Thermal Inactivation of Clostridium perfringens Enterotoxin

H. S. NAIK\textsuperscript{1} and C. L. DUNCAN\textsuperscript{2*}

Food Research Institute and
Department of Food Microbiology and Toxicology
University of Wisconsin, Madison, Wisconsin 53706

(Received for publication July 29, 1977)

ABSTRACT

Thermal inactivation studies of Clostridium perfringens enterotoxin revealed that biological activity was destroyed within 5 min at 60 °C, whereas about 10% or less residual serological activity could be detected even after 80 min of exposure at 60 °C in saline or in phosphate buffer, pH 7.0 or 8.0. Loss of serological activity was more rapid at 60 °C at pH 5.4 or 6.0 than at pH 7.0 or 8.0. Flocculation of enterotoxin was visible in phosphate buffer after 20 min of exposure at 60 °C, pH 5.4, 70 min at pH 6.0 but not at all at pH 7.0 or 8.0. Rapid loss of serological activity also occurred at 60 °C in cooked turkey, chicken gravy, beef gravy as well as in S. 10, and 20% bovine serum albumin and gelatin. Up to about 12% of the heat-inactivated serological activity could be recovered by treating toxin in the food samples with urea for 1 h at room temperature. However, serological activity of toxin heated in phosphate buffer could not be reactivated by urea treatment.

The enterotoxin of Clostridium perfringens is produced during sporulation (2, 3, 5) and has a molecular weight of approximately 35,000 with an isoelectric point of 4.3. Although this toxin is not normally thought to be preformed in foods, recent evidence indicates that in some outbreaks preformed toxin in foods may be responsible for symptoms that occur earlier than 5-6 h after ingestion of contaminated food (9, 10, 11). It has been well-documented that cell-free enterotoxin experimentally administered to human subjects and monkeys can induce food poisoning symptoms (4, 13, 15). Recently we have demonstrated preformed C. perfringens enterotoxin in various foods inoculated with enterotoxigenic strains (8), as well as in food from a documented C. perfringens outbreak. Because of the potential involvement of preformed toxin in a food poisoning outbreak, the present investigation was made to determine the thermal stability of C. perfringens enterotoxin in different environmental conditions.

\textsuperscript{1}Present address: Central Food Technological Research Institute, Cheluvamba Mansion, Mysore-570013, India.

\textsuperscript{2}Present address: Campbell Institute for Food Research, Campbell Place, Camden, New Jersey 08101.

MATERIALS AND METHODS

Toxin production

C. perfringens type A strain, National Collection of Type Cultures (NCTC) 8239 was grown in Duncan and Strong (DS) sporulation medium (J) for 6-8 h. Cells were harvested and sonicated to produce crude cell extract (CCE) (J, 12). Enterotoxin in the CCE was quantified by counterimmunoelectrophoresis as described by Naik and Duncan (7).

Heat inactivation of enterotoxin in foods

Ten μg of enterotoxin/g was added to moist cooked turkey (g), pH 6.4 and marinated with a mortar and pestle. Fifty grams of this mixture were distributed into half-pint blending jars which were then heated in a water bath at 60 °C. One-milliliter quantities of commercially canned chicken and beef gravy, pH 6.46 and 6.07, respectively, containing 10 μg of enterotoxin/ml were placed in 13 x 100-mm test tubes and also heated at 60 °C. Come-up time was determined using a thermocouple. Samples were removed from the water bath at 0, 5, 10, 20, 30, 40, 50, 60, 70 and 80-min intervals. Samples were immediately diluted 1:2 with ice-cold distilled water to stop further inactivation. Samples were then centrifuged at 16,300 x g for 30 min and the supernatant fluids were analyzed for serologically active toxin by CIEP (7).

Heat inactivation of enterotoxin in cooked turkey dialysate

Two hundred grams of ground cooked turkey were dialysed against 800 ml of distilled water for 72 h at 4 °C. The dialysate was concentrated to 200 ml using carbowax 20,000. Enterotoxin was added to the dialysate at a concentration of 10 μg/ml and 1-ml quantities were placed in 13 x 100 mm test tubes. These tubes were then heated at 60 °C for intervals similar to those used for cooked turkey. Toxin heated in 0.85% saline served as a control. Residual serological as well as biological activity was measured using CIEP (7) and the guinea pig skin test for erythema activity (12), respectively.

Heat inactivation of enterotoxin in saline

Enterotoxin, 10 μg/ml in saline, was distributed in 1.0-ml quantities in 13 x 100 mm test tubes. Thermal inactivation was carried out at 45, 50, 55, 60, 65, and 70 °C as described above.

Effect of different concentrations of proteins on thermal inactivation of enterotoxin

The effect of 5.0, 10.0 and 20.0% bovine serum albumin (BSA) and gelatin at pH 6.8 on thermal inactivation of enterotoxin at 60 °C was determined. Heating conditions were similar to those described above. The effect of 0.1 M sodium phosphate buffer at pH 5.4, 6.0, 7.0 and 8.0 on thermal inactivation of enterotoxin was also measured in a similar fashion.
Recovery of heat-inactivated enterotoxin by treatment with urea

Heat-inactivated toxin in various menstrua was mixed 1:1 with 8 M urea and held for 1 h at room temperature. Urea was removed by dialysis at 4 °C for 72 h before assaying for serological and biological activity.

RESULTS AND DISCUSSION

Heat inactivation of enterotoxin in foods

After 10 min of heating enterotoxin at 60 °C in cooked turkey (Fig. 1), there was a 75.0% loss of serological activity and a 95% loss after 20 min. A subsequent gradual decrease in serological activity occurred after 20 min with no detectable toxin being present after 80 min. The come-up time to 60 °C in the 50-g quantity of turkey used per sample was 32 min. Even though 95% of the serological activity was lost at this time, the residual activity detected up until 70 min would indicate the presence of either a more heat resistant form of the toxin or that perhaps conformational changes occurred in the toxin during heating which would make it more heat stable. Similar results were obtained with toxin heated in chicken or beef gravy.

Heat-inactivation kinetics of enterotoxin in cooked turkey dialysate were also similar to those in cooked turkey meat except that serologically active toxin was not detected after 60 min (Fig. 2). Although the kinetics of toxin in saline were also similar to those in turkey meat or dialysate, the residual level of toxin was higher after 20–70 min of heating in saline and serologically active toxin was still detectable after 80 min of heating. In contrast to serological activity, biological activity of enterotoxin as measured in guinea pig skin was completely destroyed within 5 min of exposure at 60 °C in saline and within 10 min of exposure in turkey dialysate. Thus, C. perfringens enterotoxin may have two or more different specific sites in the protein for biological and serological activity. Biological activity seems to be easily destroyed by heat, whereas serological activity, although also heat labile, seems to be more stable under similar heating conditions.

Recently, Granum and Skjelkvåle (6) also reported that C. perfringens enterotoxin was very heat labile with 90% of its biological activity being destroyed after only 1 min of exposure at 60 °C. They also observed that enterotoxin heated for 1 min at 55 °C (1 mg/ml in 0.02 M phosphate buffer) rapidly recovered its biological activity from 30% initially to an apparently stable level of about 50% after 4 days at 20 °C. Enterotoxin heat-treated for 5 min recovered its biological activity slowly, i.e., from 15% initially to only 20% after 6 days of storage at 20 °C. With enterotoxin heated for 15 min at 55 °C there was no recovery of biological activity from an initial 10% residual activity. We were unable to reactivate serologically active enterotoxin heated in saline at 60 °C for 80 min by storage at 20 or 4 °C.

Effect of different proteins on thermal inactivation of enterotoxin

Biologically active enterotoxin was completely inactivated after 5 min at 60 °C in 5.0, 10.0 and 20.0% BSA or gelatin at pH 6.8. Serological activity was destroyed after 10 min of heating under similar conditions.

Effect of pH on thermal inactivation of enterotoxin

Heat-inactivation of enterotoxin suspended in 0.1 M phosphate buffer at pH 5.4, 6.0, 7.0 or 8.0 is shown in
extent (results not shown). Biological activity could not be recovered by urea treatment regardless of the heating time was very slow as compared to that at pH 5.4 and 6.0. The enterotoxin flocculated after 20 min of exposure to 60 C at pH 5.4 and after 70 min of exposure at pH 6.0. At pH 7.0 the loss of serological activity with heating time was very slow as compared to that at pH 5.4 and 6.0 with only 50% of the serological activity remaining after 10 min, 25% after 20-60 min and about 12% being present after 80 min. Thus, a neutral or alkaline pH protected the serological activity of enterotoxin at 60 C, whereas at an acidic pH in conjunction with heat, the loss of serological activity was very rapid. However, biological activity was not observed after 5 min of exposure of C. perfringens enterotoxin at 60 C, no matter whether toxin was suspended at pH 5.4, 6.0, 7.0 or 8.0 in phosphate buffer. This further indicates that biological activity of C. perfringens enterotoxin is more heat-labile than its serological activity and depending upon environmental factors serological activity may be heat-stable for a reasonable period at 60 C.

**Effect of urea treatment on the recovery of enterotoxin serological activity**

Enterotoxin in 5% gelatin or BSA (pH 7.0) lost about 90% of its serological activity within 5 min of exposure at 60 C and all activity after 20 min. However, in samples treated with urea after heating in BSA a recovery of 50% of the serological activity at 5 min, 6% up to 50 min and 3% even after 80 min of exposure at 60 C was observed in the case of 5% BSA (Fig. 4). Urea-induced recovery was also observed with toxin heated in gelatin, chicken gravy, beef gravy or moist cooked turkey, though to a lesser extent (results not shown). Biological activity could not be recovered by urea treatment regardless of the heating menstruum. The urea treatment may have exposed serologically active sites for reaction with antienterotoxin which were not available as a result of heating the toxin in the presence of secondary proteins such as gelatin or BSA. It was interesting to note that urea treatment did not affect the detection level of serologically active enterotoxin heated at 60 C in phosphate buffer pH 6.0 or 7.0.

**Tatini (14) has also reported some enhancement in serological detectability of heat-inactivated staphylococcal enterotoxin A and D after urea treatment.** He also reported increased biological activity of urea-treated enterotoxin A. Our results suggest that C. perfringens enterotoxin is heat labile in foods as well as in laboratory media. Under normal food cooking conditions the toxin would most likely be inactivated. In testing for preformed enterotoxin in suspect foods from food poisoning outbreaks, it appears desirable to treat the toxin with urea for potential recovery of serologically active toxin that may have been heat-inactivated.

**ACKNOWLEDGMENTS**

The research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, Public Health Service research grant AI-11865-06 from the National Institute of Allergy and Infectious Diseases, Public Health Service research grant FD-00203-07 from the Food and Drug Administration, and by contributors to the Food Research Institute by member industries. C.L.D. is the recipient of a Public Health Service Research Career Development award AI-70721-03 from the National Institute of Allergy and Infectious Diseases. H.S.N. is the recipient of a Postdoctoral Research Fellowship from the Government of India, in the field of Veterinary Public Health.
REFERENCES

8. Naik, H. S., and C. L. Duncan. 1977. Enterotoxin formation in foods by Clostridium perfringens type A. J. Food Safety 1: