the presence of SEB was determined

serologically by a line of precipitation which formed when the enterotoxin diffused through the gel and reacted with its specific antibody. Heat treated samples were diluted 2-fold to give 1/2, 1/4, 1/8, 1/16, and 1/32 dilutions. Twenty-five microliter aliquots of consecutive serial dilutions were pipetted into outer wells of a template that was situated over a thin layer of agar; 25 µl of antiserum B (Makor Chemicals Ltd., Jerusalem, Israel) was placed in the center well. Incubation for 3 days at room temperature or 1 day at 32 C in a moist chamber was sufficient time for diffusion to occur. Results were recorded as the reciprocal of the highest dilution that showed a positive test.

RESULTS

Lee (1974) reported that protection of SEB by the recombined dialyzed (Dz) and dialysate (Ds) fractions of beef broth protein was approximately equal to that of the non-dialyzed (ND) protein. D_{110} for the ND protein was 63 min and was 60 min for the Dz + Ds combination. In addition, since protection by the Dz fraction (D_{110}=32 min) was less than that of the ND protein, this implied that the original ND protein contained a dialyzable factor which significantly affected the thermal stability of SEB. When the Ds fraction was treated with proteolytic enzymes, trypsin and chymotrypsin, the resulting Ds fraction possessed less thermal protection for SEB, thus the factor was thought to be a protein. This project involved the study of the protective factor(s) in the Ds fraction.

Nature of the Charge on the Ds Fraction

Isoelectric focusing of the Ds fraction was performed to determine the approximate isoelectric points of the proteins. With ampholytes in the 3.5-10 pH range, the stained gel had a group of bands very close together at the anodic end and a smear at the cathodic end. In gels made with ampholytes that ranged in pH from 3-6, slightly better

resolution was obtained. Some distinct bands were observed beginning at the center of the gel and spreading towards the anodic end in addition to a small smear at the top (Fig. 3). A densitometer scan at A_{280} of duplicate but unstained gels did not yield any information, probably due to interference by the ampholytes.

Characterization of the Beef Broth Ds Fraction

Ion Exchange

Since the isoelectric pH of the Ds fraction appeared to fall in the lower pH range, the Ds fraction could attach to anion exchangers in a buffer which had a pH above the isoelectric pH. On Cellex D, an anion exchange resin, the Ds fraction separated into several peaks upon linear gradient elution using a pH 7 buffer and varying ionic strength by addition of 0-0.5 M NaCl (Fig. 4). To simplify collection and concentration of peaks, stepwise elution was employed. Fraction I which did not adsorb to Cellex D comprised approximately 48% of the total protein in the Ds fraction, while fraction II which rinsed off Cellex D with 1 M NaCl comprised approximately 52% of the protein (Fig. 5).

Fraction I was subsequently separated on a cation exchange resin, Whatman CM 22. Elution of the protein from the resin by buffers of various pH values resulted in different chromatographic profiles (Fig. 6). However, to minimize alteration of the protein by a change in pH,

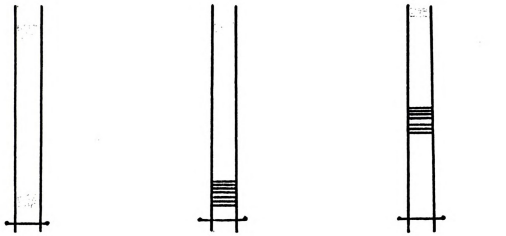


Figure 3. A schematic representation of isoelectric gels of the dialysate fraction of protein from beef broth.

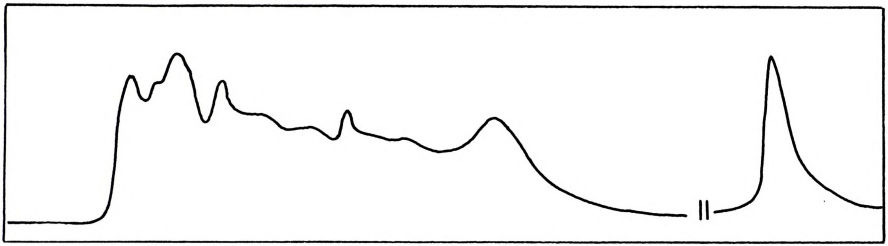


Figure 4. A chromatograph of the linear elution of the dialysate fraction of protein from beef broth from a Cellex D column.

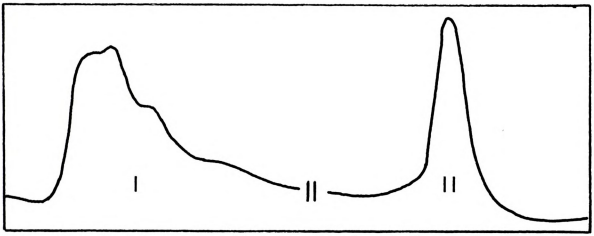


Figure 5. A chromatograph of the stepwise elution of the dialysate fraction of protein from beef broth from a Cellex D column. Fraction I did not adsorb to the resin; fraction II was eluted off using 1 M NaCl.

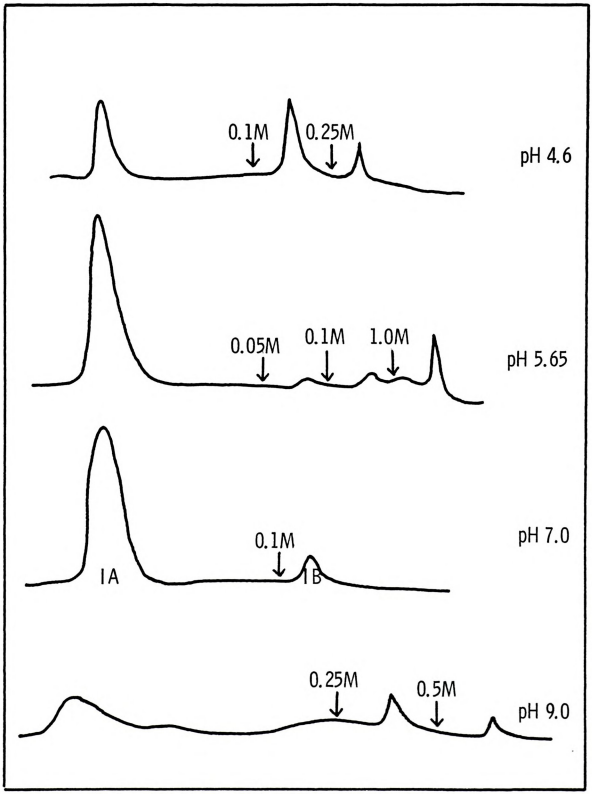


Figure 6. The chromatographs of fraction I of the dialysate fraction of protein from beef broth from Whatman CM 22 chromatography using buffers of various pH values. Fraction IA did not adsorb to the resin at pH 7.0; fraction IB adsorbed and was eluted off using 0.1 M NaCl.

separation on Whatman CM 22 was subsequently done with the pH 7 buffer. Fractions IA and IB were obtained with fraction IA approximately 92% of fraction I, and fraction IB approximately 8%.

Fraction I, II, IA, and IB were tested for their protective effect on SEB during thermal inactivation. Fractions I and II of the total Ds protein plus the Dz fraction had a 50% decrease in protection as compared to the ND protein when tested individually with SEB. When the two fractions were recombined together with SEB, protection was nearly equal to that of the original ND protein during the first 30 min, but little protection was apparent after 45 or 60 min (Table 1). Fractions IA and IB of fraction I appeared to have a protective effect equal to that of the ND protein after 15 and 30 min treatments when each was combined with SEB alone; protection at 45 and 60 min treatments was decreased when compared to ND protein (Table 2). No data were available on the recombination of IA + IB with SEB.

Gel Filtration

The Ds fraction was separated on the basis of molecular size by gel filtration on Bio-Gel P-10 (MW 1,500-20,000). The Ds fraction was previously reported by Lee et al. (1977) to have a molecular weight between 10,000 and 20,000. Elution with water resulted in very poor resolution of the Ds fraction. Resolution was slightly better using

Table 1. Heat inactivation of staphylococcal enterotoxin B at 110 C in the presence of beef broth protein[a] and protein fractions[b] after chromatography on Cellex D.

Time at 110 C min	ND	Ds + Dz	DsI + Dz	DsII + Dz	DsI+DsII+Dz
15	16^{-c}	16^{-}	8^{+}	8^{+}	16^{-}
30	8	8	4	4^{+}	8^{-}
45	4	4	2	2	1
60	2	2	0	0	0

[a]ND = non-dialyzed beef broth protein; Dz = dialyzed; Ds = dialysate

[b]DsI and DsII = fractions from Ds after Cellex D chromatography (see text for details).

[c]Numbers represent the titer of the remaining SEB activity.

Table 2. Heat inactivation of staphylococcal enterotoxin B at 110 C in the presence of beef broth protein[a] and protein fractions[b] after chromatography on Whatman CM 22.

Time at 110 C min	ND	Ds + Dz	DsIA+Dz	DsIB+Dz
15	8[c]	8	8	8
30	8	8	8	8
45	4^+	4^+	2	2
60	2	2	1	1

[a]ND = non-dialyzed beef broth protein; Dz = dialyzed; Ds = dialysate

[b]DsIA and DsIB = fractions from Ds I after Whatman CM 22 chromatography (see text for details).

[c]Numbers represent the titer of the remaining SEB activity.

0.05 M phosphate buffer, pH 7.4; the Ds fraction separated into one portion with molecular weight ≥20,000 and a portion with molecular weight <13,000 (Fig. 7).

Electrophoresis

Electrophoresis was used to distinguish the molecules in the Ds fraction by their net charge and shape. In the 7.5% disc gels, there were no distinct bands; however, a smear did appear. There were also smears in the 12% gels and in the 7.5% and 10% gels in which the samples had been applied in urea. Finally, there was a broad band in addition to a background smear in the 10% sodium dodecyl sulfate gels (Fig. 8).

Ultrafiltration

Ultrafiltration of the Ds fraction through a UM 10 (MW 10,000) membrane provided a filtrate (F) containing components with molecular weights ranging from 1,000-10,000 and a retentate (R) containing components with molecular weights ranging from 10,000-14,000. R represented approximately 88% of the total while F represented approximately 12%.

R and R fractions were subsequently tested for their protective effect on SEB during thermal inactivation. When R and F were combined separately with SEB or recombined together with SEB, protection was equal to that of the ND fraction during heating for up to 45 min (Table 3).

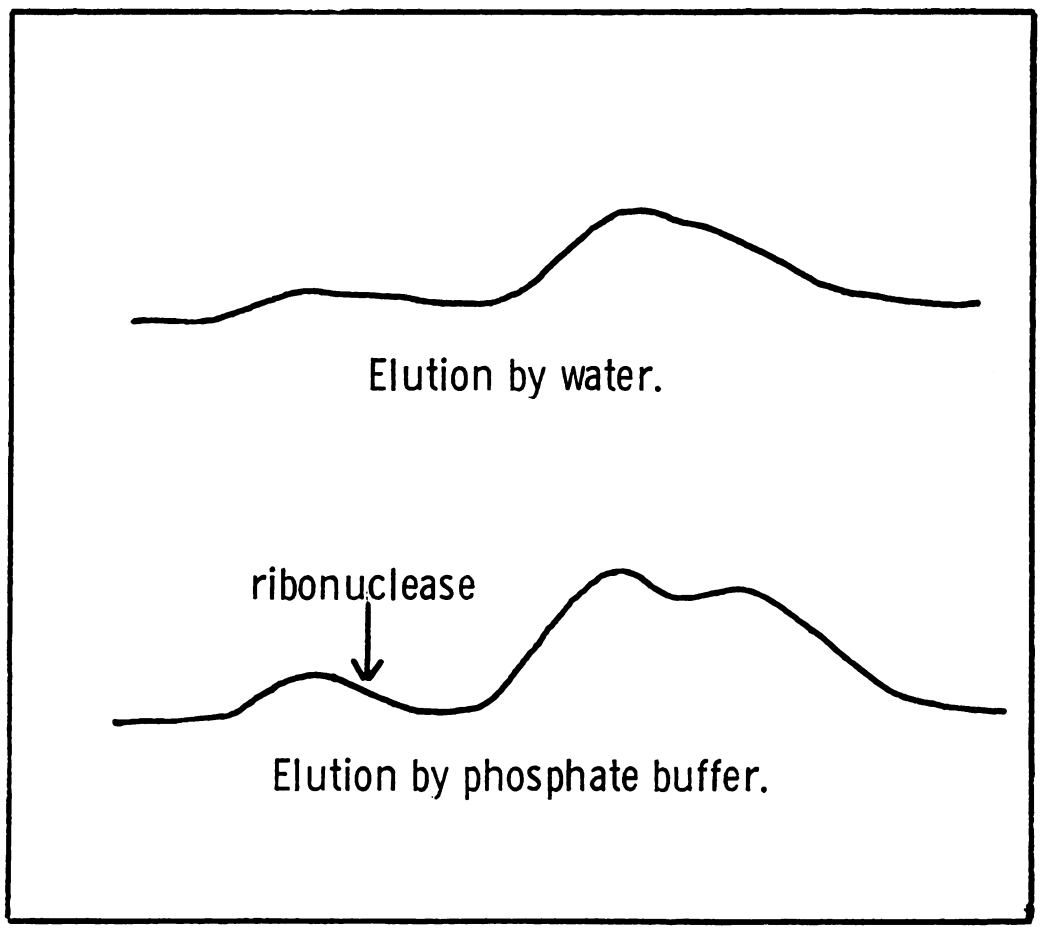


Figure 7. The gel filtration chromatographs of the dialysate fraction of protein from beef broth. Sample was applied on a Bio-Gel P-10 column and eluted with water or phosphate buffer.

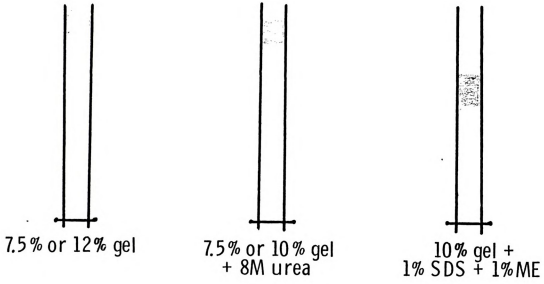


Figure 8. A schematic representation of electrophoretic gels of the dialysate fraction of protein from beef broth.

Table 3. Heat inactivation of staphylococcal enterotoxin B at 110 C in the presence of beef broth protein[a] and protein fractions[b] obtained by ultrafiltration using UM 10.

Time at 110 C min	ND	R + Dz	F + Dz	R+F+Dz
15	16[c]	8	16⁻	16⁻
30	8	8	8	8
45	4	4	4	4
60	4⁻	1	1	1

[a]ND = non-dialyzed beef broth protein; Dz = dialyzed; Ds = dialysate

[b]R and F = fractions from ND protein after ultrafiltration using a UM 10 membrane. R = retentate and F = filtrate.

[c]Numbers represent the titer of the remaining SEB activity.

Heat Inactivation of SEB in the Presence of Proteins from Different Sources

Preliminary heat inactivation tests were performed using several different sources of protein. Beef broth protein was used as a basis of comparison for the other proteins: soytone, Edi-Pro-N (a soy isolate), yeast extract, a commercial meat extract, and chuck roast bouillon protein (C.R.B.). The tests revealed that the chuck roast bouillon protein provided the greatest protection to SEB (Table 4).

Fractionation of the chuck roast bouillon protein by ultrafiltration provided two fractions, the Ds and Dz fractions. Tests to determine their protective effect on SEB during thermal inactivation revealed that the Ds and Dz fractions when heated alone with SEB resulted in a significant loss of protection as compared to ND chuck roast protein; however, the protective effect returned when the two fractions were recombined (Table 5).

Characterization of Chuck Roast Bouillon Protein

Gel Filtration

Gel filtration on Bio-Gel P-4 (MW 4,000) resulted in poor resolution of the chuck roast bouillon protein (Fig. 9). In comparing the Dz and Ds fractions, gel filtration demonstrated that the Ds fraction contained more lower molecular weight component than the Dz fraction, but there

Table 4. Heat inactivation of staphylococcal enterotoxin B at 110 C in the presence of various proteins.

Time at 110 C min	Veronal buffer	Yeast Extract	Soytone	Edi-Pro-N
15	4[a]	8^-	4	4
30	2^+	4	4^-	2^-
45	2	1	2	1
60	0	1	1	1^-

Time at 110 C	beef broth protein	Meat Extract	C. R. B. protein	C. R. B. protein +Difco beef extr.
15	8	8	32^-	16^+
30	4	4	8	8
45	2	4^-	4	4
60	1	2	4^-	4^-

[a]Numbers represent the titer of the remaining SEB activity.

Table 5. Heat inactivation of staphylococcal enterotoxin B at 110 C in the presence of chuck roast bouillon protein fractions[a].

Time at 110 C min	ND	Dz	Ds	Ds + Dz
15	16[b]	4^{+}	4^{+}	16^{-}
30	8	4	4	8
45	4^{+}	2^{+}	2	4^{+}
60	4	1	2^{-}	4

[a]ND = non-dialyzed C. R. B. protein; Dz = dialyzed C. R. B. protein; Ds = dialysate

[b]Numbers represent the titer of the remaining SEB activity.

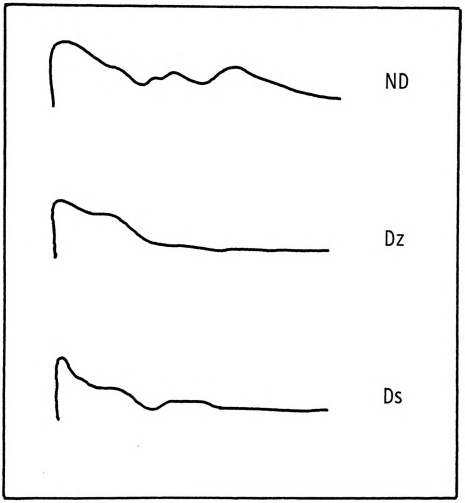


Figure 9. The gel filtration chromatographs of chuck roast bouillon protein fractions which were applied to a Bio-Gel P-4 column.

were also some very similar larger molecular weight components in both fractions.

Ion Exchange

Ion exchange on Cellex D revealed that the Dz fraction adsorbed more strongly than the Ds fraction (Fig. 10). Two fractions of the non-dialyzed chuck roast bouillon protein were obtained by Cellex D ion exchange chromatography; fraction #1, which did not adsorb to the resin, and fraction #2, which was eluted with 1 M NaCl. Fraction #1 was approximately 85% of the total ND sample; fraction #2 was approximately 15%. These two fractions were subsequently tested for their protective effect; tests revealed Cellex D chromatography resulted in complete loss of protection even when the fractions were recombined (Table 6).

Electrophoresis

The chuck roast bouillon protein and fractions, i.e. ND and Dz and Ds fractions were characterized by disc gel electrophoresis using 7.5% and 15% gels. Distinct bands were visible only in the 15% gels; the ND and Dz fractions had similar patterns and all gels contained a background smear. Using 5-25% gradient gels, the patterns were similar to that obtained in 15% gels but the bands were slightly more distinct; however, there was still a background smear. (Fig. 11).

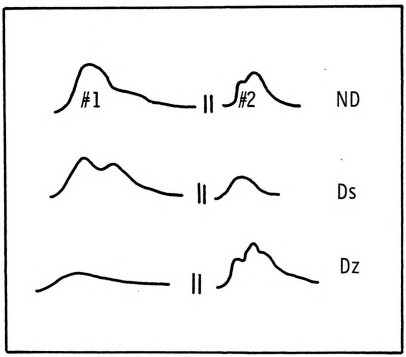


Figure 10. The ion exchange chromatographs of chuck roast bouillon protein fractions which were applied to a Cellex D column. Fraction #1 did not adsorb to the resin; fraction #2 adsorbed and was eluted off using 1M NaCl.

Table 6. Heat inactivation of staphylococcal enterotoxin B at 110 C in the presence of chuck roast bouillon protein[a] and protein fractions[b] after chromatography on Cellex D.

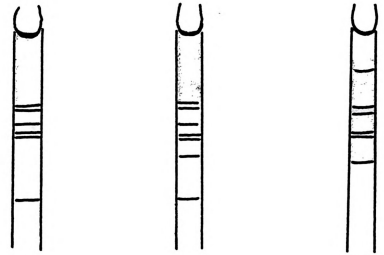
Time at 110 C min	ND	#1 + Dz	#2 + Dz	#1+ #2+ Dz
15	16[c]	8	0	1
30	8	4	0	0
45	8⁻	0	0	0
60	4	0	0	0

[a] ND = non-dialyzed C. R. B. protein.

[b] #1 and #2 = fractions from ND after Cellex D chromatography (see text for details).

[c] Numbers represent the titer of the remaining SEB activity.

15% GELS



5 - 25% GRADIENT GELS

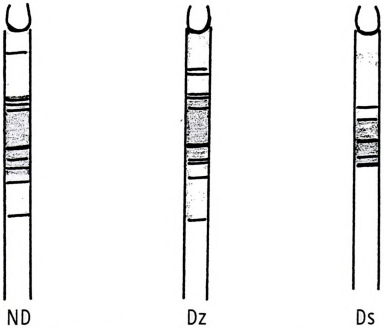


Figure 11. A schematic representation of stained bands obtained after electrophoresis of chuck roast bouillon protein fractions on 15% disc gels and 5-25% gradient disc gels.

Effect of pH, Ionic Strength, and Denaturants

pH

The chuck roast bouillon protein was tested for its protective effect on SEB during thermal inactivation at pH 4.5, 7.4, and 9.0. Data suggested that protection by this protein was provided over a broad pH range. The best protection appeared to be around the neutral pH of 7.4. At pH values of 4.5 and 9.0 there was still some protective effect although the rate of inactivation appeared to be faster whether or not the chuck roast bouillon protein was present in the system (Table 7).

Ionic Strength

Thermal inactivation of SEB in the presence of the chuck roast bouillon protein was also studied at various ionic strengths. When the ND fraction was rinsed several times with water before addition to the system, the protective effect of this rinsed ND fraction decreased, particularly with extended heat treatments; however, if the ionic strength of the system containing the rinsed fraction was increased by the addition of 1 M NaCl, the protective effect returned to the original level. No additional protective effect was evident when the ionic strength of the nontreated ND fraction was increased by the addition of 1 M NaCl (Table 8).

When the chuck roast bouillon protein was rinsed several times with water or with a NaCl solution, different

Table 7. Heat inactivation of staphylococcal enterotoxin B at 110 C at various pH values in the presence of chuck roast bouillon protein.

Time at 110 C, min	pH 4.5	pH 7.4	pH 9.0
10	[a] $8/2^-$	$16/4^-$	$8^+/4$
20	$4/2^-$	16/2	8/2
30	$4/1^-$	$8^+/2^-$	$4^-/1$
40	$4/1^-$	$8^+/2^-$	2/0

[a] Values represent titer of SEB activity remaining with protector [b] present / titer of SEB activity remaining without protector.

[b] protector = non-dialyzed chuck roast bouillon protein.

Table 8. Heat inactivation of staphylococcal enterotoxin B at 110 C in the presence of chuck roast bouillon protein with and without 1M NaCl.

Time at 110 C min	ND[a]	ND+ 1M NaCl	ND rinsed[b]	ND rinsed+ 1M NaCl
15	16[c]	16	8	16
30	8	8	4^+	8
45	4^+	4^+	4^-	4^+
60	4^-	4^-	2	4^-

[a]ND = non- dialyzed chuck roast bouillon protein

[b]ND rinsed = non-dialyzed chuck roast bouillon protein which had been rinsed several times with water.

[c]Numbers represent the titer of the remaining SEB activity.

chromatographic profiles were obtained (Fig. 12). When the C.R.B. protein rinsed with water was eluted from a Bio-Gel P-10 column with 2.2 mM veronal buffer, the chromatograph contained 3 peaks. When C.R.B. protein rinsed with a 1 M NaCl solution was eluted with 2.2 mM veronal buffer + 1 M NaCl, the chromatograph contained only 1 major peak.

Denaturants

The effects of 4 M guanidine hydrochloride (GuHCl), 6 M urea, and 1% sodium dodecyl sulfate (SDS), and 1% sodium dodecyl sulfate + 1% mercaptoethanol (ME) on the thermal inactivation of SEB in the presence of chuck roast bouillon protein were studied (Table 9). Data on control systems in which SDS were present with SEB showed that SDS itself may have afforded some protective effect to SEB; when ME was added, this protective effect was slightly less. When the chuck roast bouillon protein was added to the SDS or SDS + ME plus SEB system there was no apparent additional protective effect.

In the urea plus SEB control system, urea completely inactivated SEB. The addition of chuck roast bouillon protein had no apparent protective effect.

The system containing GuHCl and SEB appeared to have some protective effect but this diminished as the time of heat treatment increased. When the chuck roast bouillon protein was added in the presence of GuHCl, there was a substantial protective effect.

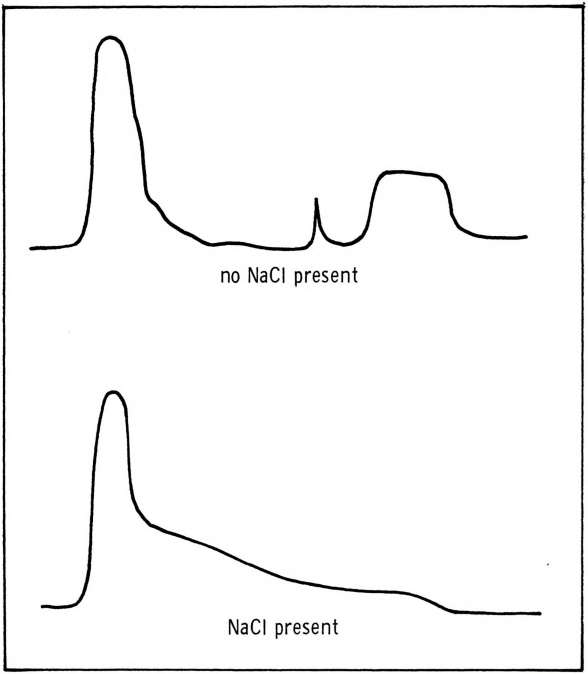


Figure 12. Chromatographs of chuck roast bouillon protein applied to a Bio-Gel P-10 column and eluted with 2.2 mM veronal buffer or 2.2 mM veronal buffer containing 1M NaCl. Samples were rinsed with water or 1M NaCl solution prior to application to the column.

Table 9. Heat inactivation of staphylococcal enterotoxin B at 110 C in the presence of denaturing agents.

Time at 110 C min	GuHCl	Urea	SDS	SDS + ME	SEB alone [b]
10	[a] 16/8	8/0	$8^{+}/8$	4/4	4
20	$8/4^{-}$	0/0	$8^{+}/8$	4/4	2^{+}
30	8/1	0/0	$8^{+}/4$	4/4	2
40	$4^{+}/1^{-}$	0/0	$8^{+}/4$	4/4	0

[a] Titer of system containing protector[c], denaturant, and SEB / titer of system containing denaturant and SEB.

[b] Titer for control system containing only SEB. Heat treatments were for 15, 30,45, and 60 min.

[c] protector = non-dialyzed chuck roast bouillon protein.

DISCUSSION

Preliminary studies involving the inactivation of staphyloccal enterotoxin B (SEB) in the presence of the non-dialyzed (ND), dialyzed (Dz), and dialysate (Ds) proteins from beef broth confirmed the results found by Lee et al. (1977) that proteins in beef broth retarded the inactivation of SEB heated at 110 C. A more detailed investigation of the nature of the protein(s) in beef broth which were involved in this protection was undertaken. Specifically, an attempt was made to isolate and characterize the component(s) active in the protection of SEB during thermal inactivation.

Characterization of Beef Broth Dialysate Protein

Since the inactivation of SEB was more rapid in the presence of dialyzed beef broth protein than in non-dialyzed beef broth protein at comparable concentrations, results indicated that a dialyzable factor was involved in the protection of SEB during heating (Lee et al., 1977). The beef broth dialysate protein was characterized by ion exchange chromatography, gel filtration, electrophoresis, and ultrafiltration. When the dialysate protein was separated into fractions I and II by ion exchange chromatography

on Cellex D, the ability of either fraction to protect SEB during thermal inactivation was apparently lost. However, when fraction I was separated on a cation exchange column, both fraction IA and IB were capable of affording only limited protection to SEB after being heated for 30 min at 110 C. Results from anion and cation exchange chromatography suggested that loss of the protective effect may be due to a detrimental effect of some aspect of the separation technique on the protein. Another explanation could be that more than a single component in the dialysate protein was necessary for maximum protection of SEB during thermal inactivation.

An indication of the number and size of the components in the beef broth dialysate protein was determined by gel filtration and electrophoresis. Both techniques resulted in poor separation of the dialysate fraction. This suggested that the components were very close in size and had similar charge. Electrophoresis using a more concentrated gel, i.e. 15% instead of 7.5% or 12%, may have increased resolution of these components.

Ultrafiltration of the dialysate protein provided a retentate and a filtrate fraction. Whether the fractions were combined separately with SEB or together with SEB, protection was equal to that of the non-dialyzed protein up to 45 min at 110 C. Interestingly, both fractions obtained by ultrafiltration had equal protective effects even though

the filtrate was only 12% of the total dialysate protein, while the retentate was 88%. This suggested that a low molecular weight component(s) of approximately 10,000 (for which the UM 10 membrane has borderline selectivity) may be involved in this protection or that more than one component was involved -- some low molecular weight component(s) passing through the membrane and some higher molecular weight component(s) being retained.

Thermal Inactivation of SEB in the Presence of Proteins from Different Sources

The beef broth contains protein of both animal and plant origin including beef broth, yeast extract, and hydrolyzed vegetable protein. In an attempt to determine the origin of the protective protein(s) in beef broth bouillon, meat extract, chuck roast bouillon protein, soytone, soy isolate (Edi-Pro-N), and yeast extract were used.

Preliminary heat inactivation tests with SEB in the presence of the various proteins revealed that chuck roast bouillon protein provided the greatest protection. This suggested that the component(s) in beef broth which were active in protection of SEB during heating might be of animal origin. The chuck roast bouillon protein even showed a slightly higher protective effect than the beef broth bouillon protein or the meat extract protein; this may be due to the fact that the laboratory-prepared chuck

roast bouillon protein did not undergo the more extreme processing given the commercial products. Addition of beef extract to the chuck roast bouillon protein did not enhance its protective effect.

Other investigations have revealed protection of staphylococcal enterotoxin by beef bouillon during thermal inactivation treatments. Denny et al. (1971) showed that SEA was more stable when heated in beef bouillon than in a pH 7.2 phosphate buffer. SEA was also more heat stable in beef bouillon than in a casamino acid medium (Humber et al., 1975). Over 50% of the original toxin activity of SEB was retained in beef broth after heating for 5 min at 100 C (Reichert and Fung, 1976). In contrast to these findings however, thermal loss of SEB serological activity was rapid in the presence of pure meat proteins, myosin or met-myoglobin, and in a ground round slurry (Satterlee and Kraft, 1969).

I am unaware of any investigations on the effect of pure vegetable protein on thermal inactivation of SEB.

Characterization of Chuck Roast Bouillon Protein

The chuck roast bouillon (C.R.B.) protein was fractionated into a dialyzed portion and dialysate. Each fraction when tested alone with SEB resulted in a significant loss of protection compared to the non-dialyzed C.R.B. protein. When the two fractions were combined together with SEB, the protective effect returned. Thus, as

previously shown with beef bouillon protein, a dialyzable factor of the C.R.B. protein appeared to be responsible for the protective effect.

C.R.B. protein and its fraction were characterized by gel filtration, gel electrophoresis, and ion exchange. Poor resolution by gel filtration with Bio-Gel P-4 (MW 4,000) was the result of the broad range in molecular weight of the components in the C.R.B. protein; the large molecular weight components eluted with the void volume. In comparing the chromatographic profiles of the Dz and Ds fractions, the Ds fraction appeared to contain more lower molecular weight components, although both fractions appeared to contain some similar larger molecular weight components. During dialysis of the non-dialyzed C.R.B. protein, the smaller molecular weight components do in fact dialyze into the dialysate fraction; some of the similar larger molecular weight components common to both fractions may be the result of borderline selectivity of the pores in the dialysis tubing.

Ion exchange chromatography of the C.R.B. protein revealed that the charges on the components in the Dz fraction differed from those in the Ds fraction since the Dz fraction adsorbed more strongly than the Ds fraction. The ion exchange fractions of non-dialyzed protein, #1 and #2, had no protective effect whether they were tested individually or recombined with SEB. Since fractions #1 and #2

comprise the ND protein, they should theoretically have shown protection to SEB when they were recombined. Loss of protection may be the result of a harmful effect of the ion exchange technique on the protein.

The best resolution of the C.R.B. protein by electrophoresis was obtained on 5-25% gradient disc gels. The Dz and Ds fractions appeared to contain some similar components. All electrophoretic patterns suggested that there was a similarity in size and charge of several of the components in the C.R.B. protein.

Effect of pH

When controls which contained only SEB were heated at 110 C at pH 4.5, 7.4, and 9.0, inactivation of SEB was rapid. In a study by Warren et al. (1974a) SEB was denatured by low pH; addition of HCl to pH <3.5 at 23 C resulted in denaturation of SEB, a basic protein, presumably due to protonation of $-COO^-$ groups involved in maintaining structure, followed by mutual repulsion between neighboring cationic groups with resulting electrostatic stress. However, in this investigation the effect of heating at 110 C alone was sufficient to inactivate SEB at pH 4.5, 7.4, and 9.0.

Upon addition of the C.R.B. protein to the system at pH 4.5, 7.4, and 9.0, inactivation of SEB was significantly retarded. The C.R.B. protein appeared to provide the greatest protection at a neutral pH. At pH 4.5 and 9.0

inactivation of SEB occurred at a slightly higher rate. In general, proteins are more stable under neutral conditions and are more likely to undergo conformational changes under extremes of pH (Atassi, 1977). In a specific study with SEB, rapid denaturation occurred at a pH value less than 3.5 (Warren et al., 1974a). When ionic strength was maintained at 0.10 during heating at 100 C, SEB was more stable at pH 6.4 than at pH 4.5 or 7.5 (Jamlang et al., 1971).

Effect of Ionic Strength

Addition of 1 M NaCl to sample systems increased the ionic strength. Non-dialyzed C.R.B. protein which had been rinsed with water using a UM 2 (MW1,000) membrane resulted in a slight loss of protection to SEB when compared to protection by untreated, non-dialyzed C.R.B. protein. This suggested that ionic strength might have an important role in protection of SEB. Scott and Stewart (1950) found that anionic compounds were the most important protective substances for Clostridium botulinum toxin heated in vegetable liquors; dialysate from vegetable liquor was equally as protective as the original vegetable liquor (Scott, 1950). Scott (1950) also discovered that multivalent anions were more effective than univalent ones; furthermore, a number of ionic substances were most protective when present in concentrations as high as 1.0 M. The protection was hypothesized to result from some form of

combination of the ion with oppositely charged centers on the protein molecule.

Chromatographic profiles of the ND C.R.B. protein rinsed in water and ND rinsed in a 1 M NaCl solution differed (Fig. 12). Elution of ND which had been rinsed with water resulted in 3 peaks, while elution of ND which had been rinsed and eluted with 1 M NaCl resulted in only 1 peak. This again suggested that ionic strength was important for protection of SEB, and addition of NaCl may have caused the proteins to associate resulting in more effective protection of SEB. Loss of the protective effect in fractions obtained by chromatographic techniques may be in part a dilution effect resulting in a decrease in ionic strength. This effect was reversible and could be regained by increasing the ionic strength.

Jamlang et al. (1971) reported that there was a gradual increase in amount of remaining SEB activity as ionic strength increased from 0.10 to 1.0 in a pH 6.4 buffer heated at 70 C. In this investigation higher SEB activity also remained after heating at 110 C in a higher ionic strength system. However, high ionic strength alone was not entirely responsible for the protection of SEB during thermal inactivation. Some protection was due to the presence of C.R.B. protein since the rinsed ND protein protected SEB to some extent.

Effect of Denaturants

The effect of denaturants on C.R.B. protein and SEB during thermal inactivation of SEB was studied in an attempt to gain insight into the chemistry of the C.R.B. protein/SEB interaction. When sodium dodecyl sulfate (SDS) was heated with SEB, SDS itself afforded some protection to SEB. Anionic detergents such as SDS were previously reported to stabilize proteins against thermal aggregation (Kinsella, 1976). Thermal inactivation of SEB in the presence of SDS + mercaptoethanol (ME), resulted in slightly less protection than when SDS alone was present. This may be explained by the fact that ME, a reducing agent, disrupted the S-S bond of SEB; hence, since the S-S bond was not intact, native refolding of the denatured toxin was not possible (Warren et al., 1974b). When ND C.R.B. protein was added to the SDS or SDS + ME plus SEB system there was no apparent additional protective effect.

In the urea + SEB system, urea completely inactivated SEB. Urea alone at high concentrations, 6-8 M, can denature protein at room temperature; this occurs when the $CO{\cdot}NH^{-}$ group in urea forms H-bonds with peptide linkages, thus competing with intrachain H-bonds which maintain native structure (Haurowitz, 1963). In addition to urea, the effect of heat probably aided inactivation of SEB. When C.R.B. protein was added, no significant protective effect was revealed.

In the presence of guanidine hydrochloride (GuHCl), some SEB activity remained after heat treatment at 110 C for 10 min; however, SEB activity decreased rapidly as the time of treatment increased. Results from the control system indicated that the denaturant itself, GuHCl, provided limited protection to SEB since SEB activity was even higher than SEB activity remaining in the system which was heated at 110 C with only SEB present. When C.R.B. protein was added to the system containing SEB and GuHCl, SEB activity remaining after heat treatment at 110 C was higher than activity remaining in the control. This suggested that the C.R.B. protein provided an apparent protective effect to SEB.

The study with the denaturants, SDS, SDS + ME, and urea did not reveal useful information about the protective factor(s) since the activity of SEB in control systems was already significantly retarded by the presence of the denaturant alone. In the study with GuHCl, the C.R.B. protein did have a protective effect towards SEB during thermal inactivation at 110 C; this revealed that the C.R.B. protein had a higher affinity than the denaturant molecule for SEB.

CONCLUSION

The isolation and characterization of the protective factor(s) from beef broth or chuck roast bouillon revealed little useful information. These were processed proteins. Due to this processing, there was probably breakdown of the protein into low molecular weight components and significant alteration of the protein; thus, characterization by various techniques was very difficult.

In addition, during the course of this research, several limitations of analysis were discovered:

a. The quantity of SEB was a limiting factor whether purchased commercially or prepared in the laboratory.

b. The determination of the activity of the protective factor(s) was time consuming and limited the number of determinations that could be made.

c. Dilution which was a common occurrence of separation techniques such as gel filtration or ion exchange may have had a harmful effect on results.

Results did suggest that several components of similar size and charge may be involved in protection of SEB during thermal inactivation. Furthermore, the protective factor(s)

was effective over a broad pH range. Finally, ionic strength enhanced protection to SEB during thermal inactivation, but was not totally responsible for the protective effect.

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