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1. AGENCY USE ONLY (Leave Blank)

2. REPORT DATE: 5 JUL 1990

3. REPORT TYPE AND DATES COVERED: Final, Thesis

4. TITLE AND SUBTITLE

Heat Stable Nuclease Contamination of Lysostaphin

5. FUNDING NUMBERS

6. AUTHOR(S)

CPT Mark Malatesta

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Thesis for Microbiology Mater's degree,prepared at the University of Alabama in cooperation with Dr. G. L. Sloan, Dr. P.A. LeBlanc and Dr. H.E. Heath.

12a. DISTRIBUTION/AVAILABILITY STATEMENT

DISTRIBUTION STATEMENT A
Approved for public release;
Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

Lysostaphin is a mixture of enzymes used by researchers to lyse Staphylococci in order to release nucleic acid material. In this study we report that these same preparations contain nucleases which may reduce nucleic acid yield unless certain precautions are taken.

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14. SUBJECT TERMS

Lysostaphin,nucleases, staphylococcus

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT: UNCLASSIFIED

18. SECURITY CLASSIFICATION OF THIS PAGE: UNCLASSIFIED

19. SECURITY CLASSIFICATION OF ABSTRACT: UNCLASSIFIED

20. LIMITATION OF ABSTRACT

Submitted by Mark Malatesta in partial fulfillment of the requirements for the degree of Master of Science in Microbiology.

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# Heat-Stable Nuclease Contamination of Lysostaphin

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Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Microbiology
in the Department of Microbiology
in the Graduate School of
The University of Alabama

TUSCALOOSA, ALABAMA

1990

## ACKNOWLEDGEMENTS

I wish to thank Dr. Gary L. Sloan for his guidance and continuous support during the course of this research. Without it none of this would have been possible. I am extremely grateful to Dr. H. E. Heath, Dr. Paul A. LeBlanc and Dr. Harriett Smith-Somerville for their technical assistance and suggestions. I wish to thank Pam Barnett for typing this thesis and her assistance in preparing for my seminars. I wish to thank Kevin Keehan, Veronica Neumann, Jeff Stiefel, the faculty, staff, and graduate students for making my two years here very enjoyable. I am forever grateful to my wife Rosemarie for her patience, encouragement, and support. Thanks.

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## INTRODUCTION

Lysostaphin is a commercially available staphylolytic preparation produced by *Staphylococcus simulans* biovar *staphylolyticus* (17). The staphylolytic component in lysostaphin is a glycylglycine endopeptidase that hydrolyzes the polyglycine crossbridges present in the cell wall peptidoglycans of staphylococci (1). This enzyme is a zinc metalloenzyme with a molecular weight of 25,000 and an isoelectric point of 9.5 (18,19). Endopeptidase is produced during the post-exponential phase of aerobic growth, and its production is coordinated with that of a micrococcolytic endo-β-N-acetylglucosaminidase (hexosaminidase) and a sulfhydryl protease (2,9,11,15). *S. simulans* biovar *staphylolyticus* has been shown to contain five plasmids, designated pACK1 through pACK5 in decreasing order of size (5). The endopeptidase is encoded on pACK1 (4,5).

Lysostaphin is commonly used to lyse staphylococcal cells to release DNA and RNA for genetic studies (3,8,10). Here we report that commercial lysostaphin preparations contain nuclease activities that decrease the yield and quality of nucleic acids in staphylococcal cell lysates and

that these losses can be minimized by the addition of the chelating agent EGTA (ethylene glycol-bis[β-aminoethyl ether]N,N,N',N'-tetraacetic acid) to the lysis buffer.

## MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. simulans* biovar *staphylolyticus* NRRL B-2628 was obtained from the Northern Utilization Research and Development Division, Peoria, IL. Stock cultures were maintained on Tryptic Soy Agar (Difco) at 4°C. Liquid cultures in modified lysostaphin production medium (12) were grown for 18 h at 37°C on a gyratory shaker (Model G25, New Brunswick) at 250 rpm. Culture supernatants were concentrated by ultrafiltration using Amicon Centriprep 10 filters with a nominal molecular weight cutoff of 10,000.

*Staphylococcus aureus* ATCC 12600 was obtained from the American Type Culture Collection. Stock cultures were maintained on Tryptic Soy Agar at 4°C. Liquid cultures in Tryptic Soy Broth without dextrose (Difco) were grown for 18 h at 37°C with shaking at 250 rpm.

Chemicals. Lysostaphin (Lots 126F0473 and 127F0801) was obtained from Sigma Chemical Co. Recombinant lysostaphin was obtained from Applied Microbiology, Inc. All other chemicals were reagent or analytical grade and were purchased from commercial sources.

Electrophoresis. Analytical polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed by a modification of the procedure of Weber and Osborn (20) with stacking gels at pH 5.8 made to a final concentration of 4% (wt/vol) acrylamide and 0.175% N,N'-methylene-bisacrylamide and with resolving gels at pH 7.2 made to a final concentration of 11.5% (wt/vol) acrylamide and 0.5% N,N'-methylene-bisacrylamide. As appropriate, boiled calf thymus DNA (10 ug/ml; Sigma) or boiled ribosomal RNA (25 ug/ml; Sigma) was incorporated into the gels just prior to polymerization. Vertical slab gels (Protean gel electrophoresis apparatus, Bio-Rad) were run at 30°C with a constant current of 100 mA for the stacking gel and 200 mA for the resolving gel. Gels were stained with a silver stain kit (Bio-Rad) or were treated to allow renaturation of nucleases as described below.

The nucleases were renatured using a modification of the method of Rosenthal and Sacks (16). The gels were washed with distilled water for 10 min and then incubated for 1 h on a rotating platform at 25°C in 500 ml of 4 mM Tris-HCl (pH 7.6) containing 2 mM $MgCl_2$. A second incubation in the same buffer was carried out overnight at 30°C. The buffer was then changed to 4 mM Tris-HCl (pH 7.6) containing 2 mM $MgCl_2$ and 2 mM $CaCl_2$, and the gels were incubated at 37°C for an additional 4 h. After renaturation, the gels were stained with 1 mg/ml ethidium bromide and allowed to develop for up to 2 h. The gels

were examined on a UV transilluminator; dark bands on a fluorescent background indicate digestion of the nucleic acid.

Molecular size standards (SDS-200 and SDS-200P) were obtained from Diversified Biotech and included phosphorylase B (95.5 kDa), glutamate dehydrogenase (55.0 kDa), ovalbumin (43.0 kDa), lactate dehydrogenase (36.0 kDa), carbonic anhydrase (29.0 kDa), lactoglobulin (18.4 kDa), and cytochrome c (12.4 kDa).

Nucleic Acid Preparation. Nucleic acids were prepared from *S. aureus* cells in the presence and absence of EGTA. Lysostaphin (25 ug/ml) was dissolved in endopeptidase buffer (12) with and without 75 mM EGTA and preincubated for 10 min at room temperature. The *S. aureus* cells were harvested, washed with endopeptidase buffer, resuspended in 250 ul of endopeptidase buffer with or without 75 mM EGTA plus lysostaphin and incubated for 30 min in a 37°C water bath. Samples were adjusted to a concentration of 1% (wt/vol) SDS, 5 M NaCl, and 2 mM EDTA and the mixture was kept at -20°C for 1 h. Precipitated material was removed by centrifugation at 4°C for 15 min, and phenol extractions were performed on each supernatant. The nucleic acids were precipitated with 2.5 volumes of 95% (vol/vol) ethanol at -20°C for 30 min and collected by centrifugation at 4°C for 15 min. The precipitate was dried and dissolved in 25 ul of 10 mM Tris hydrochloride (pH 8.0) containing 1 mM EDTA for electrophoretic analysis. Agarose gel electrophoresis

was performed as previously described (6). Agarose gels were scanned with a Biomed Instruments, Inc. Video Densitometer II.

## RESULTS AND DISCUSSION

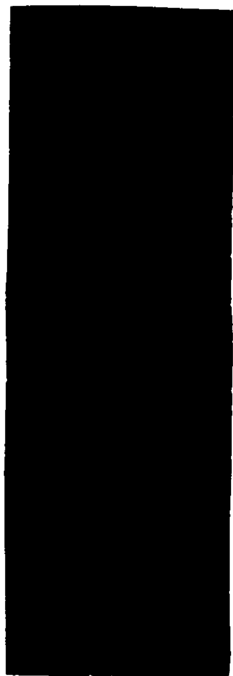
Based on SDS-PAGE analysis, *S. simulans* biovar *staphylolyticus* produces at least 14 exoproteins during aerobic growth in modified lysostaphin production medium (2,13). In addition to the staphylolytic endopeptidase, micrococcolytic hexosaminidase, and sulfhydryl protease, the organism also produces N-acetylmuramyl-L-alanine amidase, β-lactamase, phosphatase, β-hemolysin, and heat-stable DNase activities (17,19).

SDS-PAGE analysis of commercial lysostaphin preparations obtained from culture supernatants of *S. simulans* biovar *staphylolyticus* (Sigma) revealed that these preparations contain a number of exoproteins in addition to the endopeptidase (Fig. 1, lane 1). Because many investigators routinely use lysostaphin to release DNA and RNA for genetic studies on staphylococci, we tested Sigma lysostaphin to determine if it contains DNase or RNase activities that might interfere with the isolation of nucleic acids from these organisms. These analyses revealed that Sigma lysostaphin did contain several of the DNase and RNase activities found in culture supernatants from *S. simulans* biovar *staphylolyticus* (cf. Fig. 2A and 2B, lanes 1 and 2).

Figure 1. SDS-PAGE of commercial lysostaphin preparations. Lane 1, 25 ug of Sigma lysostaphin; Lane 2, 60 ug of recombinant lysostaphin (Applied Microbiology). Numbers refer to molecular weights (kDa) of the molecular size markers described in the text.

-95.0
-55.0
-43.0
-36.0
-18.0
-12.4

Figure 2. SDS-PAGE in gels containing nucleic acids. (A) SDS-PAGE in gels containing DNA. Lane 1, extracellular proteins from *S. simulans* biovar *staphylolyticus*; lane 2, Sigma lysostaphin (40 ug); lane 3, recombinant lysostaphin (60 ug) from Applied Microbiology. (B) SDS-PAGE in gels containing RNA. Lane 1, extracellular proteins from *S. simulans* biovar *staphylolyticus*; lane 2, Sigma lysostaphin (40 ug); lane 3, recombinant lysostaphin (30 ug) from Applied Microbiology. Numbers refer to molecular weights (kDa) of the molecular size markers described in the text.


-95.0
-55.0
-43.0
-36.0
-18.0
-12.4

-95.0
-55.0
-43.0
-36.0
-18.0
-12.4

Recombinant lysostaphin has recently become available from Applied Microbiology, Inc. These lysostaphin preparations are produced by a member of the genus Bacillus that contains the cloned lysostaphin endopeptidase gene from S. simulans biovar staphylolyticus. Analysis of recombinant lysostaphin preparations by SDS-PAGE showed that they also contained a number of proteins in addition to the staphylolytic endopeptidase (Fig. 1, lane 2). One DNase and several RNases were found to be associated with these preparations (Fig. 2A and 2B, lane 3).

We undertook to develop lysis procedures that would minimize the nuclease activities in commercial lysostaphin preparations without interfering with the activity of the endopeptidase. Other staphylococcal nucleases have been reported to have an absolute requirement for calcium ions (7). We therefore tested a number of chelating agents for their ability to inhibit the nucleases in lysostaphin without inhibition of the endopeptidase, which is a zinc metalloenzyme (18). We found that lysates prepared in endopeptidase buffer that contained 75 mM EGTA gave significantly better DNA and RNA preparations for both commercial lysostaphins than lysates prepared in endopeptidase buffer without EGTA. In these analyses, we lysed S. aureus ATCC 12600 cells, which contain a large penicillinase plasmid (6), with lysostaphin concentrations typically used for the lysis of staphylococci (10 to 50 ug/ml [3,8,10]). The presence of EGTA in lysates made with

Sigma lysostaphin resulted in greatly increased yields of supercoiled plasmid molecules as determined by agarose gel electrophoresis (cf Fig. 3, lanes 1 and 2). In addition, these gels showed that the yield and average molecular weights of RNA molecules were significantly increased in these lysates. Lysates prepared with recombinant lysostaphin in the presence of EGTA also had increased amounts of supercoiled plasmid molecules and RNA, and the RNA was of higher molecular weight (cf. Fig. 4A, lanes 1 and 2 and the corresponding densitometric analysis in Fig. 4B).

If commercial lysostaphin preparations are used to prepare staphylococcal cell lysates in order to isolate DNA or RNA, we recommend the following precautions be taken. Use the smallest amount of lysostaphin and the shortest reaction time possible that will give complete cell lysis, and incorporate 75 mM EGTA in the lysis buffer to inhibit nuclease activities that otherwise will lower the yield and quality of the nucleic acids in the lysate.

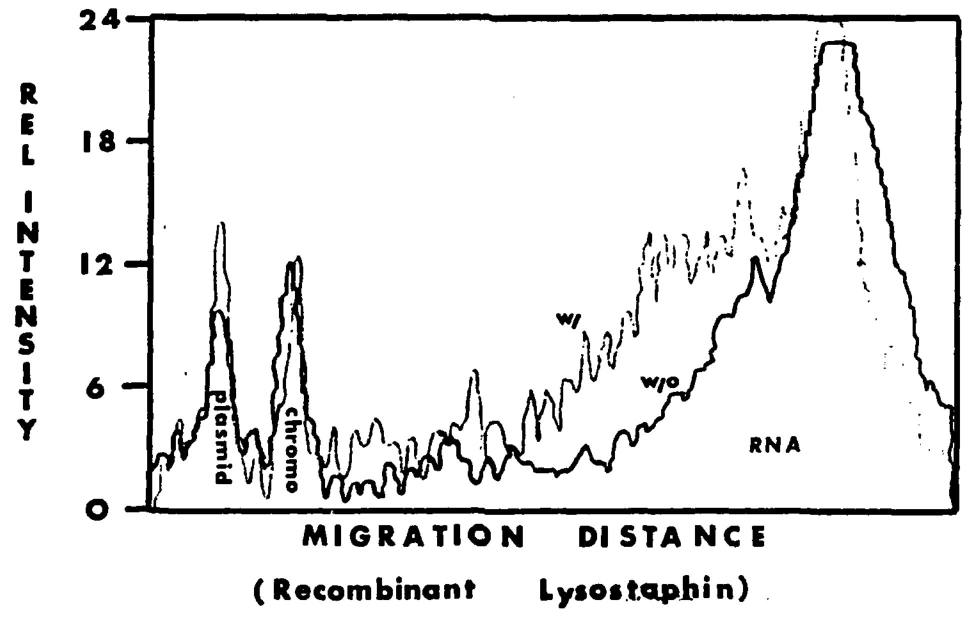
Figure 3. Electrophoresis in 1% agarose of *S. aureus* nucleic acids isolated after Sigma lysostaphin lysis. Lane 1, cell lysate prepared with Sigma lysostaphin in endopeptidase buffer; lane 2, cell lysate prepared with Sigma lysostaphin in endopeptidase buffer containing 75 mM EGTA. Abbreviations: P, penicillinase plasmid; C, chromosomal DNA; R, RNA.

P
C

R

Figure 4. Electrophoresis in 1% agarose of *S. aureus* nucleic acids isolated after recombinant lysostaphin lysis. (A) Lane 1, cell lysate prepared with recombinant lysostaphin (Applied Microbiology) in endopeptidase buffer; lane 2, cell lysate prepared with recombinant lysostaphin in endopeptidase buffer containing 75 mM EGTA. (B) Densitometric scan of the agarose gel in (A). The solid line represents the nucleic acids present in lysates made with EGTA in the lysis buffer; the dashed line represents the nucleic acids present in lysates made in the same buffer without EGTA. Abbreviations: P, penicillinase plasmid; C, chromosomal DNA; R, RNA.

P
C
R


PLASMID & RNA
RECOVERY WITH & WITHOUT
EGTA
REL INTENSITY
24
18
12
6
0
plasmid
chromo
w/
w/o
RNA
MIGRATION DISTANCE
(Recombinant Lysostaphin)

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