GEORGIA INSTITUTE OF TECHNOLOGY — OFFICE OF CONTRACT ADMINISTRATION

PROJECT ADMINISTRATION DATA SHEET

[X] ORIGINAL [ ] REVISION NO. ____

Project No. G-33-L06 GTRC/GIT DATE 8 / 25 / 83

Project Director: Dr. J. C. Powers School/XXX Chemistry

Sponsor: PHS/NIH National Heart, Lung, and Blood Institute

Type Agreement: Grant No. 5R01HL22530-06

Award Period: From 8/1/83 To 10/31/84 (Performance) 10/31/84 (Reports)

Sponsor Amount:	This Change	Total to Date
Estimated:	$ 69,087	$ 69,087
Funded:	$ 69,087	$ 69,087

Cost Sharing Amount: $ 3,636 Cost Sharing No: G-33-382

Title: "Active Site Studies on Blood Proteases"

ADMINISTRATIVE DATA

OCA Contact Frank Huff X4820

1) Sponsor Technical Contact:

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Defense Priority Rating: N/A Military Security Classification: N/A

(or) Company/Industrial Proprietary: ____

RESTRICTIONS

See Attached NIH Supplemental Information Sheet for Additional Requirements.

Travel: Foreign travel must have prior approval – Contact OCA in each case. Domestic travel requires sponsor approval where total will exceed greater of $500 or 125% of approved proposal budget category.

Equipment: Title vests with N/A; none proposed.

COMMENTS:

Follow-on to G-33-L05. Includes $1,333 unobligated balance from prior budget period (year 05). Award dated 8/2/83 authorizes $46,243 in direct costs.

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FORM OCA 4-383

GEORGIA INSTITUTE OF TECHNOLOGY OFFICE OF CONTRACT ADMINISTRATION

SPONSORED PROJECT TERMINATION/CLOSEOUT SHEET

Date 7/22/85

Project No. G-33-L06 School/~~Lab~~ Chem

Includes Subproject No.(s)

Project Director(s) J. C. Powers ~~GTRC~~/ GIT

Sponsor DHHS/PHS/NIH/NHLBI

Title Active Site Studies on Blood Proteases

Effective Completion Date: 10/31/84 (Performance) 1/31/85 (Reports)

Grant/Contract Closeout Actions Remaining:

- [ ] None
- [X] Final Invoice or Final Fiscal Report
- [ ] Closing Documents
- [X] Final Report of Inventions
- [ ] Govt. Property Inventory & Related Certificate
- [ ] Classified Material Certificate
- [ ] Other

Continues Project No. G-33-L05 Continued by Project No.

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ACTIVE SITE STUDIES ON BLOOD PROTEASES

FINAL REPORT (8/1/83 through 10/31/84)

HL22530-06 (G-33-L06)

by

James C. Powers

School of Chemistry

Georgia Institute of Technology

Atlanta, GA 30332

Inventions. None to report.

Publications Since the Last Report.

"Kinetics of Hydrolysis of Peptide Thioester Derivatives of Arginine by Human and Bovine Thrombins," R. R. Cook, B. J. McRae, and J. C. Powers (1984) Arch. Biochem. Biophys. 234, 82-88.

"Peptide Thioesters and 4-Nitroanilides as Substrates for Porcine Pancreatic Kallikrein," J. C. Powers, B. J. McRae, T. Tanaka, K. Cho, and R. R. Cook (1984) Biochem. J. 220, 569-573.

"Active Site Mapping of Bovine and Human Blood Coagulation Serine Proteases Using Synthetic Peptide 4-Nitroanilide and Thioester Substrates," K. Cho, T. Tanaka, R. R. Cook, W. Kisiel, K. Fujikawa, K. Kurachi, and J. C. Powers (1984) Biochemistry 23, 644-650.

"Mammalian Tissue Trypsin-like Enzymes. Comparative Reactivities of Human Skin Tryptase, Human Lung Tryptase and Bovine Trypsin with Peptide 4-Nitroanilide and Thioester Substrates," T. Tanaka, B. J. McRae, K. Cho, R. Cook, J. E. Fraki, D. A. Johnson, and J. C. Powers (1983) J. Biol. Chem. 258, 13552-13557.

"Benzyl p-Guanidinothiobenzoate Hydrochloride, A New Active-site Titrant for Trypsin and Trypsin-like Enzymes," R. R. Cook and J. C. Powers (1983) Biochem. J. 215, 287-294.

Progress Report.

Scientific Goals. The goal of this research was an understanding of the nature of the active sites of plasma serine proteases and other related trypsin-like enzymes. These proteins are the basis of important physiological processes such as blood clotting, fibrinolysis and the immune defense mechanism involving the complement system. The tools for the investigation were synthetic peptides. Synthetic peptides corresponding to sites which are cleaved by various plasma proteases were prepared. The binding of these peptides to plasma proteases and their rates of hydrolysis was determined. In addition we designed and synthesized small peptide thioesters which are more reactive toward serine proteases than simple peptides. These were utilized to develop more sensitive and specific assays for individual enzymes.

Subsite Mapping of Coagulation Serine Proteases. The PI has carried out extensive subsite mapping studies of the coagulation serine proteases (thrombin, factor XIIa, factor XIa, factor Xa, factor IXa, plasma kallikrein, and activated protein C) and other trypsin-like enzymes using synthetic peptide substrates. These studies have yielded two major results. First, assay systems using synthetic peptide substrates for most of the coagulation enzymes have been developed in the laboratories of the PI. Thus, highly reliable and sensitive synthetic substrates are available for most of the enzymes which are involved in coagulation. Second, studies with synthetic substrates have yielded information on the subsite preferences of the various enzymes. In general, good substrate sequences make good inhibitor sequences and the subsite information will be invaluable in choosing the appropriate inhibitor sequences to investigate in future studies of anticoagulant drugs.

Three types of substrates were utilized in the subsite mapping studies: peptide thioesters, peptide 4-nitroanilides, and fluoroescent-quench peptide

substrates. Each group of substrates has its own particular advantages and disadvantages. Peptide thioesters are extremely reactive toward serine proteases. Cleavage of the thioester bond by the enzyme yields a thiol which is continuously determined spectrophotometrically by measuring the release of a chromogenic product formed upon reaction with a thiol reagent such as 4,4'-dithiodipyridine contained in the assay mixture. Thioesters are good substrates for serine proteases and since thiol reagents can detect low levels of thiol, the substrates are quite sensitive. They are usually more reactive than the corresponding nitroanilides or aminomethylcoumarins and can detect concentrations as low as 1-5 picomolar.

At the other extreme of the spectrum are peptide substrates where the bond being hydrolyzed is a simple peptide bond. This type of substrate is extremely specific and the specificity for a particular protease is determined by the amino acid sequence of the substrate. However the hydrolysis rates are quite low compared to other types of substrates and there are no convenient ways of following the hydrolysis reaction. It is possible to follow the reaction by carrying out ninhydrin or fluorescamine assays, but these are time consuming and not suitable for a continuous assay. A very valuable tool for measuring rates is the incorporation of a fluorescent and a fluorescence quenching groups on opposite ends of the peptide chain of a substrate. Such a substrate has low intrinsic fluorescence due to internal quenching. Hydrolysis of any bond in the substrate then results in separation of the fluorophore and the quenching group with the resultant increase in fluorescence which can be followed continuously. This type of substrate has the advantage over the other two groups of taking advantage of recognition by the enzyme for both P and P' residues. These substrates are highly specific, but suffer from the inherent low reactivity of the peptide bond.

The middle group of substrates consists of activated amides of peptides and includes 4-nitroanilides, aminomethylcoumarins, methoxynapthyl amides, and aminoquinolines. Both chromogenic and fluorogenic substrates are found in this group and are widely used in clinical and research laboratories since a number of these substrates are commercially available. Activated amides are intermediate in specificity and reactivity between the peptide bond and peptide thioesters and are a good compromise in the choice of a suitable substrate for a particular proteases. However there are a number of enzymes such as factor IXa for which no suitable activated amide substrate has been discovered.

We have investigated all three types of substrates and have discovered substrates that range from highly specific fluorescent quench substrates for factor XIa to extremely sensitive peptide thioester substrates for all the trypsin-like enzymes that we have studied.

Fluorescence-Quench Substrates for Factor IXa and Factor XIa. We have utilized the published activation site sequence of bovine factor IX and X to synthesize a number of peptides specifically designed respectively as substrates for bovine factors XIa and IXa (Castillo et al., 1983). The substrates contain a fluorophore (2-amininobenzoyl, Abz) and a quenching group (4-nitrobenzylamide, Nba) that are separated upon enzymatic hydrolysis with a resultant increase in fluorescence that was utilized to measure hydrolysis rates. Some of the data is shown below.

-Gln-Val-Val-Arg*Ile-Val-Gly-Gly- factor X activation site sequence
-Glu-Phe-Ser-Arg*Val-Val-Gly-Gly- factor IX activation site sequence

Factor XIa Substrates

	k_{cat}/K_M
Abz-Glu-Phe-Ser-Arg*Val-Val-Gly-Nba	21,000 $M^{-1}s^{-1}$
Ac-Glu-Phe-Ser-Arg*Val-Val-Gly-NH_2	32,000
Abz-Glu-Phe-Ser-Arg*Val-Val-Nba	4,500
Abz-Leu-Thr-Arg*Val-Val-Gly-Nba	26,000

The kinetic behavior of factor XIa toward the synthetic peptides substrates indicates that it has a minimal extended substrate recognition site of five residues and has favorable interactions over seven subsites. The first substrate was one of the most reactive and has the factor IX activation site sequence. The last substrate, which was the most reactive fluorogenic substrate, is partially based on a second cleavage site of factor IX by factor XIa. The third substrate was the most specific and was not hydrolyzed by factors IXa or Xa or thrombin.

Factor IXa failed to cleave any of the synthetic peptides bearing the activation site sequence of factor X. However it slowly cleaved four hexa- and heptapeptide substrates with factor IX activation site sequences. The most reactive was Abz-Phe-Ser-Arg-Val-Val-Gly-Nba with k_{cat}/K_M = 550 $M^{-1}s^{-1}$.

Peptide Thioesters. We have extensively studied the reaction of peptide thioesters with trypsin-like enzymes. The subsite specificity of the various enzymes have been mapped using over 20 amino acid and peptide thioesters containing a P1 Arg residue (Powers et al., 1984; Cho et al., 1984). Most of the substrates are dipeptides and included 12 representative amino acid residues in the P2 position. Some of the substrates were extended in the P' direction to include P1' and P2' residues, and some of the substrates were extended in the P direction to include P3 and P4 residues. Kinetic measurements have been made on at least 17 substrates with the following enzymes: bovine factor IXa, Xa, XIa, XIIa, human factor XIIa, activated bovine and human protein C, bovine and human thrombin, bovine plasma kallikrein, porcine granular kallikrein, human lung and skin tryptase, bovine pancreatic trypsin, bovine acrosin, activated human C1r and C1s. This is the first time that kinetic constants have been obtained with the same set of substrates with such a large number of closely related trypsin-like enzymes (or any other group of enzymes for that matter). This was only possible because of the high sensitivity of peptide thioesters which allowed us to carry out complete kinetic studies using only trace amounts of the enzymes. The only coagulation protease which we have not studied is factor VIIa and we intend to remedy that situation as soon as we are able to obtain some enzyme.

It is not possible to discuss all of the results which we have obtained with peptide thioesters in a short space. But the following are the most interesting observations. In almost all cases, peptide thioesters are the most reactive substrates for a particular protease and are also likely the most sensitive. We have yet to find a coagulation protease that would not hydrolyze several thioester substrates. Thus, we were able to obtain kinetic data with poorly reactive coagulation proteases such as factor IXa and discovered a very useful substrate for this enzyme. Some of the enzymes such as factor IXa

hydrolyzed only a few of the substrates, while others such as factor XIa and factor XIIa hydrolyzed almost all the substrates. Thiobenzyl ester substrates are most useful for assays of purified enzymes since many of the peptide thioesters reacted with all of the proteases studied and certainly with the more reactive enzymes such as thrombin. The low specificity of thioester substrates is balanced by the fact that there are frequently no other substrates for coagulation proteases with low reactivity.

Nitroanilide Substrates. We have also studied nitroanilide substrates since they offer a compromise between peptide thioesters and peptides in both reactivity and specificity. A indication of the comparative reactivity of the various leaving groups can be gained by comparing the k_{cat}/K_M for the factor IXa hydrolysis of Z-Trp-Arg-SBzl (340,000 $M^{-1}s^{-1}$), Z-Trp-Arg-NA (NA = nitroanilide, 110), and Z-Trp-Arg-AMC (AMC = aminomethylcoumarin, 73). The thiobenzyl ester is much more reactive, but less specific.

We have used a series of fourteen tripeptide 4-nitroanilide substrates of the type Z-AA-Gly-Arg-NA and Z-AA-Phe-Arg-NA where AA = Ala, Asn, Glu, Lys, Phe, Pro, or Ser to map the S3 subsite of bovine thrombin, factor IXa, factor Xa, factor XIa, human factor XIIa, and activated bovine and human protein C. Kinetic constants for the enzymatic hydrolysis of the substrates by each enzyme were determined and used to compare the relative reactivities of the individual enzymes. Most of the enzymes reacted with all the substrates, although a few showed considerable specificity. Human factor XIIa showed the highest reactivity of all the coagulation proteases studied and was also very substrate specific. The best substrate was Z-Lys-Phe-Arg-NA with k_{cat}/K_M = 140,000 $M^{-1}s^{-1}$. Activated bovine protein C (best substrate = Z-Ser-Phe-Arg-NA), factor Xa (best substrate = Z-Glu-Gly-Arg-NA) and thrombin (best substrate = Z-Lys-Gly-Arg-NA) were the group of enzymes that showed next highest reactivity toward the substrates. Bovine activated protein C, thrombin, and factor Xa displayed relatively little substrate specificity. Activated human protein C (best substrate = Z-Ser-Phe-Arg-NA) and factor XIa (best substrate Z-Glu-Gly-Arg-NA) are moderately reactive enzymes.

Conclusion. This research has led to a better understanding of the nature of the active site structures of various plasma proteases. We have obtained basic information concerning the interaction of these proteases with their substrates and natural inhibitors, and how their activity is affected by various plasma modulators such as phospholipids. The synthetic substrates which have been developed in this research are quite sensitive and should allow assays to be developed for plasma components which are impossible currently due to the complex nature of clotting asays. This research may lead to new methods of disease diagnosis and could lead to new avenues of therapy where various specific activators or inhibitors are used to control the activity of the proteases involved in important physiological processes such as blood coagulation, fibrinolysis and complement.