GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION
SPONSORED PROJECT INITIATION

Date: 9/6/79

Project Title: Active Site Studies on Blood Proteases

Gr Cd.
NIH

Project No: G-33-L02

Project Director: Dr. James C. Powers

Sponsor: DHEW/PHS/NIH - National Heart, Lung, & Blood Institute; Bethesda, MD 20014

Agreement Period: From 8/1/79 Until 7/31/80 (02 Year)

Type Agreement: Grant No. 5 R01 HL22530-02

Amount: $40,051 PHS Funds (G-33-L02)
2,798 GIT Contribution (G-33-349)
$42,849 TOTAL

Reports Required: Annual Progress Reports with Continuation Applications; Terminal Progress Report upon Grant Expiration

Sponsor Contact Person (s):

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PHS Grants Management Official
James M. Pike, Chief

Defense Priority Rating: None

Assigned to: Chemistry (School/Laboratory)

COPIES TO:

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CA–3 (3/76)

GEORGIA INSTITUTE OF TECHNOLOGY OFFICE OF CONTRACT ADMINISTRATION

SPONSORED PROJECT TERMINATION SHEET

Date 7/12/83

Project Title: Active Site Studies on Blood Proteases

Project No: G-33-L02

Project Director: Dr. James Powers

Sponsor: DHEW/PHS/NIH - National Heart, Lung, & Blood Institute; Bethesda, MD 20014

Effective Termination Date: 7/31/80

Clearance of Accounting Charges: 7/31/80

Grant/Contract Closeout Actions Remaining:

NONE

- ☐ **Final Invoice and Closing Documents**
- ☐ **Final Fiscal Report**
- ☐ Final Report of Inventions
- ☐ Govt. Property Inventory & Related Certificate
- ☐ Classified Material Certificate
- ☐ Other _____

NOTE: Follow-on project (03 Year) - G-33-L03

Assigned to: Chemistry (School/Laboratory)

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FORM OCA 10:781

APPLICANT: REPEAT GRANT NUMBER SHOWN ON PAGE 1 →	GRANT NUMBER	
SECTION IV—SUMMARY PROGRESS REPORT	HL 22530-03	
PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR (Last, First, Initial)	PERIOD COVERED BY THIS REPORT	
Powers, James C.	FROM	THROUGH
NAME OF ORGANIZATION		
Georgia Institute of Technology	8/1/79	5/10/80

TITLE (Repeat title shown in Item 1 on first page)

Active Site Studies on Blood Proteases

1. List publications: (a) published and not previously reported; (b) in press. Provide five reprints if not previously submitted.
2. List all additions and deletions in professional personnel and any changes in effort.
3. Progress Report. (See Instructions)

Final Report
G-33-102, Powers

1. Publications - None
2. Professional Personnel - No Changes
3. Progress Report

Objectives. The objectives of the present research are to 1) design and synthesize specific substrates for assaying blood proteases, 2) determine the kinetic parameters for reaction of various blood proteases with the substrates using purified enzymes, 3) study the nature of the extended substrate binding site in plasma proteases and 4) synthesize and study the reactivity of peptides corresponding to plasma protease cleavage sites.

The goals for the current year were to investigate synthetic methods for thiol ester substrates of plasma proteases (objective 1), to determine kinetic parameters using the thiol esters (objective 2), and to synthesize peptides corresponding to plasma protease cleavage sites (objective 4).

Thiol Ester Substrates. We have now shown that peptide thiol esters of the type RCO-Arg-SR can be used in sensitive assays for the serine proteases involved in clottin Hydrolysis at the thiol ester bond yields a thiol which can be detected with Ellman's reagent or other thiol reagents.

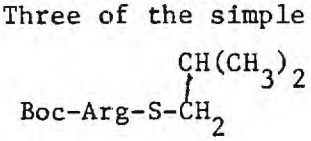
$$\text{RCO-Arg-SR} \longrightarrow \text{RCO-Arg-OH} + \text{HSR}$$

As the peptide is hydrolyzed, the absorbance at 410 nm increases as the released thiol reacts with Ellman's reagent. Since the thiol esters are good substrates for serine proteases and Ellman's reagent can detect low levels of thiol, the proposed substrates should be quite sensitive. Recently we have synthesized the thiol ester elastase substrate MeO-Suc-Ala-Ala-Pro-Val-$SCH_2C_6H_5$. Human leukocyte elastase hydrolyzes this compound very effectively and the substrate can be used to assay elastase concentration as low as 5 picomolar.

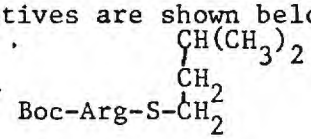
One significant advantage of thiol ester substrates is their use of P′ resides. Thus it is possible to design a substrate for an enzyme where only the new N-terminal sequence released on cleavage of its substrate is known. Thus a substrate such as Ac-Arg-SIle-Val-Gly-Gly-NH_2 could be used to study or detect enzymes that activate Factor VII. It should be noted that the Arg-SIle bond in the above substrate differs from the Arg-Ile bond in the natural substrate simply by replacement of the NH of the peptide bond with a sulfur atom.

☆U.S. GOVERNMENT PRINTING OFFICE: 1979 O— 281-217 3055

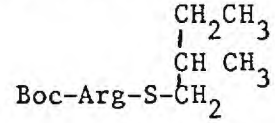
We have now completed the synthesis of a series of amino acid and peptide thiol esters. Three of the simple derivatives are shown below.



Boc-Arg-SBu-i



Boc-Arg-SPe-i



Boc-Arg-SPe-s

The structures incorporate respectively the side chains of a P_1' valine, leucine and isoleucine residue. The activation site of the zymogens of all known serine proteases involves cleavage of a Arg-Val, Arg-Ile or Arg-Leu bond. These three substrates have allowed us to study the P_1' specifically of activating serine proteases such as Factor IX_a, XI_a and XII_a.

The relative k_{cat}/K_M values for all the substrates studied are listed in the table. The substrates were studied at pH 7.5, 0.1 M HEPES buffer, 0.01 M $CaCl_2$, 9.8% v/v DMSO at 30°C with 4 clotting enzymes and trypsin. Only relative values are given in this short report, although all the kinetic parameters have been determined and will be reported in a full paper.

One highlight of the data is the discovery of two good synthetic substrates for Factor IX_a. Both the substrates (Z-Phe-Arg-SBu-i and Z-Trp-Arg-SBu-i) have a P_2 aromatic residue. No other good synthetic substrates exist for Factor IX_a. These substrates will be useful in future studies of Factor IX_a.

In addition to the Factor IX_a substrates we discovered several excellent Factor XI_a sustrates. Neither Factor IX_a or XI_a have previously been studied in any detail with synthetic substrates. We are now synthesizing tripeptide thiol esters to continue our subsite mapping studies of these two enzymes. In addition, we expect to study other enzymes such as Factor XII_a in the near future.

Peptides Corresponding to Plasma Protease Cleavage Sites. Serine proteases such as Factors IX_a, XI_a and XII_a probably recognize both a specific amino acid sequence and a specific conformation in their natural substrates. As an initial working hypothesis, I have assumed that recognition of the sequence is the most important and that synthetic substrates with the appropriate sequence should be fairly specific for individual enzymes. Thus an appropriate Factor IX_a substrate would be something with the sequence RCO-Gln-Val-Val-Arg*Ile-Val-Gly-NH_2 (*indicates the cleavage site in Factor X, Factor IX_a' substrate). Initially we have decided to concentrate all of our effort on Factor IX_a, XI_a and XII_a substrates. Very little research with synthetic substrates has been reported thus far with these enzymes and thus we may be able to gain new insights about the active sites of these enzymes. Some of the peptides which we are in the process of synthesizing are listed below:

Factor IX_a substrate (sequence of Factor X cleavage site)
Abz-Gln-Val-Val-Arg*Ile-Val-Gly-Nba

Factor XI_a substrates (sequence of Factor IX cleavage sites)
Abz-Glu-Phe-Ser-Arg*Val-Val-Gly-Nba
Abz-Lys-Phe-Ser-Arg*Val-Val-Gly-Nba

Factor XII_a substrate (P_1-P_3 corresponds to Factor XI clevage site)
Abz-Ala-Ala-Ala-Arg*Ile-Val-Gly-Nba

In each case the N-terminus of the peptide is blocked with a 2-aminobenzoyl (Abz) group and the C-terminus is protected as the 4-nitrobenzyl amide (Nba). The rate of enzymatic hydrolysis for each of these substrates will be monitored using fluorometric techniques. The aminobenzoyl group is fluorescent and the nitrobenzyl group is a quenching group. Upon hydrolysis, the quenching group is separated from the fluorescent group such that the latter freely fluoresces after excitation at an appropriate wavelength. The rate of increase in fluorescence corresponds to the rate of hydrolysis of the peptide.

For the last year we have been involved exclusively in synthesis. We have now completed the synthesis of 6-8 peptides with sequences corresponding to Factor IX_a and XI_a cleavage sites. We are now in the process of doing the kinetic studies with these peptides.

Research Goals for the Coming Year

1) Measure kinetics with the dipeptide thiol ester substrate using enzymes such as plasmin, Factor XII_a, kallibrein and $C1\bar{s}$. Synthesize tripeptide thiol ester substrates and use them to map the extended substrate binding site of Factor IX_a and XI_a.

2) Continue the synthesis of fluorescent peptide substrates corresponding to Factor IX_a and XI_a cleavage sites. Measure the kinetics of their reaction with various blood clotting serine proteases.

Significance. The plasma proteases that are being investigated in this research are involved in a number of important physiological process such as blood coagulation, fibronolysis and kinin formation. A number of clinical assays are used on the determination of the activity of various plasma proteases mostly using clotting assays. The synthetic substrates which will be developed in this research should be quite sensitive and specific and should allow assays to be developed for plasma component which are impossible currently due to the complex nature of clotting assays. Since they are spectrophotometrically or spectrofluorometrically based, they would be useable in most clinical labs, even those without extensive instrumentation.

The proposed research would lead to a better understanding of the nature of the active site structures of various plasma proteases and how they are affected by various plasma modulators such as phospholipids. This should give us a better understanding of various coagulation disorders. This in turn could lead to new avenues of therapy where various specific activators or inhibitors are used to control the activity of certain plasma proteases.

The undersigned agrees to accept responsibility for the scientific and technical conduct of the project and for provision of required progress reports if a grant is awarded as the result of this application.

May 12, 1982
Date

James C. Powers
Principal Investigator