GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION
SPONSORED PROJECT INITIATION

Date: August 21, 1978

Project Title: Active Site Studies on Blood Proteases

Project No: G-33-L01

Project Director: Dr. James C. Powers

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

Agreement Period: From 8/1/78 Until 7/31/79 (01 Year)

Type Agreement: Grant No. 1 R01 HL22530-01

Amount: $45,949 New PHS Funds (G-33-L01)
4,460 GIT Contribution (G-33-331)
$50,409 TOTAL

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

Sponsor Contact Person (s):

Technical Matters

Wolf W. Zuelzer (Dr. Ann Ball)
Director
Division of Blood Diseases and Resources
National Heart, Lung, and Blood Institute
Bethesda, MD 20014

Phone: (301) 496-5911

Contractual Matters
(thru OCA)

Mr. Geoffrey Grant
Grants Management Officer
Grants Operation Branch
Division of Extramural Affairs
National Heart, Lung, & Blood Institute
Bethesda, MD 20014

Phone (301) 496-7255

Defense Priority Rating: None

Assigned to: Chemistry (School/Laboratory)

COPIES TO:

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- Project Code (GTRI)
- Other

CA-3 (3/76)

GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION

SPONSORED PROJECT TERMINATION

Date: September 6, 1979

Project Title: Active Site Studies on Blood Proteases

Project No: G-33-L01

Project Director: Dr. James C. Powers

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

Effective Termination Date: 7/31/79 (01 year)

Clearance of Accounting Charges: 7/31/79

Grant/Contract Closeout Actions Remaining:

_ Final Invoice and Closing Documents
_ Final Fiscal Report
_ Final Report of Inventions
_ Govt. Property Inventory & Related Certificate
_ Classified Material Certificate
x Other Annual Report of Expenditures (01 year) due no later than 10/31/79.

NOTE: FOLLOW-ON PROJECT IS G-33-L02 (02 YEAR).

Assigned to: Chemistry (School/Laboratory)

COPIES TO:

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Other_____

CA-4 (1/79)

SECTION IV

APPLICANT: REPEAT GRANT NUMBER SHOWN ON PAGE 1 →

GRANT NUMBER HL22530-02

SECTION IV—SUMMARY PROGRESS REPORT

PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR (Last, First, Initial)
Powers, James C.

PERIOD COVERED BY THIS REPORT

FROM	THROUGH
8/1/78	5/1/79

NAME OF ORGANIZATION
Georgia Institute of Technology

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)

1. None

2. The project has just been started. Two graduate research associates, Brian McRae and Mario Castillo, began work on the project during the year. Charles Vencill, a graduate research associate, will begin this summer.

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 program of study has just been started and goals for the current year were to investigate synthetic methods for thiol ester substrates of plasma proteases (objective 1) and to synthesize peptides corresponding to plasma protease cleavage sites (objective 4).

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's substrate). Intially 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

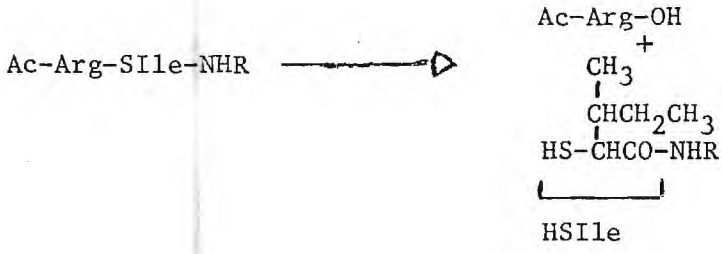
☆ U. S. GOVERNMENT PRINTING OFFICE : 1978 260-899/3198

Factor XII_a substrate (P_1-P_3' corresponds to Factor XI cleavage 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.

The synthesis of three shorter peptides (Abz-Val-Val-Arg*Ile-Val-Gly-Nba, Abz-Val-Val-Arg*Ile-Val-Nba, Abz-Val-Arg*Ile-Val-Gly-Nba) has already been completed. The reactivity of the three peptides toward Factor IX_a, X_a, and XI_a were measured by Dr. K. Kurachi and Dr. E. Davie at the U. of Washington. Although they were effectively hydrolyzed by trypsin, they were poor substrates for Factors IX_a, X_a and XI_a. These findings inspired the synthesis of peptides with an extended N-terminus such as Abz-Glu-Val-Val-Arg*Ile-Val-Gly-Nba which is in progress. We believe P_4-P_3' interactions may critical for substrate recognition by the various clotting enzymes.

Specific Substrates-Thiol Esters. Peptide thiol esters such as Ac-Arg-SIle-NHR (SIle = $-SCH_2(C_4H_9)CO-$) should be useful in sensitive assays for the serine proteases involved in clotting. Hydrolysis at the Arg-SIle bond would yield a thiol which can be detected with Ellman's reagent.

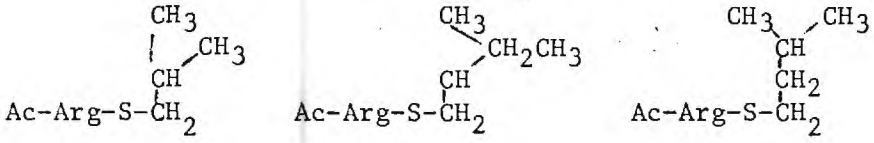


As the peptide is hydrolyzed, the absorbance at 410 nm increases as the released thiol reacts with Ellman-s reagent. Since thiol esters are good substrates for serine proteases and Ellman's reagent can detect low levels of thiol, the proposed substrates should be quite sensitive. Recently we have synthesized 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 concentrations 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 sulfuratom.

Our first synthetic goal was Ac-Arg-SIle-Val-Gly-NH_2. The synthesis of HSIle-Val-Gly-Gly-NH_2 (HSIle = $HSCH_2(C_4H_9)CO-$) was readily accomplished. How-

ever we have thus far been unable to couple this thiol with a number of blocked arginine derivatives. We then decided to work out the synthetic methods with a series of simplier arginine thiol esters (1, 2 and 3).



1 2 3

The structures incorporate respectively the side chains of a P_1' valine, isoleucine and leucine 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 substrate will thus allow us to study the P_1' specificially of activating serine proteases such as Factor IX_a, XI_a and XII_a.

We have completed the synthesis of 1 and are currently working on the synthesis of 2 and 3. Purfication of reaction products is our major difficulty. We expect high pressure liquid chromatography to be extremely useful in this regard and are currently constructing a system to be used in the purification of arginine peptides. Once we have worked out the methods for the synthesis and purification of 1, 2 and 3, we will return to the synthesis of larger thiol esters such as Ac-Arg-SIle-Val-Gly-Gly-NH_2.

The kinetics of reaction of 1 with trypsin (K_{cat}/K_m = 2.9 X 10^6 M^{-1} s^{-1}) have been measured at pH 7.5, but no kinetic work has been yet done with any of the clotting factors.

Research Goals for the Coming Year.

1) Complete the synthesis of several fluorescent peptide substrates and measure the kinetics of their reaction with various blood clotting serine proteases.

2) Develop methods for the synthesis and purification of arginine thiol esters. Study the reaction of simple derivatives with various clotting factors and begin the synthesis of longer arginine thiol esters.

Significance. The plasma proteases that will be investigated in the proposed research are involved in a number of important physiological processes such as blood coagulation, fibrinolysis and kinin formation. A humber of clinical assays are based 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 usable 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 a s phospholipids. This should give us a better understanding of various diseases such as coagulation disorders and tumors

which release the plasminogen activator. 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 17, 1979
Date

James C. Powers
Principal Investigator or
Program Director

Prepared for the Science Information Exchange.
Not for publication or publication reference.

U. S. Department of
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE

NOTICE OF RESEARCH PROJECT

PROJECT NO. (DO NOT USE THIS SPACE)

TITLE OF PROJECT

Active Site Studies on Blood Proteases

GIVE NAMES, DEPARTMENTS, AND OFFICIAL TITLES OF PRINCIPAL INVESTIGATORS OR PROJECT DIRECTORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT.

James C. Powers
Professor of Chemistry
School of Chemistry

NAME AND ADDRESS OF APPLICANT INSTITUTION

Georgia Institute of Technology
Atlanta, Georgia 30332

SUMMARY OF PROPOSED WORK—*(200 words or less—Omit Confidential data.)*

In the Science Information Exchange summaries of work in progress are exchanged with government and private agencies supporting research in the bio-sciences and are forwarded to investigators who request such information. Your summary is to be used for these purposes.

The objective of the proposed research is to prepare a series of sensitive and specific substrates for assaying blood proteases such as Factors VII_a, IX_a, X_a, XI_a, XII_a, plasmin, thrombin, kallikrein and the plasminogen activator. These proteins are the basis of important physiological processes such as blood clotting, fibrinolysis, the complement system and kinin release. Mapping of the extent of the extended substrate binding region in these proteases will be undertaken with synthetic substrates. The proposed research will attempt to determine the reasons for the high degree of substrate specificity of plasma proteases. The synthetic substrates also will be used to study how other plasma components such as phospholipids modulate the activity of these enzymes. The proposed research may lead to new methods for disease detection or to new mechanisms for the control or modulation of plasma protease activity in various disorders.

PROFESSIONAL SCHOOL *(medical, dental, etc.)* WITH WHICH THIS PROJECT SHOULD BE IDENTIFIED	SIGNATURE OF PRINCIPAL INVESTIGATOR	DATE
College of Sciences and Liberal Studies	James C Powers	5/17/79

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SUPPORTING AGENCY

METHOD OF SUPPORT *(Check one)*

☐ Agency Staff (Intramural) ☐ Negotiated Contract ☐ Special Project Grant ☐ Research Grant ☐ Other (Specify)

FUNDS OBLIGATED CURRENT F.Y.	NUMBER OF FUTURE YEARS TENTATIVELY ASSURED	BEGINNING DATE	ESTIMATED COMPLETION DATE

PHS-166 (Formerly NIH-1946)
REV. 9-74