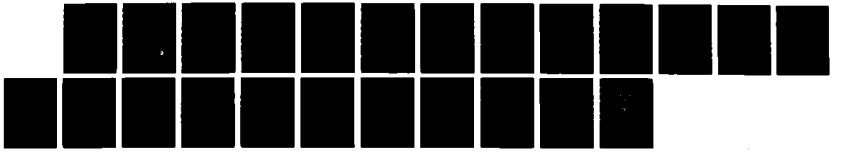
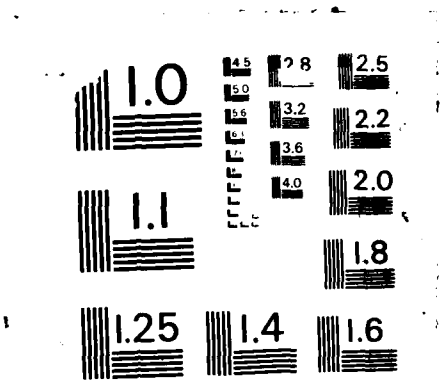
AD-A180 171 DEGRADATION OF ALBUMIN BY TRYPSIN: POTENTIAL APPLICATION OF HIGH-PERFORMA..(U) CHEMICAL RESEARCH DEVELOPMENT AND ENGINEERING CENTER ABERDEEN.. 1/1

UNCLASSIFIED P DIAMANDARAS ET AL. AUG 87 CRDEC-TR-87071 F/G 6/1 NL





MICROCOPY RESOLUTION TEST CHART

NATIONAL BUREAU OF STANDARDS - 1963 -

(2)

CHEMICAL RESEARCH, DEVELOPMENT & ENGINEERING CENTER

CRDEC-TR-87071

DEGRADATION OF ALBUMIN BY TRYPSIN: POTENTIAL APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) AND DIFFERENTIAL SCANNING CALORIMETRY (DSC) FOR MONITORING THE DEGRADATION OF PROTEINACEOUS TOXINS BY PROTEOLYTIC ENZYMES

by Penelope Diamandaras
William E. White, Ph.D.
Ilya Elashvilli

RESEARCH DIRECTORATE

August 1987

U.S. ARMY ARMAMENT MUNITIONS CHEMICAL COMMAND

Aberdeen Proving Ground, Maryland 21010-5423

Disclaimer

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

Distribution Statement

Approved for public release; distribution is unlimited.

UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE

AD-A182 171

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED	1b RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3 DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution is unlimited.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) CRDEC-TR-87071	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a NAME OF PERFORMING ORGANIZATION CRDEC 6b OFFICE SYMBOL (*If applicable*) SMCCR-RSB	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (*City, State, and ZIP Code*) Aberdeen Proving Ground, MD 21010-5423	7b ADDRESS (*City, State, and ZIP Code*)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION CRDEC 8b. OFFICE SYMBOL (*If applicable*) SMCCR-RSB	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c. ADDRESS (*City, State, and ZIP Code*) Aberdeen Proving Ground, MD 21010-5423	10 SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. PROJECT NO. 1L162706 TASK NO. A553F WORK UNIT ACCESSION NO

11 TITLE (*Include Security Classification*) Degradation of Albumin by Trypsin: Potential Application of High-Performance Liquid Chromatography (HPLC) and Differential Scanning Calorimetry (DSC) for Monitoring the Degradation of Proteinaceous Toxins by Proteolytic Enzymes

12 PERSONAL AUTHOR(S)
Diamandaras, Penelope; White, William E., Ph.D.; and Elashvilli, Ilya

13a TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (*Year, Month, Day*)	15. PAGE COUNT
Technical	FROM 85 Dec TO 86 Feb	1987 August	21

16 SUPPLEMENTARY NOTATION

17 COSATI CODES			18. SUBJECT TERMS (*Continue on reverse if necessary and identify by block number*)
FIELD	GROUP	SUB-GROUP	Trypsin Protein digestion HPLC Differential scanning calorimetry
06	01		
20	13		

19 ABSTRACT (*Continue on reverse if necessary and identify by block number*)

Albumin was selected as a model protein for developing assay systems for monitoring degradation by proteolytic enzymes. Using a diode array detector, high-performance liquid chromatography was ideal for following albumin digestion by trypsin. The albumin peak gradually diminished and was replaced by several peaks corresponding to shorter peptides. In contrast, differential scanning calorimetry was of no value in following the disappearances of the protein. A thermal transition resulting from some confirmational change occurred at 52 °C but did not change upon digestion of the protein.

20 DISTRIBUTION/AVAILABILITY OF ABSTRACT ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ OTIC USERS	21 ABSTRACT SECURITY CLASSIFICATION UNCLASSIFIED
22a NAME OF RESPONSIBLE INDIVIDUAL TIMOTHY E. HAMPTON	22b TELEPHONE (*Include Area Code*) (301) 671-2914 22c OFFICE SYMBOL SMCCR-SPS-T

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted
All other editions are obsolete

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

PREFACE

The work described in this report was authorized under Project No. 1L162706A553F. This work was started in December 1985 and completed in February 1986.

The use of trade names or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

This report has been approved for release to the public.

Blank

CONTENTS

Blank

LIST OF FIGURES

Blank

DEGRADATION OF ALBUMIN BY TRYPSIN: POTENTIAL APPLICATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) AND DIFFERENTIAL SCANNING CALORIMETRY (DSC) FOR MONITORING THE DEGRADATION OF PROTEINACEOUS TOXINS BY PROTEOLYTIC ENZYMES

1. INTRODUCTION

Proteinaceous toxins including botulinum, Staphlococcus enterotoxins A and B, tetanus, cholera, and ricin are potent potential biological warfare agents. Recent advances in biotechnology (e.g., recombinant DNA or gene splicing) increase the number of exploitable proteinaceous toxins and thus the need for effective and safe decontaminants. Proteolytic enzymes can degrade these under mind, physiological conditions.

To evaluate proteolytic enzymes as possible decotaminants, an assay procedure must be developed. Methods that involve measuring particular amino acids or peptide bonds are not very promising because these parameters do not change significantly during enzymatic digestion. This paper describes the feasibility of two methods for monitoring the digestion of proteins.

2. INSTRUMENTATION

High performance liquid chromatography (HPLC) and differential Scanning Calorimetry (DSC) are two analytical techniques that measure parameters associated with the entire protein.

The ULTROCHROM GTi HPLC marketed by LKB in Sweden is especially designed for work with biological systems. Surfaces exposed to the sample and buffers are made of inert titanium alloys and polymers instead of stainless steel in order to eliminate corrosion that can be catalyzed by aqueous salt solutions. The diode array detector provides a full spectrum (190-370 nm) every second in real time.[1]

The Toyo Soda GlasPac TSK G3000SW gel filtration column separates biopolymers by size. The packing material contains holes of various sizes that were generated by crosslinking. Some proteins are too large to enter these cavities and therefore elute from the column first. Smaller proteins enter some of the larger cavities and elute later. The smallest peptides are retained the longest inside the cavities and therefore elute last.[2]

A differential scanning calorimeter measures the heat (enthalpy) absorbed or liberated by a sample during a thermal transition or reaction. The instrument contains reference and sample compartments that are heated at a constant predetermined rate. When a sample undergoes an endothermic transition, additional heat must be supplied in order to maintain the constant

prescribed temperature increase. Thus, the instrument measures the difference in heat supplied to the sample and reference as a function of temperature. Endothermic transitions and increases in heat capacity are represented by upscale departures from the ordinate baseline, and exothermic reactions are represented by downscale departures from the baseline. Values are read directly in millicalories per second.[3]

When proteins are heated, they undergo thermal transitions that are dependent on solvent and ionic strength. If a protein has been cut into smaller fragments, the characteristic transitions resulting from conformational changes should disappear.[4]

3. PROCEDURES

3.1 High-Performance Liquid Chromatography.

Reference chromatograms were made of a 20 µl sample of albumin, prepared by dissolving 10 mg/ml of Bovine Serum Albumin (crystalized and lyophilized by Sigma-A4378) in 0.1M tris (0.1M NaCl) buffer adjusted to pH 7.8 and a 20 µl sample of trypsin, made by dissolving 10 mg/ml of trypsin (crystalized and lyophilized by Worthington-TRL6259) in tris buffer. Trypsin, a pancreatic enzyme, produced peak proteolytic activity at pH 7.8.[5]

A reaction mixture containing 10 mg/ml of albumin and 0.5 mg/ml of trypsin in tris buffer was prepared and incubated at 38 °C. A 20 µl sample of this reaction mixture was injected after 0, 1, 3, and 5 hours. After the 5-hour injection, one sample was refrigerated for 72 hours to retard further digestion, while another sample remained at 38 °C to allow further digestion to occur. All chromatograms were made on a LKB Bromma GTi high-performance liquid chromatograph, using a 20-µl sample, 0.50 ml/min flow rate, and a LKB GlasPac TSK G3000SW, 8 x 300 mm column. A tris buffer containing 10 mM of tris, 0.1M of NaCl and adjusted to pH 7.1 using HCl was used as the column effluent.

3.2 Differential Scanning Calorimetry.

Samples were prepared by procedures identical to those for the HPLC study. Reference thermograms were made using approximately 0.025 ml albumin (10 mg/ml), trypsin (10 mg/ml) in tris buffer (10 mM, pH 7.8). The time study was repeated using a reaction mixture containing 10 mg/ml albumin and 0.5 mg/ml of trypsin in a tris buffer. The scans were performed on a Perkin Elmer DSC, Model 4, using air as the reference. The following parameters were incorporated into all thermograms:

T final	150 °C
T initial	30 °C
heating rate	10 °C/min.

4. RESULTS

Reference chromatograms for albumin (MW 65,000) and trypsin (MW 24,000) are shown in Figures 1 and 2, respectively. The albumin chromatogram consisted of two peaks. The minor component, eluting at 16 min (8 ml elution volume), was either some dimer or impurity. The major component corresponding to the molecular weight of albumin eluted at 17.5 min (8.75 ml). Trypsin eluted later at 26 min (13 ml). These retention times remained constant during the study.

The chromatogram of the reaction mixture at 0 hour of incubation (Figure 3) revealed three distinct peaks that were attributed to albumin and trypsin. After a 1-hour incubation period (Figure 4), the first peak produced by albumin (16 min) had disappeared, and new peaks appeared later during a span of 20 to 37 min. These peaks were attributed to the peptides formed from trypsin's digestion of albumin. They continued to increase in size during further incubation. The chromatogram at 72 hours (Figure 5) indicates that all the albumin had been digested. Figure 6 illustrates the changes in albumin concentration as a function of time. Initially, the concentration decreased rapidly from 10-6.5 mg/ml, but then the rate slowed after 5 hours.

The reference thermograms of the albumin standard (Figure 7) and trypsin (Figure 8) revealed a simular endothermic transition at approximately 52 °C. When the two proteins were mixed, an endothermic transition again occurred at 52 °C. There was no change in this transition after the reaction mixture had incubated for 3 hours (Figure 9). The major transition that occurs around 100 °C results from vaporization of the solvent. Therefore, when aqueous solutions are used, the maximum temperature for observed transitions is about 80 °C unless considerable effort is made to balance the reference and sample and to use special cups that are designed to contain vapor samples.

5. DISCUSSION

Trypsin was selected as the model proteolytic enzyme because it is well characterized and is quite specific. Because it cleaves proteins only at the basic amino acids like lysine and arginine, trypsin produces fewer peptides than less specific proteases. Consequently, monitoring the progress of digestion is facilitated. Trypsin's moleular weight of 24,000 is considerably less than that of albumin (65,000). Therefore, the two proteins elute from the gel filtration column at different times.

The HPLC produced two peaks for the albumin standard and another distinct peak for the trypsin standard. The position of the peaks was consistent with the molecular weights of the proteins. Albumin, which is larger, eluted more quickly than trypsin. The small peak in the albumin chromatogram was due to a

contaminant and was the first to disappear when the sample was incubated with trypsin. The ultraviolet spectrum of the peak was also consistent with a protein contaminant.

The HPLC has successfully provided a means for qualitating and quantitating the digestion of albumin. By measuring the area under the specific peak representing a protein, it is possible to calculate the amount of protein remaining undigested. This method can thus be utilized as an assay procedure for the decontamination of proteinaceous toxins.

Results obtained on the DSC showed that albumin and trypsin underwent reproducible endothermic transitions at 52 °C. When the protein was incubated with the proteolytic enzyme, there were no changes in the thermograms. Because enzymatic digestion was demonstrated in previous experiments using the HPLC, the absence of any new thermal transitions could not be due to a lack of enzymatic activity. The differential scanning calorimeter thus failed to reveal changes in the characteristic thermogram of albumin after digestion. Therefore, differential scanning calorimetry has shown to be of limited value as an assay tool for monitoring the digestion of proteins.

6. CONCLUSIONS

Using a gel filtration column, HPLC provides an excellent assay procedure for the detection of protein digestion via proteolytic enzymes. This assay will be utilized in subsequent studies to investigate the use of proteolytic enzymes as possible decontaminants of proteinaceous toxins. In contrast, differential scanning calorimetry is of limited value.

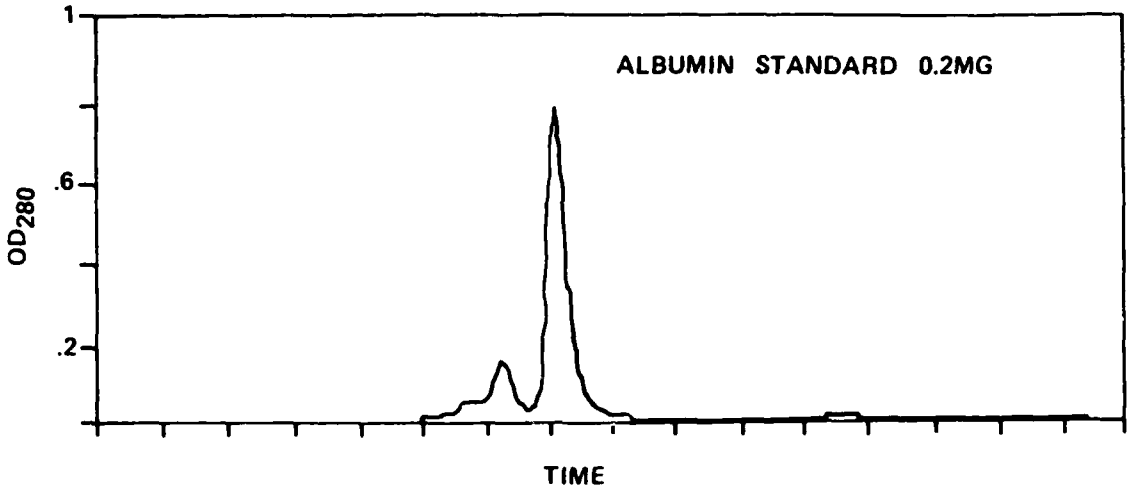


Figure 1. Albumin Standard

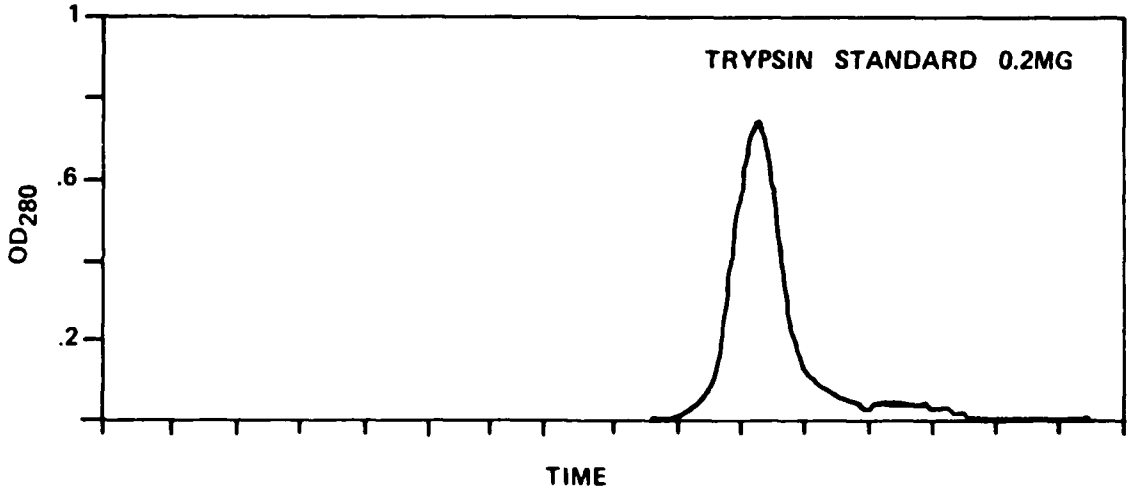


Figure 2. Trypsin Standard

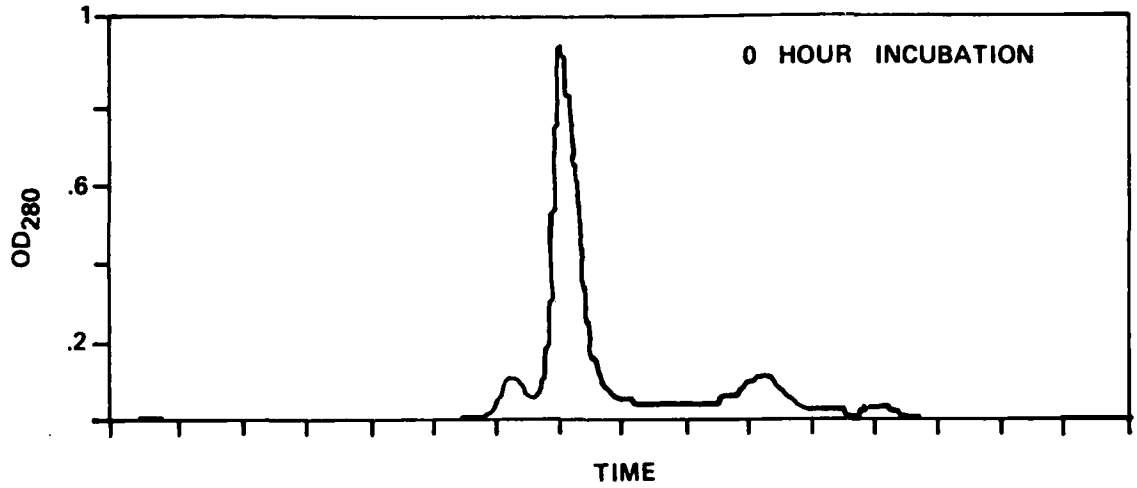


Figure 3. Reaction Mixture at 0 Hour Incubation, Containing 0.2 mg of Albumin and 0.0 mg of Trypsin

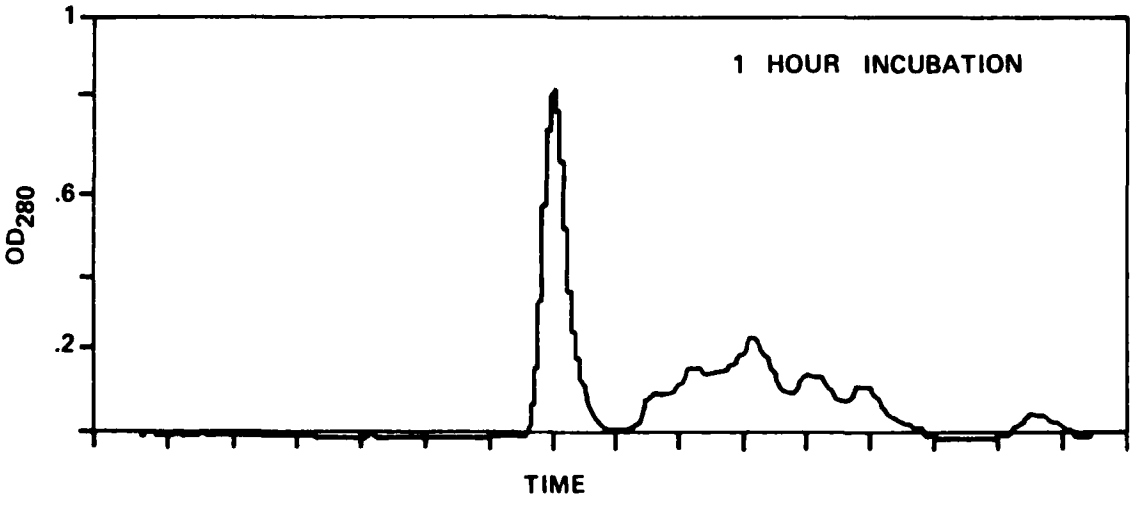


Figure 4. Reaction Mixture at 1 Hour Incubation

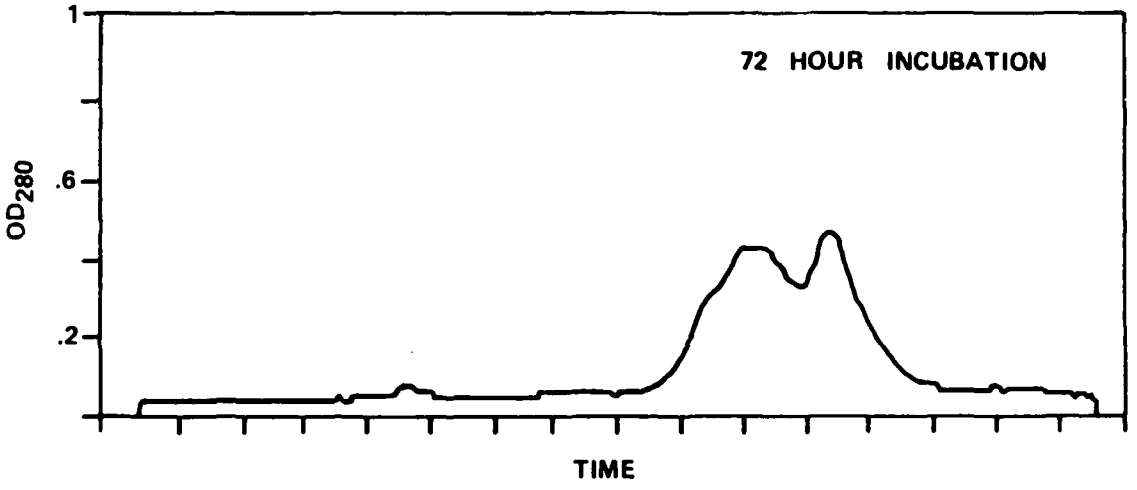


Figure 5. Reaction Mixture at 72 Hours Incubation

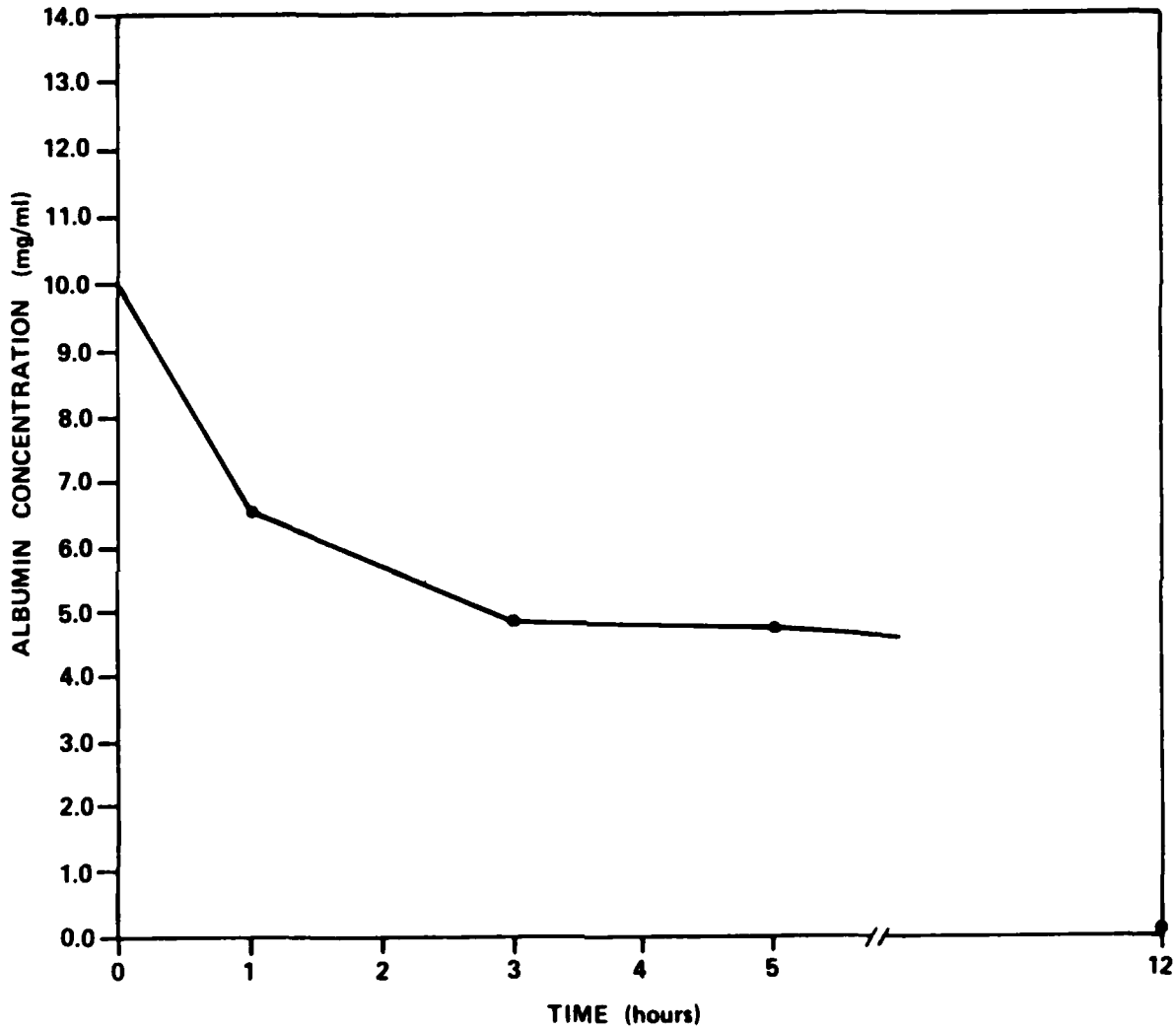


Figure 6. Albumin Concentration (mg/ml)

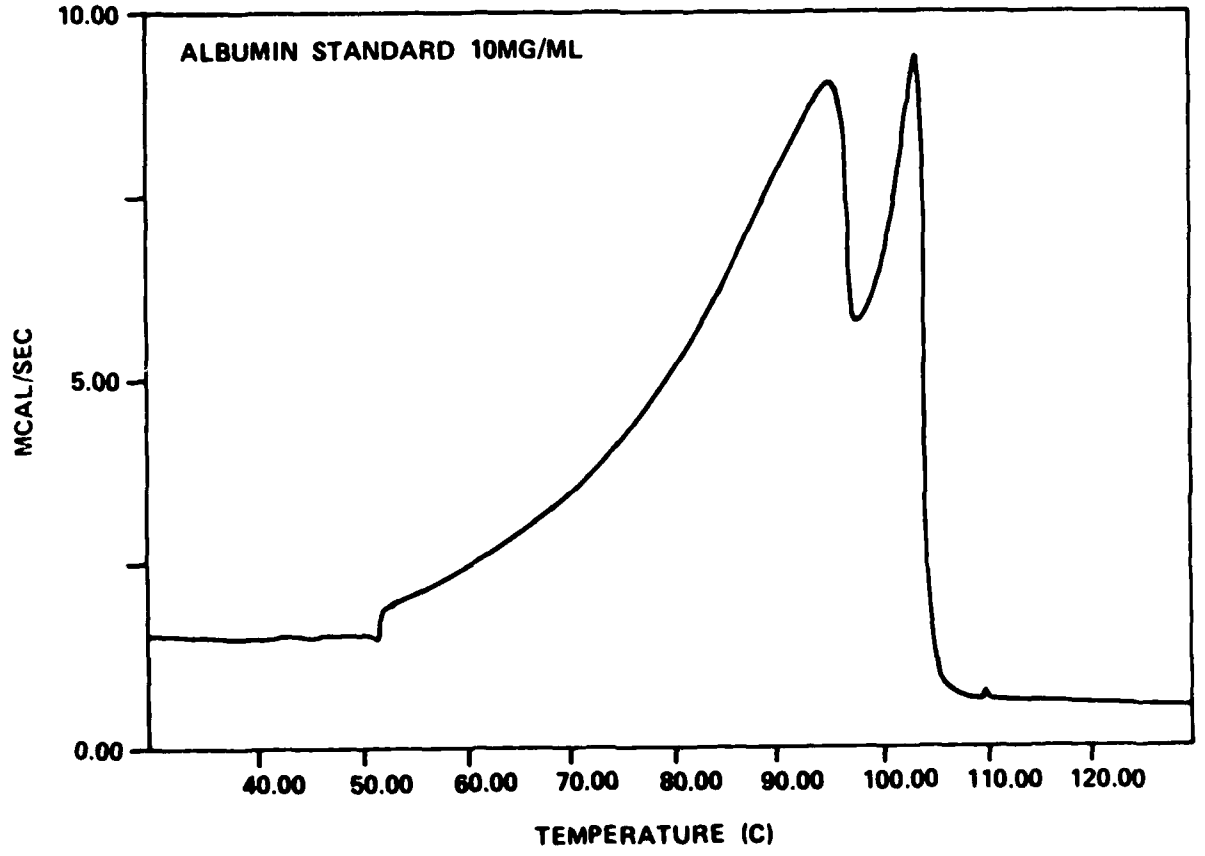


Figure 7. Thermogram 10 mg/ml of Albumin

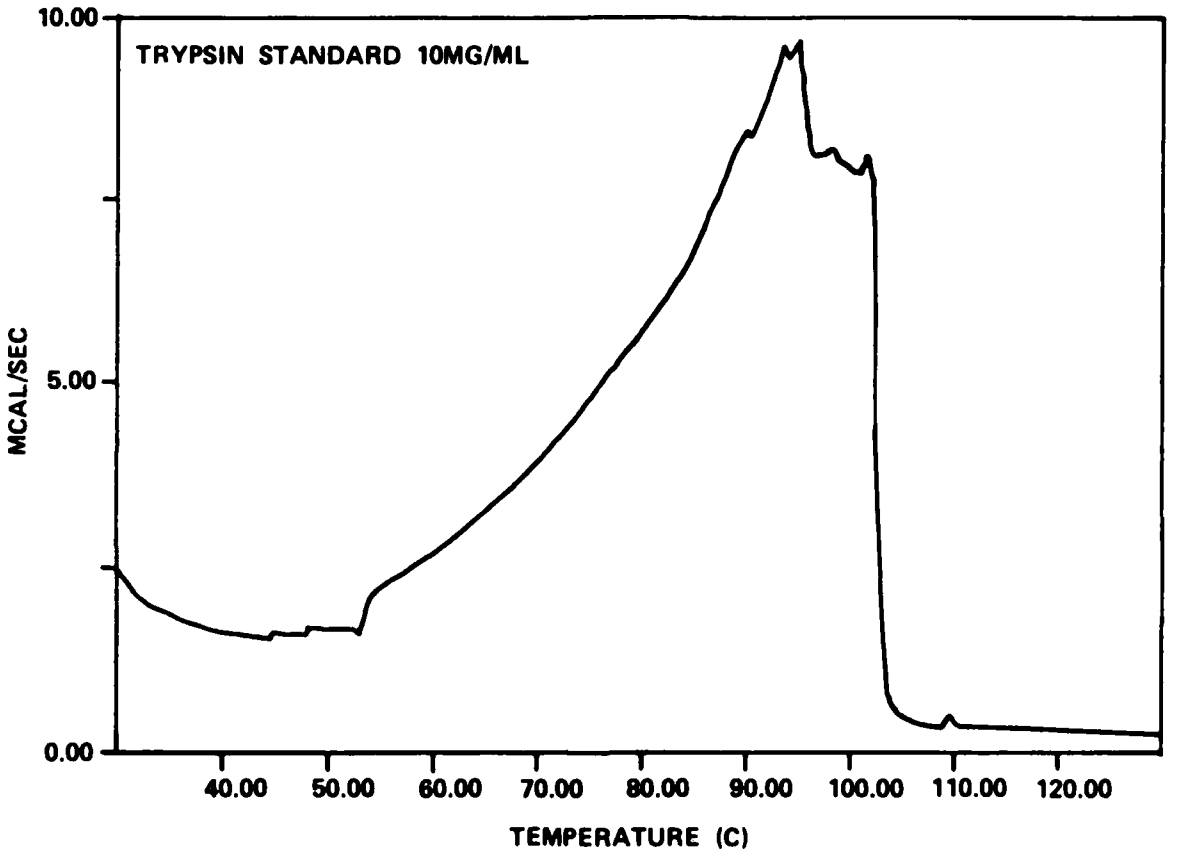


Figure 8. Thermogram for 10 mg/ml of Trypsin

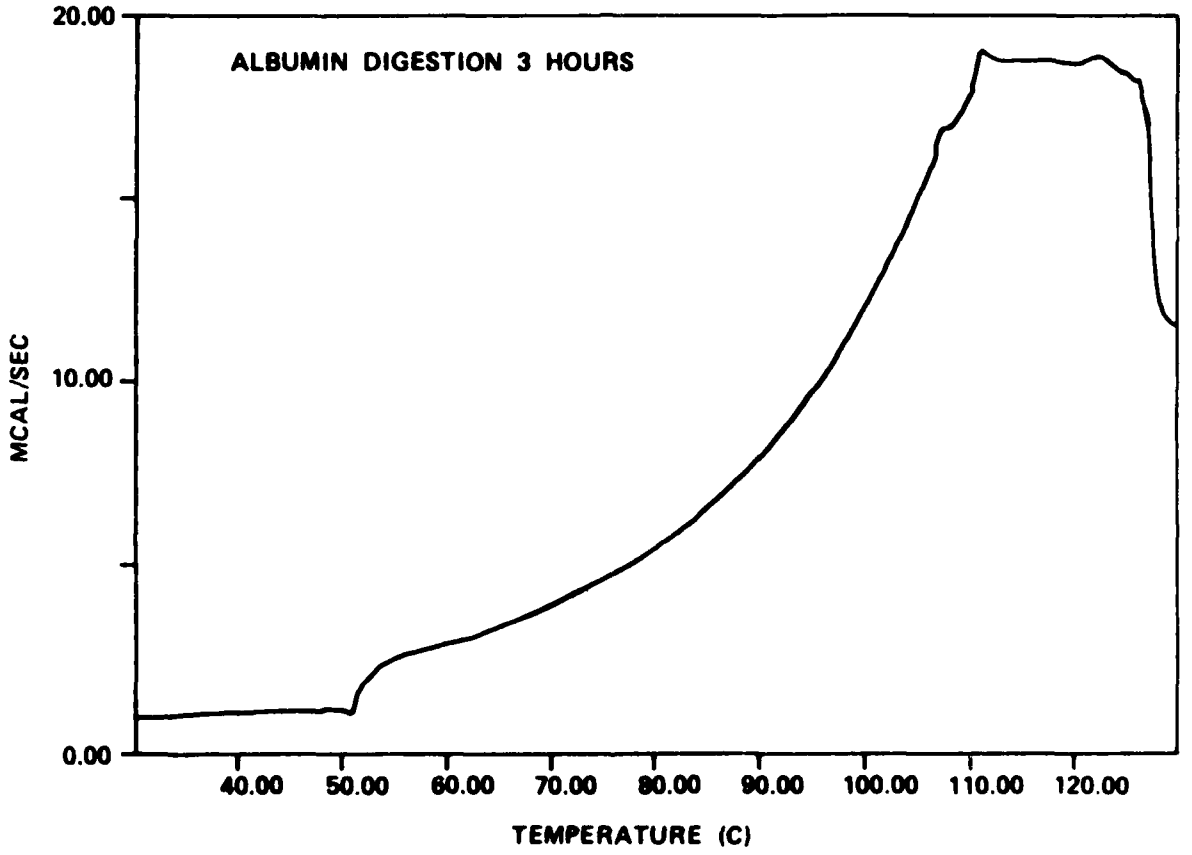


Figure 9. Thermogram Reaction Mixture at 3 Hours

Blank

LITERATURE CITED

1. The Bioseparation System for FPLC and HPLC, LKB-Produkter, Sweden, 1985.

2. Heftmann, E., eds., Chromatography, p 328. Reinhold Publishing Corp., New York, 1961.

3. Perkin Elmer Model DSC-4 Insruction Manual, p 2-1-3, Perkin Elmer Company, 1981.

4. Jacobson, A. L., and Braun, H. "Differential Scanning Calorimetry of the Thermal Denaturation of Lactate Dehydrogenase," Biochemica et Biophysica Acta 493, 142 (1977).

5. Fersht, A. Enzyme Structure and Mechanism, p 17, W. H. Freeman and Company, New York, 1977.

NOTE: For further reference, see Mahler, H. R., and Cordes, E.H. Biological Chemistry, 2nd Ed., pp 56, 77, and 766, Harper and Row, New York, 1966.

END

FILMED

FEB. 1988

DTIC