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Title: GROWTH AND ENTEROTOXIN PRODUCTION OF STAPHYLOCOCCUS AUREUS IN PAPAIN-TREATED BEEF AND HAM

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Margy Woodburn, Ph. D.

Growth and enterotoxin production of Staphylococcus aureus in papain-treated beef and ham were studied. In addition to an untreated control, four treatments were used in the beef experiment including two levels of papain, a commercial tenderizer preparation, and commercially tenderized beef. Two levels of papain treatment and an untreated control were used for the ham study. Both raw and cooked samples were tested. Proteolysis was determined by 280 nm readings in a spectrophotometer for total UV absorbing materials and for UV absorbing materials in a trichloroacetic acid soluble meat solution. The third method, a more sensitive analysis, used trinitrobenzene sulfonic acid to determine the amount of free amino nitrogen present. With the latter method some differences in the amount of amino groups present could be detected among the various treatments.

Strain S-6 which produces both enterotoxins A and B was used for the experiment. The inoculum level for studies of meat slices held at 30 and 42 C was 1×10^7 colony forming units per gram. Sampling times for the number of colony forming units and for enterotoxin production by microslide and Oudin assays were 5, 8, and 24 hr.

The action of papain did not significantly affect the number of colony forming units nor the amount of enterotoxin produced in treated versus untreated control meat samples. The number of colony forming units at 30 C increased at a slower initial rate than at 42 C although higher numbers of viable cells were detected after 24 hr in the samples incubated at 30 C.

Cooked samples supported a faster initial growth than raw samples. Earlier and greater enterotoxin B production occurred when cooked samples, especially of beef, were the substrates. Enterotoxin B concentrations in cooked beef held at 42 C were estimated to be 0.06, 0.9 - 1.0, and 0.2 - 2 μg per g for 5, 8, and 24 hr respectively. Raw beef samples contained no detectable enterotoxin until after 24 hr (0.2 - 0.9 μg per g). At 30 C approximately 1 μg per g of enterotoxin B was detected in the cooked samples and only 0.02 μg per g in some raw samples after 24 hr. The cooked and "not fully cooked" hams were similar in support of growth and enterotoxin production. Enterotoxin B concentrations present in "not fully cooked" samples held at

42 C were approximately 0 - 0.9 μg per g at 5 hr, 0 - 1 μg per g at 8 hr, and from 0.05 - 1.8 μg per g at 24 hr. Enterotoxin levels in the cooked samples were 0.2 - 0.9 μg, 0.25 - 1 ug, and 1 - 6.2 μg per g after 5, 8, and 24 hr respectively.

Isolated samples, positive for enterotoxin A (0.05 μg per g), were detected in cooked beef held at 42 C for 24 hr. Detectable amounts (0.05 - 0.2 μg per g) were found, however, in both the cooked and "not fully cooked" cured hams, especially at the higher incubation temperature.

Growth and Enterotoxin Production of *Staphylococcus aureus* in Papain-Treated Beef and Ham

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INTRODUCTION

Staphylococcal enterotoxin is responsible for one of the most common types of food poisoning in the United States. Meat and poultry served as the food source in over 50% of the food-borne outbreaks reported as due to *Staphylococcus aureus* in 1970 by the Center for Disease Control (1971). The 102 reported outbreaks involved 4, 699 people. Forty-five of these outbreaks were traced to mishandling in food service establishments, thirteen in the home, five in food processing plants, and thirty-nine were classified as "unknown." However, because the illness is short-term, many cases go unreported each year.

High protein foods serve as an ideal substrate for *S. aureus* growth. It appears that the production of enterotoxin in food is a function of growth. Meat is a rich protein source, contains minerals and vitamins, has a high moisture content and some fermentable carbohydrate, and is at a favorable pH for growth of the bacteria.

Tenderness is the most important palatability factor in consumer acceptance studies of beef (Bratzler, 1971). Current practices of enzymatic tenderization as a means of producing a more tender piece

of meat result in the breakdown of protein to peptones and peptides. Enzyme tenderizer preparations, used commercially and in the home, usually contain the plant enzyme papain. Papain is often combined with sodium chloride in the tenderizer preparation. With heating of the meat, the enzyme becomes active and proteolysis begins.

Recent research reports indicate that greater microbial growth can be observed in the presence of peptides as opposed to the presence of proteins or free amino acids (Lechowich, 1971). Wu (1968) found this to be true for S. aureus. Certain peptides were reported to stimulate both growth and enterotoxin B production.

In general, staphylococci are inhibited by other microorganisms. Natural flora in raw meat have an inhibitory effect upon the growth of S. aureus (Dack and Lippitz, 1962). Once the meat is cooked, however, it is highly susceptible to contamination and then becomes an even more favorable substrate than laboratory media for the growth of staphylococci (Genigeorgis, Savoukidis, and Martin, 1971).

Researchers, studying the various conditions affecting S. aureus, have shown that the optimum growth temperature for staphylococci is 35 to 37 C and that 4 to 8 hr of growth in a rich food source at this temperature is required for the production of enterotoxin (Dack, 1956). S. aureus will grow, however, over a much wider temperature range. Enterotoxin may also be formed at other temperatures but at a slower rate or decreased final concentration. The temperature range has

implications in food systems. A temperature above 60 C (140 F) or below 7 C (45 F) is recommended for the control of S. aureus (U. S. Public Health Service, 1962). If a contaminated food is held within this range, growth and enterotoxin production may occur. Foods involved in food-borne illnesses have often been inadequately chilled or left at room temperature for a time. Hodge (1960) reported that 94% of the staphylococcal food poisoning outbreaks during an intensive year's study involved leftover foods.

The growth of S. aureus does not alter the color, odor, or flavor of foodstuffs which makes sensory detection of the possible presence of enterotoxin impossible. Current laboratory detection techniques are limited in their sensitivity and cannot always detect enterotoxin in foods which have been known to cause food poisoning.

The objective of this study is to determine the growth and enterotoxin production of S. aureus in papain-treated beef and ham held at two temperatures.

REVIEW OF LITERATURE

For staphylococcal intoxication to occur, Bryan (1970) has outlined five conditions. There must be a source of an enterotoxin-producing strain of Staphylococcus aureus; the organism must be transferred to a food; the food must be capable of supporting growth; a temperature range suitable for growth and toxin production must be maintained for a length of time; and, finally, there must be consumption of a sufficient amount of enterotoxin.

Source

S. aureus is an ubiquitous organism in man's environment; man is the chief carrier. Staphylococci can be found in the nasal passages, on the skin, and surrounding open cuts, sores and infected areas. Over 50% of the strains isolated from humans are capable of producing enterotoxins which are the specific causative agent of the food poisoning. Recent reviews by Bergdoll (1970), Baird-Parker (1971b), and Minor and Marth (1972) describe five antigenically distinct enterotoxins which have been identified and labeled as A, B, C, D, and E. Enterotoxin B is produced in larger amounts (200-500 μg per ml) than enterotoxin A (5-6 μg per ml) or enterotoxin C (60 μg per ml) in test systems.

Contamination

Contamination of a food occurs when the food is handled by a carrier of pathogenic S. aureus and the organisms are subsequently transferred to the food. Equipment which has become contaminated and poor sanitation practices may also contribute to the dissemination.

Meat as a Substrate

Meats are ideal substrates for the growth of staphylococci as evidenced by the high number of food-borne illnesses traced to them as reviewed in the introduction. Growth and enterotoxin studies in meat will be discussed later because of the time-temperature relationship.

The pH of meat is 5.3 to 6.5 (Lechowich, 1971). S. aureus will grow over a pH range of 5 to 8. In general, strain S-6 produces a slight decrease in pH in the media during the lag phase followed by a steady increase to pH 8 or more during the logarithmic phase (Marland, 1966). In a study with cooked hams (beginning pH 6.2) inoculated with strain 196, the pH was unchanged on those samples incubated at 4 C and little growth occurred. After 5 hr at 30 C the pH dropped and finally reached a pH of 5.4 after 50 hr (McDivitt and Husseman, 1957). Morse, Mah, and Dobrogosz (1969) reported an increase in enterotoxin B production

above pH 6.4 but a repression at pH 5.0. Reiser and Weiss (1969) report that the production of enterotoxin A is more tolerant of pH than enterotoxins B or C. Enterotoxin A is produced at a pH of 5.3. Higher yields of enterotoxin B were obtained at a pH of 6.8 than at pH 6.0 or 5.3. Although Genigeorgis, Foda, Mantis, and Sadler (1971) reported growth of staphylococci at pH 4.0, the optimum for growth occurred at pH 5.0 to 6.5. They reported the optimum pH for enterotoxin C production was 5.5 to 6.5.

Fresh meats have a moisture content of 65 to 75% (Rice, 1971). Cooked beef contains 50 to 60% water; smoked cured hams will have a 40 to 50% moisture content. Water, as well as nutrients, is necessary for biological activity of S. aureus.

The protein content of meat is 15 to 20% which will supply the required amino acids for bacterial growth. Although S. aureus has some proteolytic ability, it more readily utilizes peptides and peptones (Lechowich, 1971). Wu (1968) found that certain prolyl peptides stimulated growth and enterotoxin B production. More enterotoxin was obtained from digests of protein than from synthetic media. The stimulation of enterotoxin B production from pancreatic digests has also been reported by Drye and Mah (1969) and Markus (1969). Kihara and Snell (1960) suggested that peptides are more readily utilized than free amino acids and that the higher efficiency of peptide utilization was indirectly due to the transport system. Work reported by

Schroder and Busta (1972) suggests that proteolytic action due to the growth of Bacillus subtilis in sodium caseinate or isolated soy protein was stimulative to the growth of Clostridium perfringens. Enzymatic digestion of these food proteins by trypsin and pepsin also produced a stimulative effect on growth of C. perfringens.

No research has been reported in the literature on the growth of S. aureus in meats which have undergone proteolysis due to enzyme treatment. Enzyme tenderizer preparations are used commercially and in the home to produce a more tender piece of meat. Tenderization involves the hydrolysis of muscle fiber proteins and connective tissue. The protein in muscle fiber is chiefly myosin and actin with the proteins, collagen and elastin, composing the intercellular substance. Collagen is present in larger amounts and can be partly disintegrated in a moist matrix by heat; elastin is not greatly affected by boiling water. Three types of enzymatic tenderizers have been studied in relation to muscle protein: plant, pancreatic, and fungal. Plant enzymes, which include papain, bromelain, and ficin, are currently found to be the most effective. In 1958 Wang et al. used microscopic analyses to demonstrate that enzymes of different origins preferentially attack different muscle components. Thus preparations may be composed of a combination of enzymes for greater effectiveness.

Papain and bromelain hydrolyze peptide amides and esters especially at bonds involving the basic amino acids, leucine or glycine

(deBecze, 1970). Miyada and Tappel (1956) reported that during tenderization there is a conversion of soluble protein nitrogen to non-protein nitrogen due to the hydrolysis of actomyosin. They compared the hydrolysis of beef proteins by various proteolytic enzymes using rehydrated freeze-dried, ground beef. Papain and ficin actively digested the elastin. Bromelain, ficin, trypsin, Rhozyme P-11, and papain digested collagen. Kang and Rice (1970) have also compared the relative effectiveness of some of the tenderizing enzymes on various meat fractions. The degree of hydrolysis was measured by analysis of terminal amino groups with trinitrobenzene sulfonic acid according to the Habeeb method (1960). Papain had the greatest activity on both the salt soluble and water soluble meat fractions when compared with bromelain, ficin, and trypsin.

Papain is obtained from the Carica papaya plant by tapping the mature unripened fruits (deBecze, 1970). One-third of the papain imported into the United States is used in the home. Five percent of the commercial beef is now tenderized with enzymes by meat packers (Underkofler, 1968). Papain is the most commonly used enzyme in tenderizer preparations because of its stability and also its proteolytic ability. It withstands a higher temperature than most without being inactivated (deBecze, 1970). In early papain research, Gottschall and Kies (1942) found 55-75 C to be the most favorable temperature for the activity of the enzyme. Tappel et al. (1956) suggested that the

heat labile muscle proteins are first denatured and then hydrolyzed by papain for the maximum effect. Papain is inactive at room temperature. Tappel and his coworkers reported that they found no difference in holding papain-treated meat before cooking and in cooking the treated meat immediately after enzyme application. In an experiment with unground meat at 55 C, Gottschall and Kies (1942) reported that the most rapid proteolysis occurred during the first 4 hr. By 24 hr disintegration had taken place; however, none of the papain was destroyed.

Arshad et al. (1964) have also reported that a greater rate of increase in soluble protein occurs in the initial cooking of papain-treated beef. Tenderizing power is associated with the increase in the percentage of soluble protein. This group of researchers found that a temperature of 50-70 C produced a greater percentage of soluble protein than did temperatures of 30-50 C or 70-90 C. In Gottschall and Kies' (1942) research, the partial cooking of meat before the addition of enzyme did not increase the rate of proteolysis at any temperature. The rate of proteolysis was increased, however, when the beef was ground.

Above 85 C, the enzyme is denatured and inactivated (Gottschall and Kies, 1942). In his review, deBecze (1970) suggested that by heating to 68 C the enzyme is destroyed. Enzyme tenderizers must be inactivated during cooking in order to prevent the meat from

becoming mushy or butyrous in texture. This is especially important if the meat is to be held and reheated.

Enzyme preparations may be incorporated into meats in one of several ways:

a. Applying a powder preparation to the surface and forking,

b. Dipping cuts into a dilute enzyme solution (frequently used by restaurants and frozen cut packers),

c. Injecting a few ppm (5-30) into the animal before slaughter (ante-mortem),

d. Injecting enzyme solution into the animal before rigor mortis sets in (post-mortem),

e. Pumping (by hose or needle) the solution into wholesale cuts, and

f. Adding enzyme to rehydrating liquid for freeze-dried meats.

Surface application or dipping results in a migration of only 2 to 5 mm from the surface. Forking or some other means of increasing distribution is necessary for greater effectiveness (Mier et al., 1962). Ante-mortem injections result in a uniform distribution of the enzyme under properly controlled conditions (Huffman et al., 1967; Underkofler, 1968).

Beef is the most common type of meat currently receiving enzyme treatment; however, experimental research has also involved

poultry, rabbit, mutton, and ham. Dry cured (country style) hams tend to be less tender than processed pumped hams. Smallings et al. (1971) tested the effect of ante-mortem injection of papain on the tenderness and quality of dry-cured hams. The treated muscles were more tender than the controls. In addition, their research findings support the fact that there is more variation among hams than within. Each muscle acts independently in its response to tenderizing effects of papain. Rogers, Kemp, and Varney (1965) used an electric pump to inject enzymes into the femoral artery through a hose and needle and obtained similar results with dry cured hams.

Conditions for Growth and Enterotoxin Production

Recognizing the widespread existence of S. aureus, environmental conditions become an important basis for control. Once cooked, protein foods become extremely susceptible to contamination.

S. aureus is a facultative anaerobe; however, there is more rapid and greater growth under aerobic conditions. The organism will grow anaerobically if fermentable carbohydrate is present (Bryan, 1968). Baird-Parker (1971b) reported that excess aeration or oxygen levels decrease the amount of enterotoxin produced without affecting the growth rate. No growth occurs in the absence of carbon dioxide (Angelotti, 1969).

Time and temperature studies have been reported by various researchers. Optimum growth temperature for staphylococci is 35 to 37 C (Angelotti, 1969). Angelotti, Foter, and Lewis (1961a) described the growth range as between 6.5 and 45 C (44-114 F) in ham salad and chicken à la king. Minor and Marth (1972) report that a number of 5×10^7 organisms per ml must be reached before enterotoxin is detected. Enterotoxin production is a function of growth and requires 4 to 8 hr growth at 37 C in a rich food source (Dack, 1956; Reiser and Weiss, 1969). Enterotoxin production has been demonstrated, however, in brain heart infusion broth at temperatures of 45 C (Tatini et al., 1971), and, as reported by Segalove and Dack (1941), at as low a temperature as 18 C after 3 days incubation in veal infusion broth. Using protein hydrolysate as a growth medium, Marland (1966) reported growth and enterotoxin production between 15.2 and 43.2 C. MacLean, Lilly, and Alford (1968) found that although there was equivalent growth of S. aureus in cured meats, there was 15-30 times greater enterotoxin B production at 37 C than at 16 and 20 C.

Staphylococci can withstand both drying and freezing; however, the organism is destroyed by cooking if heated to 65 C (150 F) for 12 min (Angelotti, Foter, and Lewis, 1961b). Enterotoxin B can withstand boiling temperatures for 60 min (Lechowich, 1971). Satterlee and Kraft (1969) found that with enterotoxin B, in the presence of

isolated meat proteins, thermal inactivation occurred at 60, 80, and 100 C but was at a faster rate at 80 C than 60 or 100 C.

Most strains grow well in 10% sodium chloride and some will survive concentrations of 20% (MacLean et al., 1968; Genigeorgis, Savoukidis, and Martin, 1971). Sodium chloride has a greater effect on enterotoxin production than on growth in cured meats (MacLean et al., 1968). This is not true for sodium nitrate or sodium nitrite unless they are used in combination with sodium chloride. Using brain heart infusion broth, Genigeorgis, Martin, Franti, and Riemann (1971) found that the effect of sodium chloride varied with the pH level and the strain of S. aureus. There was a decreased rate of growth as the sodium chloride concentration increased from 0 to 20%. In another study, Genigeorgis and Prucha (1971) reported that the production of enterotoxin C was not inhibited by levels of pH and sodium chloride normally found in processed meats, but that vacuum packaging may be the limiting factor. Lechowich, Evan, and Niven (1956) studied the effect of curing ingredients and procedures on the survival and growth of staphylococci in and on cured meats. The organisms survived normal curing operations until the smoking operation (58 C internal temperature reached).

In general, S. aureus growth is inhibited by the presence of other microorganisms. Natural flora of raw meat will usually outnumber and have an inhibitory effect on staphylococci. Casman,

McCoy, and Brandly (1963) found there was little or no growth after 72 hr incubation at 30 C when staphylococci inoculum was mixed with raw ground beef. Growth and enterotoxin A production, however, was demonstrated when the surface of sterile raw and cooked beef and pork were inoculated. Cooking destroys most bacterial contaminants in food including staphylococci. Once the natural flora have been destroyed, the meat is susceptible to contamination and growth by S. aureus, and, thus, production of enterotoxin. According to McCoy and Faber (1966) there is greater inhibition of staphylococcal growth at 25 C than at 35 C by other microorganisms. Bacillus cereus, however, tended to stimulate growth and enterotoxin production in meat. Dack and Lippitz (1962) introduced S. aureus into slurries of frozen pot pies. After incubation for 18 hr at 35 C the natural flora (Lactobacillus) had an inhibitory effect upon the growth of the staphylococci. The two strains that Silverman and Cohen (1971) used in a study with rehydrated beef stew competed with the indigenous flora at 20, 30, and 40 C. Neither enterotoxin A nor B was produced.

Consumption

The consumption of a contaminated food, which was handled under conditions allowing growth and enterotoxin production, may result in illness. Casman and Bennett (1965) found 50-200 million organisms in foods implicated in poisoning incidences.

A current problem in food microbiology research is the detection of enterotoxin levels which can cause food-borne illness. Susceptibility of individuals varies (Dack, 1956). The presence of staphylococcal enterotoxin can be detected with human volunteers, with test animals, or serologically. Obvious problems are encountered with the first two. Few controlled human tests have been conducted. Raj and Bergdoll (1969) used three subjects and found that 20 - 25 μg of pure enterotoxin B were capable of producing symptoms in man. Rhesus monkeys are frequently used for testing purposes; however, they are less than one-half as sensitive as man to enterotoxin B (Schantz et al., 1965).

Angelotti (1969) estimated that 1 - 4 μg of enterotoxin B will cause symptoms of staphylococcal intoxication (nausea, vomiting, cramping, and diarrhea). In an average food portion of 100 g, this implies that a level of 0.01 to 0.04 μg per g may bring on the illness. Baird-Parker (1971a) estimated that if the sensitivity of man averages 0.1 μg per lb (0.2 μg per kg body weight), a 150 lb adult could be made ill with the ingestion of 15 μg or less of enterotoxin. Bergdoll reported that individuals who became ill after eating contaminated cheese had ingested as little as 1 μg enterotoxin A in 20 g of cheese (Bergdoll, 1970). Animal studies show that dosages of approximately 5 μg of enterotoxins A, B, or C can cause emesis in rhesus monkeys weighing 2-3 kg.

Emphasis has been placed on the development of practical and effective serological techniques. Quantitative detection today relies on precipitation with specific antibodies in gel. The Ouchterlony microslide method as described by Crowle (1958) and modified by Casman et al. (1969) is based on double gel diffusion and will detect quantities as low as 0.1 μg per ml. The Oudin single gel diffusion technique can be used to detect quantities of 1 - 2 μg per ml after 7 days at 25 C (Hall, Angelotti, and Lewis, 1963). Both methods are affected by the type of diluent and the concentrations of antiserum and reference enterotoxin.

Recovery of quantitative amounts of S. aureus enterotoxin from foods implicated in food poisoning outbreaks requires extensive extraction and concentration procedures. Detection of enterotoxin in food as outlined by Casman and Bennett (1965) involves three steps: 1) separation of enterotoxin from insoluble food constituents, 2) separation of enterotoxin from soluble extractives, and, 3) concentration of the eluates. The use of gel filtration caused a slightly higher recovery of enterotoxin but did not permit the degree of concentration that was possible with the use of carboxymethylcellulose. With cooked meats, 38% of enterotoxin A and 48% of B were recovered.

In the method of Hall, Angelotti, and Lewis (1965) extraction began with food slurries of 90 ml. After homogenization with an equal volume of saline, the samples were heated 25 min at 50 C and then

allowed to stand 30 min at room temperature before centrifugation. The samples were then passed through Amberlite adsorption columns and concentrated by dialysis. Recovery was 27% for enterotoxin A and 42% for enterotoxin B. Genigeorgis, Riemann, and Sadler (1969), following in part the procedure outlined by Hall, Angelotti, and Lewis, made use of a direct means of analyzing cured meat samples. Samples were homogenized with saline, heat treated, and centrifuged as above. The supernatant was then used for quantitative testing. Heating hastens the precipitation of insoluble phosphates to eliminate interference in detection techniques.

MATERIALS AND METHODS

Preparation of Meats

Beef

A 22.7 kg (50 lb) round of beef was obtained through a local market. It was handled as aseptically as possible and cut into 13 roasts, weighing 1.1 to 1.6 kg (2.5 to 3.5 lb). The roasts were packaged in plastic bags and frozen until needed.

The commercially tenderized meat (ProTen, Swift and Co., Stockton, Calif.) was available through a local distributor in two 4.5 kg (10 lb) chuck roasts. These were each aseptically cut into blocks of meat averaging 1.3 kg (3 lb) and frozen.

The beef roasts were thawed for 36 hr in a refrigerator at 4.5 C (40 F) prior to use. Four papain treatments and one untreated control sample were used in the beef experiment. Papain (Nutritional Biochemicals Corporation, Cleveland, Ohio) was tested at two levels: low level papain treatment at 2 mg per 454 g (1 lb) beef and a high level treatment at 1 g per 454 g. A commercial meat tenderizer preparation (Adolph's Meat Tenderizer, Burbank, Calif.) was used at a level of 2.1 g per 454 g which is equivalent to the one-half tsp per lb recommended on the label. This prepared tenderizer is composed of salt, sugar, tricalcium phosphate, papain, and vegetable oil.

To treat the roasts with the two levels of papain and the commercial tenderizer preparation, the enzyme was dissolved in 5 ml distilled water and applied to the meat surface with a pipette. Forking was accomplished with a 6 prong ice pick at 2 cm intervals (2.5 cm depth penetration). Five milliliters of distilled water were forked into the control roast. The roasts were allowed to stand for 30 min at room temperature.

The commercially tenderized meat received no laboratory treatment.

Ham

Three cured and smoked whole hams were obtained through the cooperation of Dr. Walter Kennick, Associate Professor, Oregon State University, Animal Science Department. In preparation, 2 mg papain per 454 g for the low level treatment of one ham and 1 g per 454 g for the high level treatment of a second ham were included in the curing solutions. The third ham served as the control. The ingredients composing the curing solution (15.9 kg salt : 3.6 kg sugar: 3.2 kg commercial cure compound) were dissolved in water to give a 65 to 70 saltmeter reading. Ten percent by weight was added in the pumping ratio. The hams were smoked for 18 hr to reach an internal temperature of 61 C (142 F). For reporting purposes these hams will be referred to as "not fully cooked."

The control and low level treated hams were cut into four blocks, each approximately 1.4 to 1.8 kg (3 to 4 lb) in weight and boneless. During the smoking process, the high level papain-treated ham underwent considerable hydrolysis and could not be cut into roasts. The hams were stored in a refrigerator at 4.5 C (40 F) until the day of the experiment.

Preparation of the Samples

Eight sterile samples (0.6 cm thick, total of 30 g) were cut from each roast with an electric knife. The blades were sterilized in 70% ethanol and flamed. Before the sterile samples were cut, about 2 cm were trimmed from one end and four sides of each uncooked roast. The slicing method was consistent so that each piece was a cross section of the roast. Each slice represented an inner as well as an outer portion of the roast. The slices were held in a sterile jar until they could be weighed.

Six samples of each treatment were needed for the microbiological study. The 30 g sterile samples were randomly selected, weighed in sterile pint jars, and refrigerated overnight.

Two 10 g samples from the remaining slices were weighed in sterile jars and refrigerated for the determination of proteolysis.

The remaining blocks of meat were cooked on racks in aluminum pans in a 149 C (300 F) oven to an internal temperature of 82 C (180 F).

This final temperature insured the inactivation of the papain. After standing at room temperature for about 1 hr, the ends and sides of each roast were again trimmed (2 cm) using aseptic techniques and representative cooked samples (0.6 cm thick, 30 g) were cut. Six 30 g samples were aseptically weighed into sterile pint jars and refrigerated. Two 10 g samples, representative of the block of meat, were refrigerated for proteolysis determinations.

Growth and Enterotoxin Studies

Inoculation

Staphylococcus aureus strain S-6, which produces both A and B enterotoxins, was obtained from Dr. Merlin Bergdoll (University of Wisconsin, Madison). The culture was maintained on porcelain beads (Hunt, Gourevitch, and Lein, 1958) and stored in the refrigerator. For the inoculum, beads were transferred to 50 ml of 4% NZ Amine Type NAK broth (Sheffield Chemical, Norwich, New York) supplemented with 0.00005% thiamine and 0.001% niacin (Reiser and Weiss, 1969) and incubated at 37 C for 24 hr.

On the day of inoculation, the jars with the meat samples were removed from the refrigerator and placed in a water bath for equilibration with the incubation temperature. One-half milliliter of inoculum (1×10^7 colony forming units per g) was pipetted onto the

surface of the meat and distributed as evenly as possible. Two flasks with 50 ml of 4% NAK broth, pH 6.8, were inoculated and used as laboratory medium controls.

Growth Studies

After inoculation, sterile cotton gauze covers replaced the metal lids. Samples were incubated in a water bath at 30 ± 1 C (86 F) and at 42 ± 1 C (108 F). There were three samples representing each treatment incubated at each temperature in addition to a control flask with NAK broth.

One jar of each treatment was randomly removed from both the 30 and 42 C incubators after 5, 8, and 24 hr. At each sampling time, the jars were immediately placed in an ice bath. Sampling order was randomized. In general, the methodology used was that of Genigeorgis, Riemann, and Sadler (1969). A slurry was prepared by adding 30 ml sterile 0.1% peptone (Difco, Detroit, Michigan) water to each 30 g meat sample and blending at low speed for 5 min in an electric blender (Osterizer, Milwaukee, Wisconsin). Blender blades were sterilized in 70% ethanol for a minimum of 10 min followed by rinses in 3 beakers of sterile distilled water. Fifteen milliliters of sterile phosphate buffered saline (0.06 M, pH 7.2) were added to the slurry and swirled to mix. After dilutions were made, the sample was plated with plate

count agar (Difco, Detroit, Michigan) by the pour plate method. Plates were incubated for 24 hr at 37 C. A Quebec Colony Counter was used to determine the number of colony forming units (CFU).

Enterotoxin Assay

Approximately 30 ml of the slurry were poured into 40 ml Pyrex centrifuge tubes and heat treated at 50 C (ham for 30 min and beef for 40 min). Lag time was 10 min. These were then allowed to stand at room temperature for 30 min followed by centrifugation at 1000-1200 x g for 30 min. The supernatant was decanted and refrigerated for enterotoxin determination (Genigeorgis, Riemann, and Sadler, 1969).

Crowle's microslide technique, as described by Casman et al. (1969) was used to detect the presence of enterotoxin. Unknown solutions were placed in the side wells and a 1 μg per ml of control enterotoxin in the top and bottom wells as the reference solution. Antiserum (1:60 dilution for B and 1:32 for A) was used in the center well. Antiserum for enterotoxin B was produced in the laboratory and antiserum for enterotoxin A was obtained from Dr. Bergdoll. The slides were incubated for 24 hr at 37 C or for 48 hr at room temperature. After removing the template, the slides were rinsed by immersing in distilled water 10 min and stained with 0.1% Thiazine Red R dye (Crowle, 1958; Casman et al., 1969).

Diluted samples as prepared above were first tested on the microslide. Negative samples were then concentrated four times with Aquacide (Calbiochem, Los Angeles, Calif.) for 24 to 48 hr at 4 C in the refrigerator, centrifuged 10 min, dialyzed against NAK broth, and reset on a microslide.

The Oudin method (Hall, Angelotti, and Lewis, 1963) was used to quantitate enterotoxin B for the positive microslide assay samples after 8 and 24 hr. Because preliminary studies showed enterotoxin concentrations after 5 hr insufficient for assay purposes, these were not tested. Only concentrated samples were used in the Oudin assay. Tubes with a final concentration of 1:30 of antiserum for enterotoxin B and the unknown solutions were incubated at 30 C in a water bath. Four readings were taken at 24 hr intervals with a cathetometer to determine the rates of migration. The slope of the readings was then compared to a standard curve for known amounts of enterotoxin dispersed in NAK broth.

Determination of Proteolysis

Three analyses were used to determine proteolysis: determination of UV absorbing material in the buffer solution of the meat sample, determination of UV absorbing material in trichloroacetic acid (TCA) soluble meat solution, and determination of free amino nitrogen with trinitrobenzene sulfonic acid (TNBS). A blank tube with buffer

and a reference tube with bovine serum albumen (BSA) (Armour Pharmaceutical Co., Kankakee, Illinois) were prepared each time. The readings were compared to their respective standard curves using BSA and expressed as equivalent mg BSA per 100 ml; in addition, values were expressed as equivalent mg glycine per 100 ml for the TNBS assay.

To each 10 g meat sample, 200 ml of 0.01 M sodium phosphate buffer (pH 6.0) were added and blended 5 min to produce the meat slurry. Duplicate samples were tested for each treatment.

To determine total UV absorbing material present, 5 ml phosphate buffer (0.01 M, pH 6.0) were added to 7 ml of the meat slurry and filtered through Whatman No. 42 filter paper. The filtrate was collected and the optical density was read at 280 nm on a Beckman Model DU Spectrophotometer (Oser, 1965). One milliliter of the filtrate was saved for the TNBS assay.

Five milliliters of 15% TCA (Matheson, Coleman, and Bell, Norwood, Ohio) were added to 7 ml of the meat slurry. This was allowed to stand 30 min before filtering through Watman No. 42 filter paper. The filtrate was collected. Optical density readings were recorded at 280 nm using the Beckman Model DU Spectrophotometer (Oser, 1965).

The free amino nitrogen assay is used to determine free amino groups of amino acids and peptides. The 2,4, 6 trinitrobenzene sulfonic

acid reacts with the free amino groups to give nitrophenyl derivatives. The intensity of the color is proportional to the quantity of $-NH_2$ reacted (Habeeb, 1965; Kang and Rice, 1970). Appropriate dilutions were made using the filtrate with buffer. Two milliliters of each test solution were pipetted into screw cap tubes. To each tube of sample, blank, and reference were added 2 ml borate buffer (0.1 M borate, 0.2 M NaCl, pH 9.0) followed by 2 ml 0.1% TNBS reagent (Eastman, Rochester, New York). The tubes were gently shaken and then incubated for 2 hr at 40 C. Two milliliters of 10% sodium dodecyl sulfonate (Fisher, Fair Lawn, New Jersey) were added to each tube to minimize precipitate formation. Two milliliters of 0.5 N HCl were added and the tubes were again shaken gently to mix. Absorption at 335 nm was read on the Beckman Model DU Spectrophotometer.

Experimental Design

Three replications were conducted for both the ham and beef studies. One ham replication was conducted each week for three weeks. The beef experiments were handled in an incomplete randomized block design. Each replication required two series of experiments. Treatments were selected randomly for each series within a replication. Sampling order of treatments was randomized for each sampling period.

A logarithmic transformation was made for the number of colony forming units of S. aureus. For analysis purposes a value was assigned to the levels of enterotoxin based on the enterotoxin concentrations detected:

Value	Sample	Microslide assay	Oudin assay (on conc. samples)	Conc. (μg/g) for minimum detection
1	4x concentrated	-	not tested	< 0.1
2	4x concentrated	±	-	0.1-0.2
3	4x concentrated	+	-	0.2-0.25
4	4x concentrated	+	minimal	0.9-1.0
12	1:1 diluted	+	-	0.2-0.9
16	1:1 diluted	+	minimal	0.9-1.0
18	1:1 diluted	+	quantitative	> 1.0

The statistical analysis consisted of a 3 x 5 x 2 x 2 x 3 factorial design for the analysis of variance of the number of colony forming units and the enterotoxin concentration in beef. The computer program *NANOVA was used for this analysis. A 3 x 3 x 2 x 3 factorial was used for the analysis of variance for the "not fully cooked" ham and a 3 x 2 x 2 x 3 factorial for the cooked ham. To test if the treatment effects were explained by the data obtained for the chemical analysis, the computer program *ANCOV2 was used. Correlations between each of the three analyses for proteolysis determination and CFU and enterotoxin were made for both beef and ham. The *SIPS program was selected for correlations between CFU and enterotoxin for each treatment at each temperature. Correlations were also calculated across treatments for specific time periods.

RESULTS AND DISCUSSION

The purpose of this study was to investigate the effect of proteolysis due to the enzyme papain in beef and ham held at two temperatures on the growth and enterotoxin production of S. aureus. Although the primary concern was with cooked meats, raw samples were also used for the study.

Substrates

Cooked beef and ham represent two common vehicles for staphylococcal food poisoning, and, thus, were chosen for the study. Both are susceptible to enzyme tenderization. Papain is the most commonly used meat tenderizer and, for this reason, was the enzyme choice. Several means of application were selected. Surface application with forking was used for three treatments on the beef roasts. This method would typify that used by the homemaker. Papain was used at two levels: the low level (2 mg per 454 g) represents normal recommended usage and the high level (1 g per 454 g) represents an excess amount of enzyme. The high level was chosen for comparison purposes since proteolysis is difficult to determine chemically with normal amounts of the enzyme present. In addition, a tenderizer preparation available commercially (Adolph's) was applied at a level of 2.1 g per 454 g (1/2 tsp per lb) as recommended on the label. A

commercially tenderized meat (ProTen) was also included in the study.

With the hams, the enzyme was included in the curing solution and incorporated into the muscle by pumping. These injections are usually performed by the meat packer. The experiment involved two levels of papain treatment; a low level was achieved with 2 mg per 454 g and a high level with 1 g per 454 g of meat.

Both the cure for the ham and the tenderizer preparation in the beef study contained salt in addition to the other ingredients. It is recognized that salt also has a tenderizing effect on meats and may affect the degree of proteolysis.

Based on preliminary studies, three methods for proteolysis determination were used in the analyses. A protein determination at 280 nm was conducted on the total UV absorbing material in the meat solution and on the UV absorbing material in TCA soluble meat solution. Measuring absorption at 280 nm is a rapid and convenient means of estimating protein content of a solution. Precipitation with TCA causes the formation of insoluble protein salts and a change in pH. This results in an alteration of the intramolecular shape. Proteins are removed as well as some intermediate-sized peptides (Oser, 1965). A more sensitive technique using 2, 4, 6 trinitrobenzene sulfonic acid was also used. In the Habeeb method (1966), the TNBS reacts with the free amino groups to give trinitrophenyl derivatives.

Readings were compared to standard curves using bovine serum albumen.

Table 1 shows results of analyses obtained in the beef study. Calculations are not on a dry weight basis; therefore, cooked and raw samples cannot be compared. As has been reported by other researchers and discussed earlier in the review of literature, variations were found within the muscle as well as between muscles. In general, differences in treatment were not apparent from the 280 nm readings. Except for the raw sample of the commercially tenderized meat, a greater degree of proteolysis was observed in the treated samples as compared to the control with the TNBS method.

As illustrated in Table 2, detection of differences was possible in the ham experiment. All the hams were smoked to an internal temperature of 61 C (142 F). This temperature allowed the enzyme to become active; therefore, some proteolysis occurred in both the cooked and "not fully cooked" hams. Proteolysis is obvious in the ham receiving the high level of papain treatment whose values greatly exceed those of the other samples. The ham with the low level of papain treatment gave similar absorbance readings to the untreated control.

By analysis of covariance, it was possible to relate the results from the proteolysis determinations to the treatment effects. In general, the treatment effect was explained by the data for each

Table 1. Soluble proteins and related compounds in control and papain-treated beef before and after cooking as determined by three procedures.

Treatment	UV Absorbing[abc] with TCA	UV Absorbing[abc] without TCA	TNBS[cde]
Raw			
Control	772	1880.	649.
Low Level Papain[f]	883	1950.	725.
High Level Papain[g]	899	1970.	852.
Tenderizer Preparation[h]	872.	1780.	726.
Commercially Tenderized Meat[i]	708.	2060.	537.
Cooked			
Control	857.	1250.	436.
Low Level Papain[f]	1020.	1350.	465.
High Level Papain[g]	963.	1380	482.
Tenderizer Preparation[h]	1010.	1270	517.
Commercially Tenderized Meat[i]	873.	1410	552.

[a]Expressed as equivalent mg bovine serum albumen per 100 ml.

[b]Absorption at 280 nm using Beckman Model DU Spectrophotometer.

[c]Each datum is an average of duplicate samples for three replications.

[d]Trinitrobenzene sulfonic acid (Habeeb, 1966).

[e]Expressed as equivalent mg glycine per 100 ml.

[f]2 mg papain per lb.

[g]1 g papain per lb.

[h]2.1 g per lb. (Adolph's Meat Tenderizer, Burbank, California).

[i]ProTen Beef (Swift and Co., Stockton, California).

Table 2. Soluble proteins and related compounds in control and papain-treated ham before and after cooking as determined by three procedures.

Treatment	UV Absorbing[abc] with TCA	UV Absorbing[abc] without TCA	TNBS[cde]
Not fully cooked			
Control	1050.	1660.	469
Low Level Papain[f]	1120.	1960.	598.
High Level Papain[g]	3440.	3770.	3220.
Cooked			
Control	1050.	1530.	435.
Low Level Papain[f]	1110.	1920.	597.

[a]Expressed as equivalent mg bovine serum albumen per 100 ml.

[b]Absorption at 280 nm using Beckman Model DU Spectrophotometer.

[c]Each datum is an average of duplicate samples for three replications.

[d]Trinitrobenzene sulfonic acid (Habeeb, 1966).

[e]Expressed as equivalent mg glycine per 100 ml.

[f]2 mg papain per lb.

[g]1 g papain per lb.

chemical analysis in the ham study. This indicates that the methods used were a measure for proteolysis due to the different treatments.

Growth and Enterotoxin Production

Organism

S. aureus strain S-6 was selected for this study because of its ability to produce large amounts of enterotoxin B as well as smaller amounts of enterotoxin A. Although enterotoxins A and C are the usual types associated with food poisoning outbreaks, strains producing enterotoxin B are often used for study purposes because of the higher yield. Often more than one type of enterotoxin can be identified in foods implicated in food poisoning cases (Baird-Parker, 1971b). Different strains of S. aureus show similar growth and enterotoxin production patterns (Reiser and Weiss, 1969). Scheusner and Harmon (1971) found little difference in the temperature range for the production of enterotoxins A, B, C, and D in brain heart infusion.

The inoculum level for this study was standarized at 1×10^7 colony forming units per gram. This level of inoculation approximates the optimum for growth and enterotoxin production (Reiser and Weiss, 1969). A high inoculum is advantageous for studies conducted at temperatures other than 37 C. The surface inoculation used would resemble an actual contamination. Although the inoculation level is

high, it could represent the amount found in a contaminating sneeze droplet or that amount transferred by handling food with contaminated hands. Because the organisms are on the surface of the meat there is adequate oxygen for growth.

Conditions

Cooked protein foods which have been contaminated by handling and allowed to remain at room temperature or refrigerated in large masses are frequently implicated in food poisoning cases (Bryan, 1970). A temperature of 30 C was selected for this research because it approximates the temperature of a warm kitchen where foods may be left to cool after preparation.

The U. S. Public Health Service (1962) recommends that a safe temperature for storing all potentially hazardous foods is below 7 C (45 F) or above 60 C (140 F). This also applies to foods held for serving or display purposes. A temperature of 42 C was chosen for this study because it falls in the "unsafe to hold" but warm temperature range. In addition, other research, as reviewed earlier, has indicated the production of enterotoxin in laboratory media at a temperature this high.

Enterotoxin has been detected by researchers after 5 hr of growth of the organism. It has been recommended that foods not be held beyond 4 hr at temperatures outside the safety zone. Preliminary

studies showed that while some growth had occurred after 3 and 4 hr at 42 C, no enterotoxin could be detected by the microslide assay. Based on literature reviews and this preliminary work, sampling was done after 5, 8, and 24 hr of growth. A growth period of 5 hr was selected as the first sampling time in order to detect early differences in enterotoxin production due to treatment. The early hours especially have implications for food systems. Maximum enterotoxin B production occurs at the beginning of the stationary phase of growth while the production of enterotoxin A begins during the logarithmic growth phase (Markus and Silverman, 1970). In preliminary studies it was observed that by 8 hr at 42 C, the organisms were in the stationary phase of growth. By 24 hr, total numbers at 42 C were decreasing; however, growth in 30 C samples was still increasing. Sampling at 24 hr was done to give a basis for comparisons with other literature reports.

Colony Forming Units and Enterotoxin Levels.

Total plate counts were made for a determination of growth. These counts represent the number of viable cells present at a specific sampling time and do not take into consideration those bacterial cells which have already died and may have produced enterotoxin before their death. A microscopic count could be used to determine total cell numbers. The term "colony forming units" (CFU) is a more

descriptive term for describing viable cell numbers than is the term "cell count." When viewed under the microscope, staphylococci appear as grape-like clusters of cells. The colonies observed after plating and incubation are the result of growth of clusters as well as of single cells.

In general, a faster initial growth was observed in cooked meat samples than in raw. S. aureus in those samples incubated at 30 C showed a slower growth rate than those at 42 C during the early hours; however, a higher number of CFU were reached by 24 hr in the meat samples held at 30 C.

Experimental data for S. aureus in raw beef are shown in Tables 3 and 4. Growth at 30 C was relatively slow during the early hours. By 8 hr there was a ten-fold increase in CFU. From preliminary observations it was known that the most rapid growth in beef occurred between 15 and 25 hr. Samples incubated at 42 C showed a rapid rate of growth by 5 hr (2×10^8 CFU per g) and then only a slight increase in viable numbers (7×10^8 CFU per g) by 24 hr.

Cooked beef samples, as shown in Tables 5 and 6, yielded approximately ten times more colony forming units at 5 and 8 hr than observed in the raw samples. A steady increase in CFU was recorded for 30 C samples. Numbers at 42 C, however, increased slightly until 8 hr with a decline in numbers by 24 hr.

Table 3. Colony forming units and enterotoxin B production after 5, 8, and 24 hr in control and papain-treated raw beef held at 30 C.

Treatment[a]	Rep.	Hours of Incubation: 5 — CFU[b] (log)	Enterotoxin: Microslide[c]	Enterotoxin: Oudin[d] (μg/g)	8 — CFU[b] (log)	Enterotoxin: Microslide[c]	Enterotoxin: Oudin[d] (μg/g)	24 — CFU[b] (log)	Enterotoxin: Microslide[c]	Enterotoxin: Oudin[d] (μg/g)
Control	I	7.49	-(-)		7.48	-(-)		9.20	-(-)	
	II	7.15	-(-)		8.04	-(-)		9.76	-(-)	
	III	7.59	-(-)		8.26	-(-)		9.70	-(-)	
	mean	7.41			7.93			9.55		
Low Level Papain	I	7.53	-(-)		7.70	-(-)		10.83	-(-)	
	II	7.28	-(-)		7.48	-(-)		9.76	-(-)	
	III	7.60	-(-)		8.23	-(-)		9.90	-(-)	
	mean	7.47			7.80			10.16		
High Level Papain	I	7.83	-(-)		8.08	-(-)		9.88	-(-)	
	II	7.79	-(-)		8.32	-(-)		10.08	-(±)	-
	III	7.92	-(-)		8.36	-(-)		9.84	-(+)	-
	mean	7.85			8.25			9.93		
Tenderizer Prep.[e]	I	7.63	-(-)		8.34	-(-)		9.58	-(-)	
	II	7.98	-(-)		8.32	-(-)		9.94	-(-)	
	III	7.58	-(-)		8.28	-(-)		9.72	-(-)	
	mean	7.73			8.31			9.75		
Commercially Tenderized Meat[f]	I	7.75	-(-)		8.45	-(-)		10.45	-(±)	-
	II	8.00	-(-)		8.46	-(-)		10.08	-(-)	
	III	7.89	-(-)		8.63	-(-)		10.11	-(-)	
	mean	7.88			8.51			10.21		

[a] Initial inoculum level of 1×10^7 CFU per gram.

[b] Colony forming units per gram expressed in logarithmic transformation.

[c] Microslide detection with results for concentrated samples shown in parenthesis (Casman et al., 1969). Detection limit was 0.2 μg/g. Four fold concentration was done if the sample were negative.

[d] Oudin in μg enterotoxin B per gram (Hall, Angelotti, and Lewis, 1963). Oudin assay done only on those samples giving a positive microslide assay after 8 and 24 hr. Detection limits for concentration samples:

1.0 μg/g - minimum (precipitation line only)
>1.0 μg/g - quantitative amount

[e] Adolph's Meat Tenderizer, Burbank, California.

[f] ProTen Beef, Swift and Co., Stockton, California.

Table 4. Colony forming units and enterotoxin B production after 5, 8, and 24 hr in control and papain-treated raw beef held at 42 C.

Treatment[a]	Rep.	Hours of Incubation: 5 — CFU[b] (log)	5 — Enterotoxin Microslide[c]	5 — Enterotoxin Oudin[d] (μg/g)	8 — CFU[b] (log)	8 — Enterotoxin Microslide[c]	8 — Enterotoxin Oudin[d] (μg/g)	24 — CFU[b] (log)	24 — Enterotoxin Microslide[c]	24 — Enterotoxin Oudin[d] (μg/g)
Control	I	7.67	-(-)		8.15	-(-)		8.11	±(+)	-
	II	8.20	-(-)		8.76	-(-)		8.53	-(-)	
	III	8.48	-(-)		8.89	-(-)		9.04	-(+)	-
	mean	8.12			8.60			8.56		
Low Level Papain	I	8.45	-(-)		8.67	-(-)		9.96	+	-
	II	8.04	-(-)		8.79	-(-)		9.08	-(+)	min.
	III	8.41	-(-)		8.90	-(-)		8.98	+	-
	mean	8.30			8.79			9.34		
High Level Papain	I	8.36	-(-)		8.71	-(-)		8.57	-(+)	min.
	II	8.40	-(-)		8.81	-(-)		8.99	±(+)	-
	III	8.48	-(-)		8.90	-(-)		8.45	-(+)	-
	mean	8.41			8.81			8.67		
Tenderizer Prep.[e]	I	8.38	-(-)		8.74	-(-)		8.28	-(±)	-
	II	8.36	-(-)		8.49	-(-)		9.18	±(+)	min.
	III	8.23	-(-)		8.81	-(-)		8.91	-(-)	
	mean	8.32			8.68			8.79		
Commercially Tenderized Meat[f]	I	8.48	-(-)		8.99	-(-)		10.48	+	<1
	II	8.52	-(-)		9.00	-(-)		9.20	+	-
	III	8.30	-(-)		9.11	-(-)		9.52	+	min.
	mean	8.43			9.03			9.73		

[a] Initial inoculum level of 1×10^7 CFU per gram.

[b] Colony forming units per gram expressed in logarithmic transformation.

[c] Microslide detection with results for concentrated samples shown in parenthesis (Casman et al., 1969). Detection limit was 0.2 μg/g. Four fold concentration was done if the sample were negative.

[d] Oudin in μg enterotoxin B per gram (Hall, Angelotti, and Lewis, 1963). Oudin assay done only on those samples giving a positive microslide assay after 8 and 24 hr. Detection limits for concentration samples:

1.0 μg/g - minimum (precipitation line only)
>1.0 μg/g - quantitative amount

[e] Adolph's Meat Tenderizer, Burbank, California.

[f] ProTen Beef, Swift and Co., Stockton, California.

Table 5. Colony forming units and enterotoxin B production after 5, 8, and 24 hr in control and papain-treated cooked beef held at 30 C.

		Hours of Incubation								
		5			8			24		
Treatment[a]	Rep.	CFU[b] (log)	Enterotoxin Micro-slide[c]	Oudin[d] (μg/g)	CFU[b] (log)	Enterotoxin Micro-slide[c]	Oudin[d] (μg/g)	CFU[b] (log)	Enterotoxin Micro-slide[c]	Oudin[d] (μg/g)
Control	I	8.52	-(-)		9.58	-(-)		9.68	±(+)	min.
	II	8.34	-(-)		9.65	-(-)		9.78	±(+)	min.
	III	8.46	-(-)		9.54	-(-)		10.18	+	min.
	mean	8.44			9.59			9.88		
Low Level Papain	I	8.40	-(-)		9.51	-(-)		10.46	-(+)	<1
	II	8.48	-(-)		9.65	-(-)		10.23	+	min.
	III	8.59	-(-)		9.60	-(-)		10.32	+	min.
	mean	8.49			9.59			10.34		
High Level Papain	I	8.36	-(-)		9.53	-(-)		9.86	+	<1
	II	8.56	-(-)		9.62	-(+)	-	9.84	+	<1
	III	8.20	-(-)		9.23	-(-)		10.26	+	<1
	mean	8.37			9.46			9.99		
Tenderizer Prep.[e]	I	8.52	-(-)		9.54	-(-)		10.23	+	<1
	II	8.54	-(-)		9.61	-(-)		10.18	+	1.4
	III	8.30	-(-)		9.49	-(±)	-	10.04	+	min.
	mean	8.45			9.55			10.15		
Commercially Tenderized Meat[f]	I	8.20	-(-)		9.26	-(-)		10.28	+	min.
	II	8.51	-(-)		9.48	-(+)	-	9.66	+	<1
	III	8.41	-(-)		9.34	-(+)	-	10.18	+	<1
	mean	8.37			9.36			10.04		

[a] Initial inoculum level of 1×10^7 CFU per gram.

[b] Colony forming units per gram expressed in logarithmic transformation.

[c] Microslide detection with results for concentrated samples shown in parenthesis (Casman et al., 1969). Detection limit was 0.2 μg/g. Four fold concentration was done if the sample were negative.

[d] Oudin in μg enterotoxin B per gram (Hall, Angelotti, and Lewis, 1963). Oudin assay done only on those samples giving a positive microslide assay after 8 and 24 hr. Detection limits for concentration samples:

1.0 μg/g - minimum (precipitation line only)
>1.0 μg/g - quantitative amount

[e] Adolph's Meat Tenderizer, Burbank, California.

[f] ProTen Beef, Swift and Co., Stockton, California.

Table 6. Colony forming units and enterotoxin B production after 5, 8, and 24 hr in control and papain-treated cooked beef held at 42 C.

Treatment[a]	Rep.	Hours of Incubation: 5 — CFU[b] (log)	5 — Enterotoxin: Microslide[c]	5 — Enterotoxin: Oudin[d] (μg/g)	8 — CFU[b] (log)	8 — Enterotoxin: Microslide[c]	8 — Enterotoxin: Oudin[d] (μg/g)	24 — CFU[b] (log)	24 — Enterotoxin: Microslide[c]	24 — Enterotoxin: Oudin[d] (μg/g)
Control	I	8.62	-(+)		8.59	-(-)		7.60	+	-
	II	9.04	-(+)		9.11	+	<1	8.54	+	<1
	III	9.08	±(+)		9.20	+	min.	8.81	+	1.6
	mean	8.91			8.97			8.32		
Low Level Papain	I	9.18	±(+)		9.56	+	<1	9.60	+	1.1
	II	8.92	-(-)		9.63	+	min.	8.48	+	2.2
	III	9.04	±(+)		9.74	+	-	9.63	+	min.
	mean	9.05			9.64			9.24		
High Level Papain	I	8.80	-(-)		8.77	-(+)	-	8.11	+	<1
	II	9.20	-(-)		9.63	+	-	8.26	+	<1
	III	9.15	±(+)		9.34	+	min.	9.11	+	min.
	mean	9.05			9.25			8.49		
Tenderizer Prep.[e]	I	8.67	-(+)		8.76	+	-	7.30	+	-
	II	9.15	+		9.80	+	min.	8.95	+	2.2
	III	9.28	+		9.28	+	-	8.60	+	1.3
	mean	9.03			9.28			8.28		
Commercially Tenderized Meat[f]	I	9.00	-(+)		9.64	+	<1	9.72	+	1.1
	II	8.99	+		9.68	+	-	9.04	+	1.1
	III	8.94	-(+)		9.15	+	min.	8.76	+	1.1
	mean	8.98			9.49			9.17		

[a] Initial inoculum level of 1×10^7 CFU per gram.

[b] Colony forming units per gram expressed in logarithmic transformation.

[c] Microslide detection with results for concentrated samples shown in parenthesis (Casman et al., 1969). Detection limit was 0.2 μg/g. Four fold concentration was done if the sample were negative.

[d] Oudin in μg enterotoxin B per gram (Hall, Angelotti, and Lewis, 1963). Oudin assay done only on those samples giving a positive microslide assay after 8 and 24 hr. Detection limits for concentration samples:

1.0 μg/g - minimum (precipitation line only)
>1.0 μg/g - quantitative amount

[e] Adolph's Meat Tenderizer, Burbank, California.

[f] ProTen Beef, Swift and Co., Stockton, California.

In analysis of variance (Appendix Table 1) it was found that all the factors (cooked versus raw, incubation time, and incubation temperature) were significant based on the calculated F values. There was also a high degree of significance due to the interactions of these factors. The high F values can partly be attributed to the large number for degrees of freedom and the small mean square for the error term.

Table 7 gives data for the "not fully cooked" ham experiment and Table 8 for the cooked ham. Because the hams had been partially cooked during the smoking process, no large differences were observed in growth patterns of the inoculated staphylococci. The high level papain-treated ham underwent considerable hydrolysis during the smoking process and had the consistency of a paste. It received no further cooking. As for its ability to support growth, it had a slightly lower count. This could be due to the difference in physical state. In addition, the pH was slightly lower (pH 6.0) than in the other meat samples (6.2 - 6.6).

The number of CFU after 5 hr was slightly greater at 42 C than at 30 C in both hams. The total number of CFU observed at 30 C after 24 hr was 10^3 times the original inoculum level; at 42 C, it was 10^2 times the starting level. The analysis of variance for CFU from ham (Appendix Tables 3 and 5) showed that while incubation time for both the "not fully cooked" and cooked samples was significant at the

Table 7. Colony forming units and enterotoxin B production after 5, 8, and 24 hr in control and papain-treated "not fully cooked ham" held at 30 and 42 C.

Treatment[a]	Rep.	30 C, 5 hr: CFU[b] (log)	30 C, 5 hr: Enterotoxin Microslide[c]	30 C, 5 hr: Enterotoxin Oudin[d] (μg/g)	30 C, 8 hr: CFU[b] (log)	30 C, 8 hr: Enterotoxin Microslide[c]	30 C, 8 hr: Enterotoxin Oudin[d] (μg/g)	30 C, 24 hr: CFU[b] (log)	30 C, 24 hr: Enterotoxin Microslide[c]	30 C, 24 hr: Enterotoxin Oudin[d] (μg/g)
Control	I	8.53	-(-)		9.57	-(+)		10.15	+	4.5
	II	8.70	-(-)		9.32	-(-)		10.38	+	min.
	III	8.66	-(-)		9.79	-(-)		10.30	+	min.
	mean	8.63			9.56			10.28		
Low Level Papain	I	8.51	-(-)		9.15	-(-)		10.30	-(±)	min.
	II	8.64	-(-)		9.53	-(-)		10.23	-(-)	
	III	8.54	-(-)		9.46	-(-)		9.95	+	<1
	mean	8.56			9.38			10.16		
High Level Papain	I	8.36	-(-)		8.97	-(-)		9.73	-(+)	-
	II	8.32	-(-)		8.88	-(-)		9.80	-(+)	-
	III	7.92	-(-)		8.30	-(-)		9.65	-(+)	<1
	mean	8.20			8.72			9.73		
		42 C								
Control	I	9.28	+		9.86	+	min.	9.81	+	1.8
	II	8.15	+		9.30	+	<1	9.82	+	<1
	III	9.32	+		9.49	+	<1	9.68	+	<1
	mean	8.92			9.55			9.77		
Low Level Papain	I	9.32	-(-)		9.56	-(+)	min.	9.98	+	<1
	II	9.32	+		9.11	+	min.	8.98	+	min.
	III	9.00	+		8.99	±(+)	-	9.08	+	<1
	mean	9.21			9.22			9.35		
High Level Papain	I	8.80	-(-)		8.72	-(-)		8.75	-(+)	-
	II	8.51	-(-)		8.61	-(-)		9.28	-(+)	-
	III	8.26	-(-)		8.51	-(+)	min.	8.81	+	-
	mean	8.52			8.61			8.95		

[a] Initial inoculum level of 1×10^7 CFU per gram.

[b] Colony forming units per gram expressed in logarithmic transformation.

[c] Microslide detection with results for concentrated samples shown in parenthesis (Casman et al., 1969). Detection limit was 0.2 μg/g. Four fold concentration was done if the sample were negative.

[d] Oudin in μg enterotoxin B per gram (Hall, Angelotti, and Lewis, 1963). Oudin assay done only on those samples giving a positive microslide assay after 8 and 24 hr. Detection limits for concentration samples:

1.0 μg/g - minimum (precipitation line only)
>1.0 μg/g - quantitative amount

Table 8. Colony forming units and enterotoxin B production after 5, 8, and 24 hr in control and papain-treated cooked ham held at 30 and 42 C.

Treatment[a]	Rep.	30 C, 5 hr: CFU[b] (log)	30 C, 5 hr: Enterotoxin Microslide[c]	30 C, 5 hr: Enterotoxin Oudin[d] (μg/g)	30 C, 8 hr: CFU[b] (log)	30 C, 8 hr: Enterotoxin Microslide[c]	30 C, 8 hr: Enterotoxin Oudin[d] (μg/g)	30 C, 24 hr: CFU[b] (log)	30 C, 24 hr: Enterotoxin Microslide[c]	30 C, 24 hr: Enterotoxin Oudin[d] (μg/g)
Control	I	8.85	-(-)		9.77	-(+)		10.26	+	1.7
	II	8.67	-(-)		9.97	-(-)		10.11	+	min.
	III	8.86	-(-)		9.96	-(-)		10.11	+	-
	mean	8.79			9.90			10.16		
Low Level Papain	I	8.54	-(-)		9.63	-(-)		10.28	-(±)	-
	II	8.65	-(-)		9.78	-(-)		10.26	-(+)	-
	III	8.28	-(-)		9.57	-(-)		9.96	-(+)	1.0
	mean	8.49			9.66			10.17		
		42 C								
Control	I	9.43	+		10.08	+	min.	10.08	+	6.2
	II	9.32	+		9.90	+	min.	9.11	+	min.
	III	9.26	+		9.89	+	<1	9.67	+	<1
	mean	9.34			9.96			9.62		
Low Level Papain	I	9.18	+		9.15	+	-	9.23	+	min.
	II	9.26	+		9.11	-(+)	min.	9.15	+	min.
	III	9.08	+		9.23	+	min.	8.84	+	min.
	mean	9.17			9.16			9.07		

[a] Initial inoculum level of 1×10^7 CFU per gram.

[b] Colony forming units per gram expressed in logarithmic transformation.

[c] Microslide detection with results for concentrated samples shown in parenthesis (Casman et al., 1969). Detection limit was 0.2 μg/g. Four fold concentration was done if the sample were negative.

[d] Oudin in μg enterotoxin B per gram (Hall, Angelotti, and Lewis, 1963). Oudin assay done only on those samples giving a positive microslide assay after 8 and 24 hr. Detection limits for concentration samples:

1.0 μg/g - minimum (precipitation line only)
>1.0 μg/g - quantitative amount

0.01 level, incubation temperature was not. The time-temperature interaction, however, was significant.

Enterotoxin was determined serologically. Casman and Bennett's (1965) extraction method using carboxymethylcellulose was compared with the more direct means of detection of Genigeorgis, Riemann, and Sadler (1969) during the preliminary studies for this research. Higher recovery rates were obtained with the latter method (73% as opposed to 27%). The direct method was adopted for the analysis of the meat samples since in preliminary enterotoxin recovery studies it was found that readings for the heat treated meat samples could be obtained by both the microslide and Oudin assays. Sensitivity of the detection procedures was equal for raw and cooked samples. Although some clouding of the gel occurred with the raw meat samples in the microslide assay, detection of precipitation lines was still possible. The migration of the pigments during the Oudin assay could be distinguished from the migration of enterotoxin after one day. Since during preliminary studies it was not possible to quantitate readings for the early hours, 5 hr samples were not tested by the Oudin assay.

Detection limits for enterotoxin B were established for both the microslide and Oudin methods, as presented in the section on experimental design. An enterotoxin concentration of 0.1 - 0.2 μg per g was the lowest amount detectable without concentration in meat by the

microslide assay. Minimal detection level by the Oudin assay represented a concentration of 0.9 - 1.0 μg enterotoxin per gram. Quantitation for the Oudin assay required greater than 1.0 μg per g in the sample. The determination of enterotoxin production in the statistical study must be interpreted only as a guide since a nonparametric system of assigning values was used for enterotoxin concentrations.

Significant differences were observed in enterotoxin levels between raw and cooked beef samples (Tables 3, 4, 5, and 6). At 30 C enterotoxin B in cooked samples was produced in quantitative amounts after 24 hr (1 μg per g), and only beginning to be in the detectable range (0.02 μg per g) in the raw samples. One sample from cooked beef in each of the treatments, high level, tenderizer preparation, and commercially tenderized, was positive when concentrated and tested by the microslide assay after 8 hr at 30 C. At 42 C, S. aureus in cooked samples had produced detectable amounts of enterotoxin B by 5 hr and up to 1 μg per g by 8 hr. All of the samples were positive by 24 hr and 13 of 15 samples gave from 0.2 μg to over 2 μg per g readings for the Oudin assay. Enterotoxin was not detected in raw samples until the 24 hr sampling period.

The analysis of variance (Appendix Table 2) for enterotoxin production in beef indicated that each of the experimental factors were highly significant at the 0.01 level, and, again, the interaction of factors was also significant.

The CFU and enterotoxin level were correlated for the high papain treatment, commercially tenderized meat, and the commercial tenderizer preparation in cooked beef held at 30 C but not for those at 42 C, perhaps due to death of cells in the latter before 24 hr.

In hams, enterotoxin production in those not fully cooked, Table 7, was observed by 8 hr in samples incubated at 42 C and in all of the 30 C samples by 24 hr. Control and low level papain-treated samples showed enterotoxin production (0.06 μg per g) after 5 hr at 42 C. The early detection could be linked to the partial cooking of the ham during the curing process. Levels could be determined by the Oudin assay from 0 - 1 μg per g at 8 hr and from 0.05 - 1.8 μg per g at 24 hr for both the control and low level papain-treated ham. The ham treated with a high level of papain showed slower enterotoxin production with a lower number of CFU present. CFU and enterotoxin level were correlated in the "not fully cooked" hams incubated at 42 C.

Cooked hams, Table 8, followed a pattern similar to beef in that production of enterotoxin occurred at 30 C by 24 hr (0.2 - 1.7 μg per g) and only in a few samples at 8 hr but was observed in all the samples held at 42 C after 5 hr (0.5 μg per g). Concentrations of 1 - 6.2 μg per g were observed after 24 hr in cooked ham incubated at 42 C. There was a high correlation between CFU and enterotoxin in cooked ham samples held at 42 C after 24 hr. All factors were found to be significant at the 0.01 level in the analysis of

variance (Appendix Tables 4 and 6) for both cooked and "not fully cooked" samples and enterotoxin values.

Since strain S-6 produces both A and B enterotoxins, the micro-slide assay was also used to test for the presence of A. Scattered positive results were obtained on concentrated 24 hr samples with no trend associated with any particular enzyme treatment. Only the cooked samples of the beef were positive (0.06 μg per g) and all but one was from meat incubated at 42 C.

In the ham, both the "not fully cooked" and the cooked samples were observed as positive for enterotoxin A. As in beef, production could not be attributed to any one treatment. In general, the 42 C held samples were more often the ones with detectable amounts (0.05 - 0.2 μg per g). However, it is interesting to note that the high level papain treatment was always positive after 24 hr at 30 C incubation. Optimum enterotoxin A production occurs at a lower pH than for enterotoxin B which could explain the effectiveness of this treatment even at lower temperatures (Reiser and Weiss, 1969).

In this study, significant differences of enterotoxins A and B production were found between cooked and raw meats which were essentially sterile. Since inhibiting microorganisms were not present, one reason for the difference may be nutritional needs. Protein denaturation, for example with cooking, appears to be the primary factor for influencing growth and enterotoxin production patterns.

Results from this study indicate that proteolysis due to enzymatic treatment does not increase markedly the capabilities of meat to support growth and enterotoxin production. Although specific growth conditions have been studied and reported by various researchers, the interaction of environmental conditions (time, temperature, nutrients, water, oxygen) is not completely understood. Although there is a greater concern with cooked meat, aseptically procured raw beef and "not fully cooked" ham were found to support growth and enterotoxin production but the levels of enterotoxin produced were much lower after 5 and 8 hr than in the cooked samples. Slower growth rates were also observed in the raw samples. The results concur with those of Casman, McCoy, and Brandly (1963), who also observed some growth in sterile raw and cooked beef and pork.

While the rates may vary, temperatures of 30 and 42 C will support both growth and enterotoxin production by S. aureus in meats. Because it was possible to detect enterotoxin after 5 hr of growth, the recommendations that perishable foods be held between 7 and 60 C for less than 4 hr(U. S. Public Health Service, 1962) may need to be reconsidered. Although detection procedures are not sensitive at very low concentrations, enterotoxin may be present after 3 or 4 hr of growth if the contamination is high. In recovery studies when pure enterotoxin B was added directly to meat samples and then analyzed, 73% was recovered so the amounts of enterotoxin actually produced

may be higher than those detected in this study. As reviewed earlier, less than 4 μg of S. aureus enterotoxin can cause food poisoning. At this level, an average serving of 100 g would contain less than 0.04 μg per g of food. This implies that any of the positive samples detected in this study as early as 5 hr (minimum detection 0.02 μg per g) as well as additional samples which were less concentrated than the detection limits would be capable of causing illness.

SUMMARY

This research was designed to investigate the effect of proteolysis due to the enzyme papain on the growth and enterotoxin production of Staphylococcus aureus because there has been some indication of increased utilization by microorganisms of substrates containing peptides. Beef and ham inoculated with strain S-6 were held at 30 and 42 C. Both uncooked and cooked meat samples were included in the study. Five treatments were used in the beef experiment: untreated control, low level papain, high level papain, a commercial tenderizer preparation, and a commercially tenderized meat. The papain and tenderizer preparation were applied to the surface and forked into the meat. For the ham experiment, in addition to the untreated control, two levels of papain were used. The enzyme was added directly to the curing solution.

Proteolysis was determined by three methods: total UV absorbing materials, UV absorbing materials in a TCA soluble meat solution, and free amino nitrogen analysis. No differences were detected in treatment levels by the 280 nm readings. The free amino nitrogen method using 2, 4, 6 trinitrobenzene sulfonic acid was more sensitive and detected differences in the degree of proteolysis in the various treatments. Treated samples gave higher readings in terms of numbers of free amino groups detected. The analysis of covariance

indicated that most of the treatment effects were explained by the data from the proteolysis determinations in the ham study.

After inoculation, the meat samples were incubated at 30 ± 1 C and 42 ± C. Sampling was done after 5, 8, and 24 hr for the number of colony forming units and enterotoxin concentration.

S. aureus on the beef samples held at 42 C had an initial growth rate ten times those at 30 C. Numbers of colony forming units at 24 hr were still increasing in the 30 C samples. Enterotoxin B production occurred earlier in the cooked samples than in the raw. Detection was possible by the microslide assay after 5 hr for the cooked beef held at 42 C (0.06 μg per g). By 24 hr, enterotoxin in nearly all the samples could be quantitated at > 1 μg per g by the Oudin method. The 30 C cooked samples were positive for enterotoxin by 24 hr (1 μg per g) although a few of the samples were also positive at 8 hr. Enterotoxin was detected in raw beef samples only after 24 hr for 42 C samples (0.2 - 0.9 μg per g) and at that time for a few of the 30 C samples (0.02 μg per g). The number of colony forming units and enterotoxin B concentrations were significantly correlated for cooked beef at 30 C but not at 42 C.

Fewer differences could be detected between the cooked and "not fully cooked" hams since both had received partial cooking during the smoking process. Colony forming units at 42 C reached a plateau (1×10^9 per g) after 8 hr. By 24 hr the 30 C samples (1×10^{10} per g)

had a greater number of colony forming units than the 42 C held samples (1×10^9). Enterotoxin B production was detected after 5, 8, and 24 hr at levels of 0 - 0.9, 0 - 1, and 0.2 - 1.8 μg per g respectively for the "not fully cooked" samples and at levels of 0.2 - 0.9, .25 - 1, and 1 - 6.2 μg per g respectively for cooked samples held at 42 C. Concentrations of 0 - 0.5 μg per g after 8 hr were detected in both raw and cooked samples incubated at 30 C with ranges of 0 - 4.5 μg for the "not fully cooked" and 0.05 - 1.7 μg per g for the cooked samples after 24 hr.

Enterotoxin A was detected in some of the concentrated 24 hr samples. Only cooked samples of beef, especially at 42 C, were positive by the microslide assay (0.05 μg per g). In both the cooked and "not fully cooked" hams, it was possible to detect enterotoxin A at concentrations of 0.05 to 0.2 μg per g.

Papain treatment in beef and ham did not appear to influence the number of colony forming units nor greatly affect the production of enterotoxin by S. aureus. Growth and enterotoxin production occurred both at 30 and 42 C in beef and ham. Although the amounts of enterotoxin detected were low, the levels obtained by 5 hr were sufficient to cause illness, if the samples were cooked.

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APPENDIX

Appendix Table 1. Analysis of Variance for number of colony forming units in beef.

Source of Variation	Degrees of Freedom	Mean Square[a]	F Value[b]
Papain treatment (P)	4	0.88	2.15
Error	8	0.41	
Cooking (C)	1	13.13	187.57**
P x C	4	0.32	4.59**
Temperature (T)	1	0.38	5.43**
P x T	4	0.22	3.14*
C x T	1	2.05	29.29**
P x C x T	4	0.07	1.00
Sampling time (S)	2	17.47	249.57**
P x S	8	0.34	4.86**
C x S	2	4.58	65.43**
P x C x S	8	0.03	0.43
T x S	2	12.87	184.00**
P x T x S	8	0.09	1.29
C x T x S	2	0.42	6.00**
P x C x T x S	8	0.06	0.86
Error	110	0.07	

Appendix Table 2. Analysis of Variance for values for enterotoxin concentrations in beef.

Source of Variation	Degrees of Freedom	Mean Square	F Value[b]
Papain treatment (P)	4	12.54	1.67
Error	8	7.53	
Cooking (C)	1	2170.83	232.92**
P x C	4	2.08	0.22
Temperature (T)	1	815.36	87.48**
P x T	4	17.29	1.86
C x T	1	151.43	16.25**
P x C x T	4	7.51	0.81
Sampling time (S)	2	1144.87	122.84**
P x S	8	8.44	0.91
C x S	2	292.20	31.35**
P x C x S	8	8.69	0.93
T x S	2	34.24	3.67*
P x T x S	8	6.18	0.66
C x T x S	2	323.60	34.61**
P x C x T x S	8	5.55	0.60
Error	110	9.32	

Appendix Table 3. Analysis of Variance for the number of colony forming units in "not fully cooked" ham.

Source of Variation	Degrees of Freedom	Mean Square[a]	F Value[b]
Papain treatment (P)	2	2.20	15.71*
Error	4	0.14	
Temperature (T)	1	0.21	3.00
P x T	2	0.01	0.14
Sampling Time (S)	2	4.78	68.29**
P x S	4	0.12	1.71
T x S	2	1.41	20.14**
P x T x S	4	0.05	0.71
Error	30	0.07	

Appendix Table 4. Analysis of Variance for the values for enterotoxin concentrations in "not fully cooked" ham.

Source of Variation	Degrees of Freedom	Mean Square	F Value[b]
Papain treatment (P)	2	216.69	30.65**
Error	4	7.07	
Temperature (T)	1	271.13	334.73**
P x T	2	39.02	48.17**
Sampling time (S)	2	425.69	525.54**
P x S	4	84.69	104.56**
T x S	2	18.02	22.25**
P x T x S	4	61.07	75.40**
Error	30	0.81	

Appendix Table 5. Analysis of Variance for the number of colony forming units in cooked ham.

Source of Variation	Degrees of Freedom	Mean Square[a]	F Value[b]
Papain treatment (P)	1	1.04	11.55
Error	2	0.09	
Temperature (T)	1	0.18	0.67
P x T	1	0.24	0.89
Sampling time (S)	2	2.36	8.74**
P x S	2	0.07	0.26
T x S	2	1.55	5.74*
P x T x S	2	0.12	0.44
Error	20	0.27	

Appendix Table 6. Analysis of Variance for the values for enterotoxin concentrations in cooked ham.

Source of Variation	Degrees of Freedom	Mean Square	F Value[b]
Papain treatment (P)	1	100.00	24.51*
Error	2	4.08	
Temperature (T)	1	961.00	227.73**
P x T	1	7.11	1.68
Sampling time (S)	2	144.53	34.25**
P x S	2	35.08	8.31**
T x S	2	19.75	4.68*
P x T x S	2	53.86	12.76**
Error	20	4.22	

[a] Logarithmic transformations of colony forming units per gram.

[b] * indicates significance at the .05 level.
** indicates significance at the .01 level.