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Peter G. Canonico

Army Medical Research Institute of Infectious Diseases
Frederick, Maryland

4 April 1973

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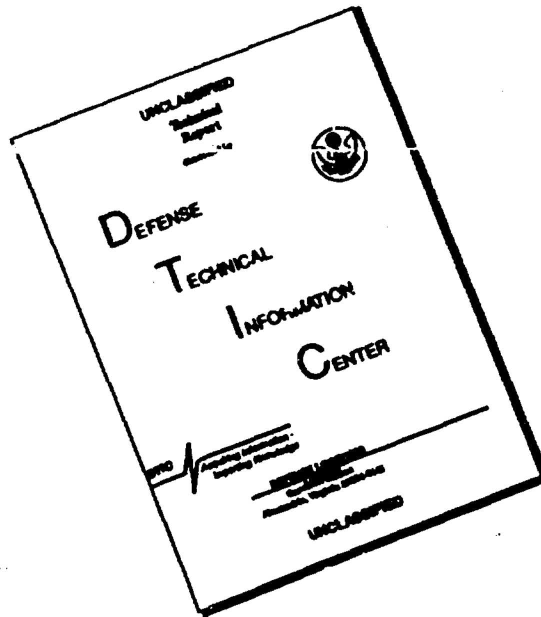

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Biochimica et Biophysica Acta, 322 (1973) 251–257
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BBA 36525

AD781367

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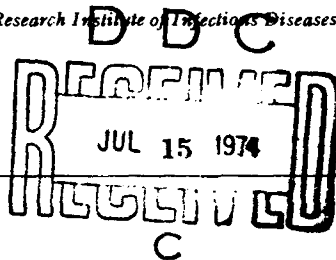
PETER G. CANONICO

United States Army Medical Research Institute of Infectious Diseases, Frederick, Md. 21701 (U.S.A)

(Received April 4th, 1973)



SUMMARY

Lysosomal proteases of rabbit liver, kidney and polymorphonuclear cells were tested for their ability to hydrolyze staphylococcal enterotoxin B, a protein exotoxin (mol. wt 28 494) of *Staphylococcus aureus*. All lysosomal preparations effectively inactivated and catabolized staphylococcal enterotoxin B *in vitro*, but only at pH values less than 3.2 and coincident with acid denaturation of toxin. Minimal digestion of staphylococcal enterotoxin B without loss of biological properties was observed at lower H^+ concentrations (pH 3.5–6.0). Denaturation of toxin by heating or performic acid oxidation markedly increased the pH range as well as the extent to which staphylococcal enterotoxin B was hydrolyzed. Iodination of staphylococcal enterotoxin B by either a chloramine T or a modified enzymatic method yielded derivatives which were more resistant to lysosomal digestion than the unmodified molecule. These results suggest that modification of a protein's native structure may either increase or decrease its susceptibility to digestion by lysosomal proteases.

INTRODUCTION

Lysosomes contain a number of proteolytic enzymes capable of hydrolyzing a variety of proteins and peptides[1]. The extensive hydrolysis of hemoglobin by lysosomal cathepsins led to the conclusion that the action of lysosomal proteases results in essentially complete hydrolysis of proteins to amino acids[2]. Other studies, however, mitigate the omnipotence of lysosomal proteolysis by demonstrating that many proteins resist hydrolysis by lysosomal proteases[3–5].

In the present study we have investigated the capacity of lysosomal proteases to hydrolyze staphylococcal enterotoxin B, a protein exotoxin produced by *Staphylococcus aureus*. Staphylococcal enterotoxin B is a single-chained protein of known amino acid sequence having a molecular weight of 28 494 (ref. 6). Native toxin is unusually resistant to proteolytic enzymes such as trypsin, chymotrypsin, rennin and papain. Its biological activity is destroyed by pepsin, but only at a pH of about 2 (ref. 7). The present study demonstrates that lysosomal proteases hydrolyze

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enterotoxin B, but the extent of digestion is dependent on the "natured state" of the molecule.

MATERIALS AND METHODS

Preparation of labeled toxin

A highly purified, lyophilized staphylococcal enterotoxin B preparation described by Schantz and coworkers[8] was used throughout these studies. ^{125}I-Labeled staphylococcal enterotoxin B (2.6 Ci/g) was prepared by a modification of the enzymatic method of Marchalonis[9] using lactoperoxidase isolated from bovine milk as described by Morrison and Hultquist[10]. Briefly, in a volume of 0.8 ml, 1 mg of staphylococcal enterotoxin B in 0.05 M phosphate buffer, pH 7.15, 10 μg lactoperoxidase and 2–5 mCi ^{125}I were reacted in the presence of 0.25 mM H_2O_2 for 10 min at room temperature. Free ^{125}I was then removed by dialysis against distilled water at 4 °C. The iodinated toxin reacted with specific antisera and migrated electrophoretically in a manner similar to native toxin. Radioiodinated toxin (2.2 Ci/g) was also prepared by the chloramine T procedure of Collins *et al.*[11].

Denatured forms of the labeled toxin were obtained by heating at 100 °C for 1 h or by oxidation with performic acid. Another denatured derivative of staphylococcal enterotoxin B was prepared by reacting radiolabeled toxin for an additional 24 h in the presence of lactoperoxidase, H_2O_2 and 1.0 mM NaI as described by Agner[12]. The resulting product was yellow, did not migrate electrophoretically nor react with specific antisera.

Preparation of lysosomal extracts

Triton WR-1339-filled rabbit liver lysosomes (tritosomes) were prepared by the procedure of Trouet as described by Leighton *et al.*[13]. Rabbit liver, kidney and polymorphonuclear leukocyte small granule fractions were prepared by adaptation of published methods[14–16]. Tritosome and small granule fraction preparations were dialyzed against distilled water to remove sucrose, freeze-thawed six times, then used as a source of lysosomal proteases without further treatment.

Enzymatic digestion of staphylococcal enterotoxin B

The incubation mixture, varying in volume from 50 μl to 2 ml depending on the purpose of the experiment, contained toxin at a final concentration of 200 μg/ml and sufficient enzyme protein to give 1 unit of cathepsin D (EC 2.4.4.23) activity* per ml of incubation mixture. Reaction mixtures were incubated at 37 °C for 12–18 h with continuous shaking. Reactions were stopped with 5 vol. cold 10% trichloroacetic acid, clarified by centrifugation and the percent of trichloroacetic acid-soluble radioactivity was assayed in a Nuclear Chicago gamma scintillation counter. In some experiments, the release of trichloroacetic acid-soluble ninhydrin positive material was determined by the manual method of Moore[17].

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* Cathepsin D activity is expressed in terms of hemoglobin digested at pH 3.8. One unit corresponds to the release of 0.1 μg tyrosine equivalents per minute.

RESULTS

Fig. 1A shows the pH profile for the digestion of staphylococcal enterotoxin B by lysosomal proteases. A pH optimum of 2.75 was found for the hydrolysis of unlabeled as well as enzymatically iodinated toxin. Hydrolysis was more extensive in citrate buffer than HCl glycine or acetate buffer (Fig. 1B). Catabolism was unaffected

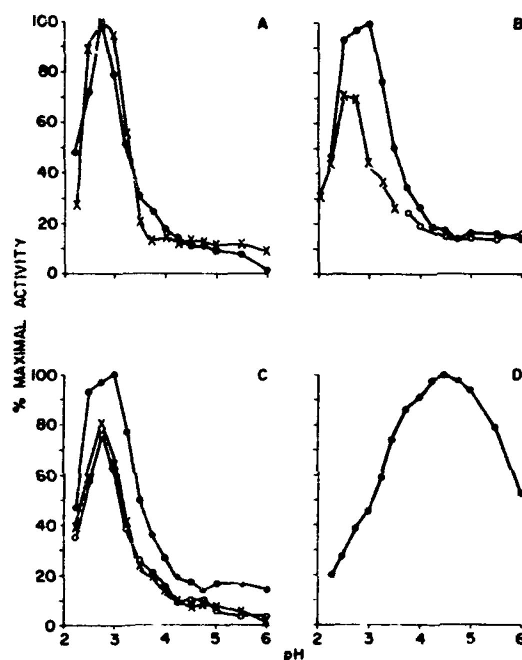

Fig. 1. pH activity profiles for the digestion of staphylococcal enterotoxin B by rabbit liver tritosome enzymes. (A) ^{125}I in trichloroacetic acid filtrate following digestion of enzymatically labeled staphylococcal enterotoxin B filtrate following digestion of enzymatically labeled staphylococcal enterotoxin B in 0.1 M citrate buffer (●—●); ninhydrin positive material in trichloroacetic acid filtrate following digestion of native staphylococcal enterotoxin B in 0.1 M citrate buffer (× — ×). (B) ^{125}I in trichloroacetic acid filtrate following digestion of enzymatically labeled staphylococcal enterotoxin B in 0.1 M citrate buffer and 5 mM cysteine (●—●); in 0.1 M glycine-HCl buffer and 5 mM cysteine (× — ×); and in 0.1 M acetate buffer and 5 mM cysteine (○—○). (C) ^{125}I in trichloroacetic acid filtrate following digestion of enzymatically labeled staphylococcal enterotoxin B in 0.1 M citrate buffer (○—○); buffer and 5 mM cysteine (●—●); buffer and 1 mM iodoacetamide (× — ×). (D) ^{125}I in trichloroacetic acid filtrate following digestion of heat denatured enzymatically labeled staphylococcal enterotoxin B. Hydrolysis performed in 0.1 M citrate buffer and 5 mM cysteine.

by 1 mM iodoacetamide (Fig. 1C), suggesting that cathepsin D is the principle protease responsible for the hydrolysis of toxin. In addition the stimulation of proteolysis and increase in pH optima in the presence of cysteine indicate that SH-dependent proteases, probably cathepsin B and C, also participate in the *in vitro* catabolism of staphylococcal enterotoxin B.

Loss of the immunoelectrophoretic properties of native staphylococcal entero-

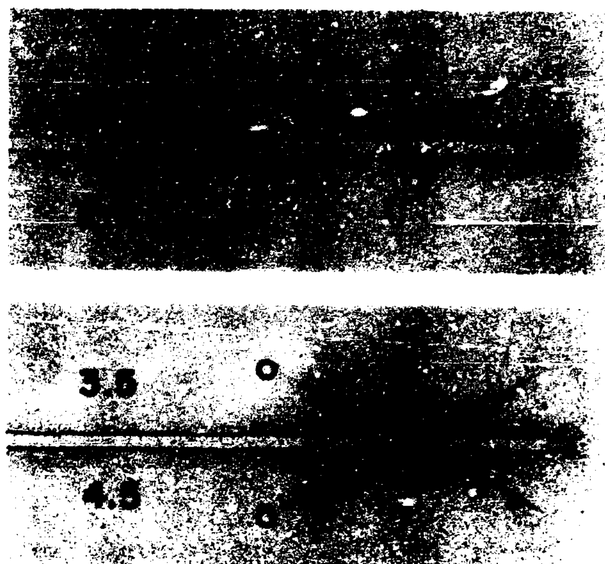

Fig. 2. Immunoelectrophoresis of staphylococcal enterotoxin B following hydrolysis by tritosome enzymes in an assay mixture containing 5 mM cysteine and 0.1 M citrate buffer at indicated pH. Immunoelectrophoresis was performed on agarose slides with 0.1 M borate buffer, pH 8.3. Electrophoresed for 60 min at 250 V and reacted with high titered goat anti-staphylococcal enterotoxin B sera. Stained with Buffalo black.

toxin B resulted after lysosomal digestion at pH 2.5 (Fig. 2). Similarly digested toxin failed to induce emesis in rhesus monkeys given an oral dose of 40 μg/kg, equivalent to 10 emetic doses (ED_{50}). When digestions were performed at pH 3.5 or 4.5 the toxin retained its immunoelectrophoretic and emetic activity, although 28 and 13% of the label was removed, respectively. The hydrolysis of staphylococcal enterotoxin B by kidney, liver and polymorphonuclear leukocyte small granule fraction proteases was similar to that observed for tritosome enzymes (Table I). Little digestion of toxin

TABLE I

In vitro HYDROLYSIS OF ENZYMATICALLY RADIOIODINATED STAPHYLOCOCCAL ENTEROTOXIN B BY LYSOSOMAL ENZYMES

The hydrolysis is expressed as percent of total ^{125}I released into trichloroacetic acid-soluble fraction after 18 h hydrolysis. Mean values of three separate experiments.

pH	*Hydrolysis (%)*			
	*Tritosome**	*Kidney***	*Liver***	*Polymorphonuclear leukocyte***
2.5†	55	40	59	49
3.5	28	20	19	16
4.5	13	14	13	6
7.5††	—	—	—	4

* Liver lysosomes isolated after Triton WR-1339 injection.
** Large granule fraction isolated by differential centrifugation of homogenates.
† Citrate buffer (0.1 M) containing 5 mM cysteine.
†† Phosphate buffer (0.1 M) containing 1.0 M KCl.

TABLE II

In vitro HYDROLYSIS OF DENATURED ^{125}I-LABELED STAPHYLOCOCCAL ENTEROTOXIN B BY LYSOSOMAL ENZYMES

The hydrolysis is expressed as percent of total ^{125}I released into trichloroacetic acid-soluble fraction after 18 h hydrolysis. Mean values of three separate experiments.

Method of denaturation	*Hydrolysis (%)*			
	Tritosome	*Kidney granule fraction*	*Liver granule fraction*	*Polymorphonuclear leukocyte granule fraction*
Performic acid oxidation				
pH 2.5	92	86	84	
pH 3.5	80	96	89	
pH 4.5	87	93	90	
100 °C for 60 min				
pH 2.5	10	27	13	46
pH 3.5	33	63	35	64
pH 4.5	44	80	44	76
pH 7.5	—	—	—	32

occurred when incubated with polymorphonuclear leukocyte small granule fraction proteases under conditions appropriate for neutral protease activation.

The hydrolysis of denatured toxin is shown in Table II. Performic acid oxidation of staphylococcal enterotoxin B resulted in extensive digestion at pH 2.5, as well as 3.5 and 4.5. Heat denaturation shifted the pH range in which the protein was hydrolyzed (Fig. 1D) with maximum digestion of toxin occurring at pH 4.5. Iodination of staphylococcal enterotoxin B by the chloramine T or modified enzymatic methods, resulted in derivatives which were highly resistant to lysosomal hydrolysis (Table III).

TABLE III

In vitro LYSOSOMAL HYDROLYSIS OF STAPHYLOCOCCAL ENTEROTOXIN B RADIOIODINATED BY ALTERNATE METHODS

The hydrolysis is expressed as percent of total ^{125}I released into trichloroacetic acid-soluble fraction after 18 h hydrolysis. Mean values of three separate experiments.

Method of iodination	*Hydrolysis (%)*		
	Tritosome	*Kidney granule fraction*	*Polymorphonuclear leukocyte granule fraction*
Modified enzymatic*			
pH 2.5	11	10	12
pH 3.5	11	10	14
pH 4.5	9	14	8
pH 7.5	—	—	10
Chloramine T**			
pH 2.5	10	11	5
pH 3.5	9	10	4
pH 4.5	8	7	3
pH 7.5	—	—	3

* Radioiodinated by the method of Agner[18] using lactoperoxidase.
** Radioiodinated by the method of Collins *et al.*[11].

DISCUSSION

The major portion of an intravenously administered dose of staphylococcal enterotoxin B is rapidly cleared from the circulation by glomerular filtration and subsequently reabsorbed and sequestered within the lysosomes of proximal tubular cells[18,19]. Most of the enterotoxin is ultimately catabolized into low molecular weight products, although, in contrast to other small proteins similarly sequestered within kidney lysosomes, the catabolism of staphylococcal enterotoxin B is considerably slower. For example, within 30 min following an intravenous injection of bovine pancreatic ribonuclease in mice, nearly 65% of the protein sequestered by the kidney is catabolized[20]. Undigested staphylococcal enterotoxin B, on the other hand, is detectable in the kidney of rat and monkey for more than 48 h after its administration[21]. Staphylococcal enterotoxin B resistance to lysosomal digestion at pH 3.5–6.0 may account for this persistance of toxin within kidney lysosomes. In addition, since toxin is readily hydrolyzed at lower pH the aforementioned hypothesis would conclude that the pH within kidney lysosomes is greater than 3.5. Such a conclusion is in accord with recent reports indicating a pH of 3.5–5.0 within secondary lysosomes of polymorphonuclear leukocytes[22,23].

The decreased catabolism of toxin at pH 3.5–6.0 is attributed to the presence of a stable molecular conformation insensitive to proteolytic attack. At pH values less than 3.5, however, an increase in the intrinsic viscosity of staphylococcal enterotoxin B, indicating a change in molecular configuration, has been observed[19]. It is proposed that this molecular change, consistent with acid denaturation, is responsible for the increaded lysosomal digestion of staphylococcal enterotoxin B at pH less than 3.5. The marked increase in pH range and extent of staphylococcal enterotoxin B catabolism following its irreversible denaturation by heating or performic acid oxidation clearly demonstrates the ability of lysosomal proteases to hydrolyze susceptible derivatives of enterotoxin at higher pH. These observations are in accord with the proposal that denaturation may be a prerequisite to lysosomal hydrolysis of certain proteins and that proteins or protein segments resistant to acid denaturation may escape lysosomal digestion[3].

Perturbation of a protein's native structure by procedures employed in topical labeling may also alter the susceptibility of the protein to catabolism. Whereas incorporation of fluorescein renders staphylococcal enterotoxin B more susceptible to hydrolysis by lysosomal enzymes[25], iodination employing strong oxidants, chloramine T or prolonged exposure to H_2O_2, yields derivatives which are more resistant to proteolysis than the native molecule. Protein derivatives more resistant than the parent protein to digestion by non-lysosomal proteases such as trypsin and papain have been reported[26,27]. These studies first suggested that an intrinsic change in conformation may result in a less digestible protein. Our data complements these observations by demonstrating that while lysosomal hydrolysis of native proteins is dependent on the presence of a susceptible structure, the susceptibility of proteins to lysosomal digestion, as in the case of trypsin, may be increased as well as decreased by modification of the native protein structure.

These conclusions are pertinent to an evaluation of studies designed to trace subcellular distribution and metabolism of topically labeled protein toxins and antigens *in vivo*. They indicate that the susceptibility of proteins to lysosomal di-

gestion may be altered by topical labeling of the molecule. Therefore, it is evident that the intracellular distribution and catabolism of labeled proteins *in vivo* may differ from non-labeled proteins. Reported differences in the *in vivo* distribution and clearance of iodinated or fluoresceinated staphylococcal enterotoxin B may, in fact, reflect differences in the susceptibility of the labeled derivatives to lysosomal directed catabolism[19].

ACKNOWLEDGMENTS

The author is indebted to Dr Virginia McGann for providing the immunoelectrophoretic data of staphylococcal enterotoxin B digests, and to Drs H. G Dangerfield and W. R. Beisel for their valuable suggestions in the preparation of the manuscript.

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