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Coagulation Enzymes of The Horseshoe Crab (*Limulus polyphemus*)

by

Joseph Chang-Ron Chen

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF CLINICAL LABORATORY SCIENCE

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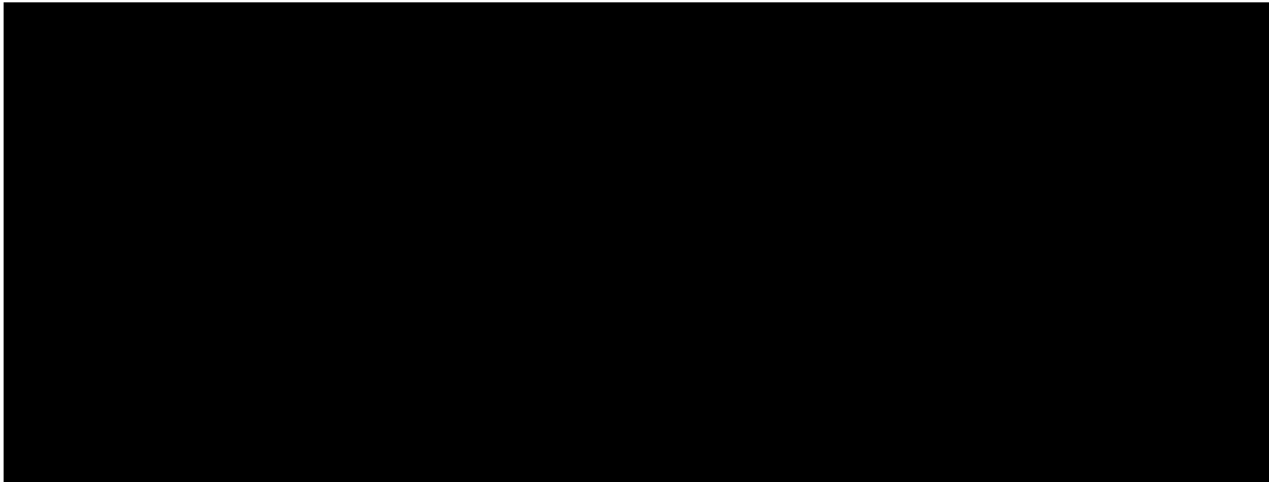

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DEDICATION

This manuscript is dedicated to my beloved mother, **Juey-In Chen,**

for her encouraging and ever-lasting love.

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INTRODUCTION

The blood coagulation system of *Limulus polyphemus*, the horseshoe crab, is contained in the granules of the amebocyte, the single type of circulating blood cell[1]. Unlike the mammalian coagulation system, all of the components of coagulation of the horseshoe crab are located within the amebocytes[2]; cell-free plasma of the hemolymph does not clot and is not required for coagulation[3]. Since horseshoe crabs live in an environment that contains many gram-negative marine bacteria, amebocytes also provide a cellular defense against those microorganisms and contribute to a disruption of hemostasis by aggregation and degranulation[1,2,4]. Degranulation of amebocytes results in release of the clotting factors which can be activated by gram-negative bacteria, endotoxin or the lipid A portion of endotoxin. In addition to these coagulation capabilities, *Limulus* amebocytes have been shown to have phagocytic, bactericidal and platelet-like aggregation properties. These findings indicate that the amebocyte may be a precursor of the mammalian monocytic, neutrophilic and platelet cell lines.

Bacterial endotoxin, an amphiphilic macromolecule at the outer membrane of gram-negative bacterial cell wall, is termed lipopolysaccharide (LPS)[5]. Chemically, LPS consist of a lipid component, termed lipid A, linked to a hydrophilic polysaccharide portion[6]. The polysaccharide portion, which is also subdivided into the O-specific chain and the core oligosaccharide, is responsible for the antigenic properties. The lipid A component expresses all of the physiological properties of intact endotoxin[7]. LPS has been demonstrated not only to activate blood coagulation, but also to activate human kallikrein and complement systems[8-10]. LPS also causes a wide spectrum of acute pathophysiological effects in susceptible hosts, including humans. These effects include fever, hypotension, disseminated intravascular coagulation (DIC), and the Shwartzman reaction. Biological analyses have demonstrated that polysaccharide free, lipid A is responsible for activation of the *Limulus* amebocyte clotting system[7,11,12], while the

polysaccharide portion was demonstrated to lack any endotoxic properties. The mechanism by which coagulation is activated by endotoxin, a molecule with no known enzymatic capabilities, is unclear.

The use of N-ethyl maleimide (NEM) to prevent amebocyte aggregation [13, 14] allows preparation of washed *Limulus* amebocytes without their disruption. Subsequently, lysates of amebocytes can be prepared. *Limulus* amebocyte lysates, prepared from NEM washed amebocytes, lysed in sterile, pyrogen-free distilled water and centrifuged to remove cellular debris[13], contain all of the necessary clotting factors and are gelled by endotoxin. The process of gelation can be observed visibly as the lysate solution, which is initially clear, progresses through stages of flocculation and increasing viscosity to a semi-solid gel[15]. During this process, protease zymogens have been shown to be activated[16-18]. The relationships between the generation of activated enzyme(s) in *Limulus* lysate by endotoxin and the visible changes of gelation of *Limulus* lysate, however, have not been described.

Gelation of *Limulus* lysate is the basis of the most sensitive assay, termed "Limulus Test", for bacterial endotoxins. The *Limulus* Test has been correlated with a wide variety of other biological assays for endotoxin, including pyrogenicity, mitogenicity, complement activation, chick embryo lethality, dermal Shwartzman reaction and tissue factor generation. [19-22]. Since there is a gradual change from a clear sol to an opaque gel in this assay, one can quantitate the concentration of endotoxin by measuring increasing turbidity over time[23] and comparing the rate of increase in turbidity with that produced by a series of known endotoxin dilutions in normal saline[15]. The *Limulus* Test has been used for detecting pg-ng/ml range concentrations of endotoxin in clinical and industrial settings and also been used as a model for mammalian coagulation [3, 11, 13, 24-27].

Progress in studying endotoxin-mediated coagulation systems has been made in *Limulus polyphemus* and several other related species of horseshoe crabs (*Tachypleus tridentatus*, *Tachypleus gigas* and *Carcinoscorpius rotundicauda*). Utilizing techniques to maintain strict endotoxin-free conditions[13,15] and several chromatographic methods[16,28,29], a series of blood coagulation factors with enzymatic activities have been identified in *Limulus*. The isolation, purification and characterization of some of the amebocyte clotting proteins from *Limulus* have provided evidence for a cascade of enzymatic activities[16,29-31]; similar proteins have been described in some of the related horseshoe crab species[32-36]. Currently, a cascade of at least three coagulation proteins has been identified and characterized in *Limulus*: a clottable protein designated "coagulogen" and the proteases, clotting enzyme and clotting enzyme activator[16,29]. A fourth factor, Protease N, has been incompletely described[29].

Studies involving gelation of *Limulus* lysate have demonstrated that appropriate concentrations of endotoxin or carboxymethylated forms of β-D-glucan can initiate the enzymatic cascade related to gelation[28,37,38]. Once formed, the gel does not lyse spontaneously[13], nor after the addition of plasmin[39]. Although some laboratories reported that the gel is insoluble in 8M urea or 2-mercaptoethanol[28,40] some authors have indicated it is soluble in 30% acetic acid or 6M urea[39,41]. The reason for these disparate observations is unclear, but suggests the need for further detailed studies.

The *Limulus* clottable protein, coagulogen, has been purified by gel filtration chromatography and comprises approximately 50% of soluble lysate proteins[28,32]. It is highly basic (pI=10) and has a molecular weight reported to range from 20-27,000 daltons, with no detectable carbohydrate. During activation of *Limulus* amebocyte lysate by bacterial endotoxin, coagulogen undergoes limited proteolytic cleavage, releasing a small C-peptide (M.W., 5,500) followed by formation of a coagulin gel[42]. Either purified *Limulus* clotting enzyme or trypsin can gel coagulogen[41,42]. Endotoxin and

carboxymethylated *B*-D-glucans, both of which can initiate the gelation of *Limulus* lysate, do not react directly with coagulogen.

Limulus clotting enzyme is a trypsin-like serine protease which proteolytically activates coagulogen. *Limulus* clotting enzyme also has restricted specificities towards synthetic peptide substrates which are very similar to those of mammalian factor Xa, but with different relative activities for those substrates[16]. Clotting enzyme has been isolated by a number of chromatographic techniques and has an estimated monomer molecular weight varying from 40,000 to 150,000 daltons[18,30,31,43]. It is an acidic (pI=5.5), heat labile protein and can be inactivated at 80°C[30]. A third protease coagulation factor termed factor B[32] or Proactivator[16] has recently been detected by fractionation of horseshoe crab lysate on heparin-Sepharose. Factor B isolated from *Tachypleus* has a molecular weight of 64,000[44] while purified proactivator isolated from *Limulus* was reported to have a molecular weight of 50,000, based on heparin-Sepharose chromatography[29]. Purified activator or activated factor B can stimulate proclotting enzyme activation by limited proteolysis but neither has demonstrable enzymatic activity for several peptide chromogenic substrates[29,44]. A fourth protein isolated by chromatography on heparin-Sepharose has been designated Protease N[29]. This factor was shown to have serine protease activity and postulated to be involved in a proteolytic activation of the proactivator molecule. Protease N has not been further characterized. Contemporary concepts about the biochemistry of coagulation in *Limulus* are shown in Figure 1.

In 1977, Nakamura et al. described the use of a peptide chromogenic substrate as a sensitive assay for *Limulus* clotting enzyme[45]. Chromogenic substrates also are widely utilized for the detection of mammalian clotting factors and other research purposes[27,46,47]. A commonly used family of chromogenic substrates is composed of a sequence of amino acids forming a small oligopeptide with a p-nitroaniline (pNA)

group at the COOH-terminal. The p-nitroaniline group is cleaved by a protease if the enzyme has specificity for the amino acids adjacent to the chromophore. Release of the pNA from the colorless chromogenic substrate generates free pNA which is yellow and can be measured spectrophotometrically at 405 nm. The concentration of activated enzyme(s) needed to obtain suitable hydrolysis of a chromogenic substrate may be less than the concentration required to form a gel[46]. Therefore chromogenic substrates are very useful for detection of enzyme activation.

The studies described in this report were designed to investigate (a) the time-course of production of activation of the enzymes in *Limulus* lysate following incubation with bacterial endotoxin and correlation of enzyme activation with visible changes of gelation, (b) separation of enzymatic activities from *Limulus* lysate by chromatographic techniques, e.g., high performance liquid chromatography (HPLC) and fast protein liquid chromatography (FPLC), (c) identification of specific enzymes from (b) by a gel electrophoretic technique, using gelatin copolymerized in an SDS-polyacrylamide gel[48,49]. Starting material for the chromatographic separations was an activated enzyme pool, termed "supernatant", which was obtained from endotoxin-mediated gelled lysate, centrifuged to remove clotted protein(s). This sample preparation was chosen to ensure optimal activation of enzymatic activities. Furthermore, this procedure accomplished the complete removal of coagulogen in one simple step, while retaining activated proteins in the supernatant. Thus, utilizing supernatant decreased by approximately one half the concentration of protein used as starting material for the chromatographic separations. Furthermore, a major non-enzymatic protein (coagulogen) was completely separated from the starting material, thus simplifying the protein purification protocol and avoiding any potential damage to the chromatographic columns from coagulogen forming a gel during the separations.

Despite the development of highly complex hemostatic mechanisms during

evolution[50], there remain remarkable similarities between the simpler primitive *Limulus* coagulation system, as described above, and the more complex mammalian systems. Amebocytes contain a clottable protein and a series of coagulation serine proteases, which constitute an enzymatic coagulation cascade similar to that of the plasma coagulation system in mammals[3]. Therefore, an understanding of blood coagulation in *Limulus* may serve as a useful model for studying the mechanisms by which endotoxin activates various enzymatic cascades, especially blood coagulation. Further isolation and characterization of the enzymatic system in amebocyte lysate may lead not only to a better assay for endotoxin but also provide valuable insights into other biologic effects of endotoxin.

MATERIALS AND METHODS

Materials. Chromogenic substrates S-2160, S-2222, S-2251, S-2302, S-2337, S-2338, S-2422, S-2423, S-2444 and S-2586 were obtained from KabiVitrum, Mölndal, Sweden (courtesy of Dr. Petter Friberger). Pyrogen-free water and 0.9% bacteriostatic sodium chloride were purchased from Travenol Laboratories, Deerfield, IL, U.S.A. Tween 20, triton X-100, tris-HCl, urea, Coomassie brilliant blue R-250, sodium chloride, and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma Chemical company, St, Louis, MO, U.S.A. Gelatin (type I) was obtained from Gibco Diagnostics, Madison, WI, U.S.A. Endotoxin, lipopolysaccharide B from *E. coli* 026:B6, was prepared by Difco Laboratories, Detroit, MI, U.S.A. Stock solutions of endotoxins with final concentration of 100 ug/ml were kept at -20°C and warmed at 37°C for 10 min before use. Glassware and serological pipettes were rendered endotoxin-free by autoclaving followed by heating at 180°C for 4 hr[13]. *Limulus* amebocyte lysate was prepared according to the methods described previously by Levin et al[13].

Limulus Amebocyte Lysate Assay for Endotoxin. Determination of appropriate concentrations of endotoxin to produce different, visible gelation changes, was evaluated by a previously described method[2]. A series of endotoxin concentrations were prepared by 1:10 dilutions in normal saline from a stock solution of endotoxin (100 ug/ml) to provide a range of endotoxin concentrations from 10 ug/ml to 10^{-6} ug/ml. Assay mixtures consisted of 50 ul *Limulus* lysate and 50 ul of diluted endotoxin incubated at 37°C. The process of gelation was observed carefully as negative (clear sol), flocculation (F), increased viscosity (V) or a semi-solid gel (G) at 15 min, 45 min, 1 hr, 2 hr and 3 hr after incubation of lysate with endotoxin.

Preparation of Activated Enzyme Pool(Supernatant). *Limulus* lysate was incubated with excess endotoxin (at final concentrations of 0.1-1.0 ug/ml) at 37°C for 3 hr , and then at room temperature for 2-18 hr. Separation of supernatant from clotted

protein(s) was performed by centrifugation at 20,000 rpm for 20 min at 4° C.

Assay of Enzymatic Activities for Chromogenic Substrates. The protease activities in the supernatant were characterized using several chromogenic substrates from KabiVitrum. Lyophilized chromogenic substrates were reconstituted with 0.1 M tris-HCl (pH 8.0) in pyrogen-free water. Some chromogenic substrates, i.e., S-2160, S-2337 and S-2222, required sonication for 30 min to dissolve completely. Stock solutions of chromogenic substrates, each 0.5 mM, were kept at 4° C. Supernatants or partially purified protein fractions from chromatographic separations were incubated with 0.1-0.5 mM chromogenic substates at 37° C. After incubation for various times, 20-80% acetic acid was added to stop the reaction. p-Nitroaniline groups, released from the peptide substrates by protease activities, were visible as yellow color and assayed spectrophotometrically by absorbance at 405 nm.

Solubility of Limulus Gel in Urea. 200 ul of *Limulus* amebocyte lysates were incubated with 10 ul bacterial endotoxin (in various concentration ranging from 0.01-1.0 ug/ml) at 37° C for various times, up to 2.5 hr after formation of a semi-solid gel. Different stages of gelation of endotoxin-treated lysate were assessed visually. Then, an appropriate volume of 8 M urea (final concentration, 6 M) was added to samples with different degrees of gelation; after addition of urea, the samples were subjected to varied degrees of agitation such as stirring with a spatula or Vortex agitation for 0.5-2 min. The residual turbidity following incubation with urea was measured spectrophotometrically at 650 nm. Enzymatic activities were detected with S-2444 and S-2423 after adding 80% acetic acid to the sample mixtures containing urea. All turbidity disappeared immediately after addition of acetic acid.

Gel Permeation Chromatography, G-100. Three ml of supernatant, which was obtained by centrifugation of endotoxin-treated lysate as described above, was applied to a column (1.6 x 93 cm) of Sephadex G-100 (Pharmacia) equilibrated with 0.1 M tris-HCl

(pH 7.7) at room temperature and eluted at an approximate flow rate of 46 ml/hr under a hydrostatic pressure of 30 cm. 100 aliquots of fractions were analyzed for enzymatic activities using 0.1 mM chromogenic substrate S-2423.

Fractionation of Activated Enzymes by Ion-Exchange Chromatography. Supernatant, produced as described above, was concentrated approximately ten-fold with a YM-10 hydrophilic membrane (Amicon Corp., Danvers, MA). 500 ml of concentrated supernatant was fractionated by ion-exchange fast protein liquid chromatography (FPLC), utilizing a Pharmacia model programmer (Pharmacia, Laboratory Separation Division, Uppsala, Sweden). Sample fractionation was accomplished with a Mono Q HR5/5 column (5mm x 50mm) (Pharmacia) equilibrated with 100 mM tris-HCl buffer (pH 8.5) at a flow rate of 2 ml/min and eluted with a NaCl-salt gradient of zero to 1 M in the same buffer. The column eluate was monitored for absorbance at 280 nm and eluted fractions were assayed for enzymatic activity, using 0.5 mM S-2423 as described above. Fractions, based on enzymatic activities, were pooled for further chromatography using HPLC.

Analysis of Protein Fractions by Gel Permeation-HPLC. After fractionation of supernatant by FPLC, pooled protein fractions were further characterized by gel permeation high performance liquid chromatography (GP-HPLC), utilizing a Waters HPLC model (Waters Associates, S. San Francisco, CA). This allowed enhanced visualization of minor components in the protein fraction and estimation of molecular weights of enzymatic activities. Samples from the Mono-Q column were prepared for gel permeation by buffer exchange with 10 mM tris-HCl, pH 7.0, employing a centricon-10 microconcentrator unit (Amicon Corp.) and filtration through a Millipore 0.22 um filter. Samples were then chromatographed at a flow rate of 0.6 ml/min on a TSK G3000SW gel permeation column (LKB Instruments, Inc., Pleasant Hill, CA) equilibrated with the above buffer. Column eluate was monitored for absorbance at 280

nm and assayed with 0.5 mM S-2423 to determine the protease activity. Fractions from the HPLC column were further analyzed by SDS-PAGE as indicated below.

Analysis of Protein Fractions by SDS-PAGE. Protein fractions from chromatographic separations were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli, as previously described[51]. The slab gel system consisted of a 10% separating gel and a 3% stacking gel. Samples applied to the gel were electrophoresed through the stacking gel at 20 mA until the samples reached the running gel. The current was then increased to 35 mA and electrophoresis was continued until the bromophenol blue tracking dye was approximately 1.5 cm from the bottom of the gel. Protein bands were stained either with Coomassie brilliant blue R-250 by the method of Fairbanks et al[52], employing the modifications of omitting step 3 and using a final destaining solution which contained 10% isopropanol in addition to 10% acetic acid[53], or stained with silver stain[54].

Non-denaturing Polyacrylamide Gel Electrophoresis. For detection of enzymatic activities from amebocyte lysate supernatants, non-denaturing disc polyacrylamide gel electrophoresis was performed employing a 3% stacking gel and 10% separating gel in tris-HCl buffer (pH 8.5). Duplicate samples were electrophoresed at a constant current 1.5 mA/gel, for 16 hr at 4°C. After electrophoresis, samples were diveded into two groups; one group of samples were stained with Coomassie blue R-250 as described above. The duplicate samples were cut into 1 cm pieces to elute proteins in 0.1 M tris-HCl (pH 8.0) for 6 hr at 4°C. Enzymatic activity in each 1 cm gel section was assayed using chromogenic substrates S-2423, S-2422, S-24444, S-2337 or S-2222.

Gelatin Substrate SDS-PAGE. Supernatants, protein fractions from FPLC-Mono Q, and protein fractions from GP-HPLC were characterized by SDS-polyacrylamide gel electrophoresis employing copolymerized gelatin. Gelatin substrate SDS-PAGE was performed according to Heussen and Dowdle[48] using the staining procedure of

McKerrow et al[49]. Ten-percent polyacrylamide, 0.1% SDS slab gel (1.5 mm) was polymerized in the presence of 0.1% type I gelatin, with a 3% stacking gel without gelatin. The procedure of electrophoresis was that of Laemmli[51]. Samples were prepared for electrophoresis by incubation with 1% SDS at 37°C for 3 hr without the addition of reducing agents. A tris-glycine running buffer, pH 8.3, was used. Samples were electrophoresed at 20 mA per gel within the stacking gel and at 35 mA per gel in the resolving gel. After electrophoresis, the gel was washed with gentle agitation in 100 ml of 2.5% Triton X-100 for 2 hr to remove SDS. The gel then was incubated in 100 mM glycine-NaOH, 2 mM calcium chloride, pH 8.5 for 18-24 hr at 37°C. Then, the gel was fixed (stopping the enzymatic reaction) for 1 hr in 50% trichloroacetic acid at room temperature, stained with 0.25% Coomassie brilliant blue R-250 for 1 hr, and finally destained in 45% methanol, 10% acetic acid until proteolytic bands that cleared the dye from the gel were optimal for photography (4-12 hr).

SPECIFIC OBJECTIVES

A. Determine which of a battery of chromogenic substrates will detect protease activities in supernatants of *Limulus* amebocyte lysates.

B. Describe the temporal pattern of enzyme activation after incubation of lysate with endotoxin and correlate with stages of visible gelation.

C. Define the best conditions for preparation of an activated enzyme pool (supernatant from gelled lysate, centrifuged to remove clotted protein(s)) for subsequent studies:

   1. Time of incubation with endotoxin to completely activate the enzymatic cascade

   2. The effect of stirring during incubation on recovery of enzymatic activities, minimizing loss of enzymes in the insoluble pellet

   3. The effect of incubation of activated lysate with chemical agents such as detergents, salt, or EDTA on recovery of enzymatic activities

   4. Storage of the activated enzyme pool to maintain activities for subsequent experiments

D. Investigate the potential presence of factor XIII-like crosslinking activity in gelled lysate

E. Obtain partial separation of enzymes present in *Limulus* lysate

F. Evaluate partially purified enzymatic activities and correlate activities with protein bands by electrophoretic techniques

   1. Non-denaturing polyacrylamide gel

   2. Gelatin substrate gel

RESULTS

Demonstration of protease activities for chromogenic substrates in supernatant.

Enzymatic activities in *Limulus* amebocyte lysate supernatant were assayed utilizing 10 synthetic chromogenic substrates. Protease(s) in the supernatant had significant activity toward six of the tested chromogenic substrates (Table 1), including substrates used for the assay of mammalian thrombin, Xa and urokinase. In this series of assays, there was one group of chromogenic substrates which developed bright yellow color in 5 min, one chromogenic substate (S-2444) in 20 min, and S-2160 only slowly achieved a faint yellow color after 1 hr at 37°C. The different affinities of the enzyme(s) in the supernatant for different chromogenic substrates provided a good basis for further examination of the time course of enzyme activation of endotoxin-treated lysate.

The pattern of enzyme activation in *Limulus* lysate after addition of endotoxin.

Endotoxin, individual chromogenic substrates, and *Limulus* lysate were combined and incubated at 37°C. The six chromogenic substrates described in Table 1 were assayed in this study. Three distinct enzymatic activities were present, based upon the reaction rates with chromogenic substrates (Figure 2). Substrates S-2337, S-2423, S-2222 and S-2422 were all cleaved at nearly identical initial rates. Enzymatic activity(s) for those substrates rapidly increased prior to visible gelation. Maximum OD levels reached were a function of the total amount of the chromogenic substrate added. Plateau levels indicated complete cleavage of the substrates; addition of more substrate resulted in additional generation of p-nitroaniline. Differences in the plateau levels were present, in part, because of variable solubilities of the chromogenic substrates, explaining the greater maximum OD detected with S-2337. S-2444 was cleaved distinctly more slowly after an apparent lag period, and demonstrated maximum activity during the period in which a visibly viscous solution became a gel. S-2160 was cleaved at a very slow rate (Figure 2).

Since the four rapidly appearing parallel activity curves detected by the chromogenic substrates S-2337, S-2423, S-2222, or S-2422 demonstrated extremely rapid enzymatic rates, it was impossible to determine if there were any differences in substrate affinity in this group. For this reason, a much smaller concentration of pre-activated enzyme (1% of that used in Figure 2) was assayed with the same chromogenic substrates, to test whether the activities could be dissociated on the basis of proteolytic rates. Excess quantities of endotoxin were used in this experiment to ensure optimal activation of *Limulus* proteases. Enzymatic rates for the four chromogenic substrates S-2337, S-2423, S-2222, and S-2422 remained identical (see Figure 3), further suggesting that all four substrates were measuring the same enzyme(s).

Optimal conditions for preparing an activated pool of enzymatic activities.

Experiments were performed to determine the conditions for maximal recovery of lysate enzymes in supernatant. Special attention was paid to maximize the activity for S-2160 (the weakest activity in whole supernatant). Studies included the time required for optimal generation of enzymatic activities, investigation of stirring or addition of salts or detergents to aid in extracting enzymes bound to coagulin gel into the supernatant, and optimal conditions for storing supernatant.

1 (a). **Time of incubation required for preparing activated enzyme pool.** The time course experiment in Figure 2 indicated that the enzymatic activity detected by chromogenic substrate S-2160 was generated at a very slow rate. In addition, since there was an apparent lag phase for both S-2160 and S-2444 activities, we suspected that a long incubation time might be required for the reaction of endotoxin with *Limulus* lysate to go to completion, with resultant activation of all of the enzymes in lysate. Maximal activation of all enzymatic activities detected by the chromogenic substrates was obtained by incubating *Limulus* lysate with excess endotoxin (final concentrations of 0.1-1.0 ug/ml endotoxin) at 37°C for 3 hr, and then at room temperature for 2 hr. Separation of supernatant from clotted protein was performed by centrifugation at 20,000

rpm for 20 min at 4° C.

1 (b). **Gelatin substrate gel patterns.** Activation of *Limulus* enzymatic activities was investigated by gelatin substrate SDS-PAGE. Supernatants were prepared from endotoxin-treated *Limulus* lysates following incubation with endotoxin for 5 min, 10 min, 20 min, 1hr or 3hr. Addition of SDS and centrifugation at 4° C stopped any subsequent enzyme activation. After electrophoresis, incubation at 37° C with glycine to facilitate protease activity for the gelatin, and staining and destaining, multiple bands of protease activity were detected (Figure 4). Estimated molecular weights for the enzymatic activities were 60-70,000 daltons, 120,000 daltons and 140,000 daltons. The clear proteolytic bands demonstrated no visible bands when stained with Coomassie blue. Therefore, it was concluded that the protein concentrations associated with enzymatic activity were under the detectable limit of Coomassie blue stain (1-10 ug). Despite the large range of incubation times for these samples, the gel patterns of clear proteolytic bands were identical for the various samples. No new activity bands were detectable after 5 min incubation with endotoxin (Figure 4).

2. **The effect of stirring on recovery of maximal enzyme activities in the supernatant.** To determine the effect of stirring, a sample mixture consisting of lysate and endotoxin was stirred at a mild speed with a stirring bar from the start of the incubation at 37° C. A duplicate sample mixture was incubated undisturbed. Supernatant was then separated from gelled coagulin by centrifugation (20,000 rpm, 20 min), and enzymatic activity for S-2160 determined. Stirring during incubation of *Limulus* lysate with endotoxin resulted in approximately 20% of total enzymatic activity (calculated from OD value after 30 min incubation) in the supernatant and 80% in the pellet (Figure 5). The unstirred control had the same distribution of enzymatic activity as the stirred samples. Therefore, stirring did not significantly increase the amount of enzymatic activity detected by chromogenic substrate S-2160 in the supernatant.

3. **Chemical agents used to alter the distribution of the enzyme(s) activities, as detected with S-2160, between supernatant and pellet.** Several chemical agents, including high (4.0M) and low (0.8M) concentrations of NaCl, non-ionic detergents (Tween 20, Triton X-100) and EDTA, were added to samples of *Limulus* lysate incubated with endotoxin. Additions of chemical agents were made either at zero time or at 1.5 hr after gelation. Recovery of enzyme(s) by salt (4.0 M or 0.8 M NaCl), when added simultaneously with endotoxin at zero time, produced the best yield of enzyme(s) activity in supernatant, as detected by S-2160 (Table 2). There was an approximately 30% increase in the S-2160 activity as compared to the control (see Figure 5). Non-ionic detergents or EDTA did not prevent trapping of enzyme activity in the insoluble pellet.

4. **Storage of the activated enzyme pool to maintain maximum activities for subsequent experiments.** Aliquots of a large activated supernatant pool prepared from endotoxin-treated *Limulus* amebocyte lysate were stored at 4°C, -72°C, or at -72°C with the addition of glycerol (final concentration, 25%). Enzyme activities in the supernatants were assayed by chromogenic substrates S-2423, S-2444 or S-2160 weekly for two months. These three chromogenic substrates detected the distinct enzyme activities shown in Figure 2. For S-2160 (see Figure 6 a, b, c.), at 4°C (a) and at -72°C (b), activity gradually declined during the eight week period of observation. Samples with added glycerol, stored at -72°C (c), had negligible S-2160 activity by one week. S-2423 (rapid enzyme) and S-2444 (intermediate enzyme) activities were unaffected by storage conditions during the 8 weeks of study. The data showed that the enzymatic activities detected by S-2423 and S-2444 did not decline during an eight week storage period, while the activity detected by S-2160 gradually declined. This suggested that the enzyme which cleaved S-2160 was a different protein from the enzyme(s) that cleaved S-2423 and S-2444.

Investigation of the presence of factor XIII-like crosslinking activity

In the time course experiment (Figure 2), the initiation of the early enzymatic activity (as measured by S-2423) proceeded any visible changes in the solution by 15 min. However, the activity curve detected by chromogenic substrate S-2444 paralleled the visible changes as gelation proceeded. The parallel between initiation of S-2444 activity and visible changes in the gel suggested the possibility that a crosslinking enzyme, detected by S-2444, was contributing to solid gel formation, as has been observed for the activity of human Factor XIII on a fibrin clot. In order to investigate this possibility, the following experiments compared the time course of initiation of early enzyme activity (as measured by S-2423) and intermediate enzyme activity (as measured by S-2444), and the time course of increasing turbidity. Urea insolubility (gel turbidity not diminished by the presence of this denaturing agent) was used to evaluate the presence of a chemically crosslinked coagulin gel. In the following experiments, relatively low concentrations of endotoxin were utilized such that changes in the visible appearing of the gel mixture occurred slowly (45-55 min necessary for gelation). This allowed comparison of the initiation of the early enzyme and intermediate enzyme activities in relation to the development of flocculation, viscosity and solid gel formation.

Fragility of the gel was assayed from the prepared sample composed of lysate, endotoxins and a chromogenic substrate (S-2423 or S-2444), incubated at 37°C for various times up to 3 hr. After incubation for various times, urea (final concentration, 6M) was added, The sample mixtures were subjected to different degrees of agitation such as mild agitation with a spatula for 10-20 sec (Figure 7a), moderate agitation with a spatula for 1 min (Figure 7b), and vigorous mixing with a Vortex for 2 min (Figure 7c). When the generation of enzymatic activity was monitored using S-2423, the increase in turbidity was observed after the initiation of this early enzymatic activity (Figure 7a). Whereas enzymatic activity for S-2423 was at a maximal rate after 5 min of incubation, significant increase in turbidity was not present until after 15-30 min, when the change

from flocculation (F) to viscous (V) states occurred. Subsequently, turbidity markedly increased when gelation (G) occurred.

In contrast, when the generation of enzymatic activity was measured using S-2444, the increase of turbidity paralleled the enzymatic activity detected by S-2444 (Figure 7b). The increase of turbidity during the transformation from flocculation to viscosity was less sharp than that seen in Figure 7a. This difference in the rate of change of turbidity may have resulted from more vigorous stirring which broke down the coagulin into smaller particles which contributed to less OD (light scattering) (Figure 7b).

Samples mixtures consisting of lysate, endotoxin and S-2444 or S-2423 for monitoring the initiation of enzymatic activity were incubated for various times. Then urea was added and sample mixtures were vigorously stirred with a Vortex. Figure 7c demonstrates that the initial increase of turbidity observed during the gelling stages again paralleled the initiation of the enzymatic activity for S-2444 and was significantly later than the initiation of enzymatic activity for S-2423. Only a slight increase in turbidity was observed in this experiment. This suggested that the degree of turbidity increase was largely affected by the vigorous agitation applied to the gelling sample mixtures.

In the experiments to determine urea solubility of coagulin in 6 M urea, the assay for factor XIII-like activity was negative (Figures 7a-c). The gel (coagulin) was insoluble in urea. According to the classic urea solubility assay for factor XIII activity in human fibrin clot, this suggested that a crosslinking factor existed in the coagulin after gelation had occurred.

Three major results were obtained from these series of experiments. First, increased turbidity following incubation of lysate and endotoxin is noted at approximately the same time as initiation of S-2444 activity. Second, these studies demonstrated that the degree of increase in turbidity of endotoxin-treated *Limulus* lysate was greatly affected by the stirring technique. Vigorous stirring in the presence of urea completely dissociated the solid gel, which had formed following incubation of *Limulus* lysate with excess

endotoxin for greater than 1 hr at 37°C. Third, addition of urea to the coagulin gel did not result in solubilization of the turbid protein. In assays of mammalian fibrin clots, the observation of urea-insolubility is indicative of the presence of factor XIII crosslinking activity. Overall, it is not clear from these observations whether a significant extent of chemical crosslinking occurs in *Limulus* lysate during gelation.

Chromatographic separation of enzymatic activities by G-100, GP-HPLC or FPLC-Mono Q.

Gel permeation chromatography on G-100. Using gel permeation liquid chromatography with Sephadex G-100, supernatant proteins were separated into four distinct fractions (figure 8). Peaks II(tubes 20 to 23) and IV (tubes 33 to 43) had no detectable enzymatic activity utilizing any of the three chromogenic substrates S-2423, S-2444, or S-2160. Peak I had the strong enzymatic activity for S-2423 (OD value of 0.56 at 405 nm after 2 hr incubation) but had no activity for S-2444 or S-2160. Peak III contained much weaker enzymatic activity for S-2423 (OD value of 0.13 after 2 hr incubation) and had no activity for S-2444 or S-2160. Peak IV which had the highest OD value, was further analyzed with SDS-PAGE and UV absorption spectrum. When 75 ul of this high OD fraction was electrophoresed on an SDS gel, no protein bands were detected with Coomassie blue. The UV absorption spectrum of this peak showed the maximal absorbance at 258 nm and a ratio (A_{260nm} / A_{280nm}) of approximately 2, which is characteristic of nucleic acids. Therefore, this suggested that peak IV contained mostly low molecular weight nucleic acids and that this simple chromatographic procedure could provide an initial step to eliminate much of the non-enzymatic material.

Ion-exchange FPLC. Supernatant was concentrated 10 fold with an Amicon YM-10 hydrophilic membrane. 500 ul of concentrated supernatant (approximately 20-25 mg protein), was applied to a Mono Q column. Separation was based on anionic exchange, and proteins were eluted with a linear 0-0.3 M NaCl gradient followed by a 1 M NaCl elution. A very complex chromatogram resulted, with multiple discrete peaks eluted

(Figure 9). Based on analysis of supernatant by gelatin substrate gels (see Figure 4), proteases are probably in low concentration (undetectable with Coomassie blue stain). Thus, representative areas through the entire elution pattern required testing for enzymatic activity. Twelve representative fractions were examined with chromogenic substrate S-2423 (Figure 9). Enzymatic activities were detected in six of the fractions which eluted between 0.05 M and 0.21 M NaCl (F1-F6). Enzymatic activity was not detected in the material which was not retained by the column or in the material eluted by high salt (1 M)*. The samples that demonstrated positive enzymatic activity were assayed for their ability to gel coagulogen. For these assays, *Limulus* lysate was heated at 60°C for 30 min. These conditions inactivate the clotting enzyme without damaging coagulogen[55], thus resulting in a coagulogen preparation that does not gel until exogenous clotting enzyme is added. Aliquots of this material were added to the fractions eluted between 0.05 M and 0.2 M NaCl from Mono Q and incubated at 37°C. All the sample mixtures formed visible flocculation within 2 hr. This suggested the *Limulus* clotting enzyme had been separated from non-binding material and high salt fractions by charge, but was not localized in one single peak. Two representative protein fractions, which had been eluted from a Mono Q column at 0.08 M (F2) and 0.18 M (F5) NaCl, respectively, and both of which demonstrated S-2423 enzymatic activity, were rechromatographed on a Mono Q column under the same conditions. Chromatograms of both protein fractions demonstrated reproducible elutions at approximate 0.08 M and 0.18 M of NaCl, respectively. This established that distinct chromatographic separation had occurred and enzyme(s) eluted at low salt concentrations were not contaminated with the enzymes eluted at higher salt concentration. These fractions were then further purified by gel permeation high performance liquid chromatography (HPLC).

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* Some other Mono Q fractionations had detectable enzymatic activity in fractions eluted by 1 M NaCl.

Gel-permeation HPLC. The six fractions derived from Mono Q chromatography, that had demonstrated enzymatic activity (as described above), were further purified by gel-permeation HPLC. Gel-permeation HPLC separated proteins by size and allowed enhanced visualization of minor components in the protein fractions. After fractionation by HPLC, enzymatic activities in collected fractions were measured using S-2423. Pooled fraction 1 from Mono Q showed two major protein peaks (Figure 10a). The elution volume of maximal enzymatic activity was 9.5-9.8 ml (estimated M.W. 160-175,000 daltons under these conditions). Pooled fraction 2 also had two major asymmetric peaks and elution volume of maximal enzymatic activity was 8.0-8.9 ml which had an estimated M.W. 250-300,000 daltons(Figure 10b). Pooled fraction 3 demonstrated one major irregular peak and the elution volume of maximal enzymatic activity was 8.0-8.8 ml (Figure 10c). Since the elution volume was close to the void volume of TSK G3000SW gel permeation column, the estimated M.W. was ≥ 300,000 daltons. Pooled fraction 4 had two major high peaks, and the elution volume of maximal enzymatic activity was 7.7-8.6 ml (estimated M.W. ≥ 300,000 daltons) (Figure 10d). Pooled fraction 5 had several complex peaks and the elution volume of maximal enzymatic activity was 7.0-7.3 ml (estimated M.W. ≥ 300,000 daltons) (Figure 10e). The last fraction, pooled fraction 6, had one high peak, one moderate peak, and four small peaks (Figure 10f). Elution volume of maximal enzymatic activity was 6.5-7.0 ml (estimated M.W. ≥ 300,000 daltons). Again, these data indicated the concentration of *Limulus* enzymes much less than non-enzymatic proteins. According to the elution volumes of enzymatic activities, all the enzymes had relatively high molecular weights. These data are presented in Table 3.

Evaluation of partially purified enzymatic activities by electrophoretic techniques.

1. Non-denaturing polyacrylamide gel.

Supernatant proteins were electrophoresed in native, non-denaturing polyacrylamide gels. Electrophoresis was performed at 4°C, 1.5 mAmp/gel for 16 hr, to avoid damage to the enzymatic activities. Following electrophoresis, the gels were cut into 1 cm pieces, minced in tris buffer, and then tested with chromogenic substrates. Duplicate gels were stained with Coomassie blue. Data from Coomassie blue stain and assays with chromogenic substrates suggested that supernatant proteins were not well separated in the native gels and the enzymatic activities were primarily detected in the upper 2 cm of the gels (Table 4). Thus, the native gel technique was not used for further identification of enzymes.

2. Gelatin substrate SDS-polyacrylamide gel.

Because of the ability to detect the proteolytic activities of enyzmes and to estimate the molecular weights simultaneously, gelatin substrate SDS-PAGE was used for the identification of enzymatic activities from fractions of the Mono Q and HPLC separations.

(A) Mono Q fractions, demonstrating enzymatic activity (see Figure 9), were analyzed by gelatin substrate SDS-PAGE. Samples were used from the chromatographic separation described in Figure 9 and also from a Mono Q fractionation in which enzymatic activity for S-2423 was detected in the high salt (1 M NaCl) fraction. Gelatin substrate gel electrophoresis (Figure 11) of the Mono Q fractions demonstrated two of the three proteolytic bands seen in the whole supernatant. A sharp band of activity with M.W. 140,000 daltons (lane 6) was present in the Mono Q fraction eluted with 1 M NaCl but not in any other of the fractions. This band had identical migration to the slowest migrating band of whole supernatant (lane 1). A separate band of activity was present in 3 of the fractions from the Mono Q. These fractions, which were eluted at 0.08 M (F2), 0.13 M (F4) and 0.21 M (F6) NaCl, demonstrated a diffuse proteolytic protein band with an estimated molecular weight 60-70,000 daltons (lanes 7, 8, 9). The molecular weights of this enzyme correlated with the fast migrating enzyme in whole

supernatant (lane 1). The proteolytic band with M.W. 120,000 daltons seen in whole supernatant was not detected in this study based on gelatin substrate gels. The proteolytic bands did not stain with Coomassie blue dye, indicating that the amount of enzyme was below 1-10 ug/ml (minimal limit of protein detected with Coomassie blue stain). These data are summarized in Table 3.

(B) Gelatin substrate SDS-PAGE also was used for the identification of enzymatic activities from fractions of gel permeation HPLC. HPLC fractions demonstrating maximal enzymatic activities in six fractions (see Figure 10a-10f) were analyzed by gelatin substrate SDS-PAGE. Duplicate fractions from HPLC were also electrophoresed with SDS-PAGE, and then stained with silver stain. Gelatin substrate electrophoresis (Figure 12(a)) of the HPLC fractions demonstrated one of the three proteolytic bands seen in whole supernatant. A diffuse proteolytic protein with an estimated molecular weight of 60-70,000 daltons (lanes 2-6) was present. Lane 1, which demonstrated enzymatic activity to S-2423 in Figure 10a, had no detectable proteolytic band probably because only a small quantity of sample (10 ul) remained for this analysis. The above data are presented in Table 3. SDS-polyacrylamide gel with silver stain (Figure 12(b)) demonstrated a faint diffuse protein band with M.W. 60-70,000 daltons (lanes d-f) which correlated with the proteolytic band seen in Figure 12(a). Lane 1 (Figure 12(b)) showed no protein band, compatible with the observation in lane 1 in Figure 12(a). The majority of protein bands with no enzymatic activity had low molecular weights (i.e., < 60.000 daltons).

DISCUSSION

The primary aim of this study was to characterize a series of blood coagulation proteins in the horseshoe crab, *Limulus polyphemus*. Currently available data indicate that the coagulation system of *Limulus* consists of a clottable protein, coagulogen, and a series of enzymes, the sequential activation of which ultimately results in an activated clotting enzyme, which then partially proteolyzes the coagulogen. Finally, gelation occurs. To date, Levin, Nakamura, and others have identified at least three enzymes which play a role in the blood coagulation cascade in *Limulus*, and all are serine proteases[16, 18, 29, 43]. Enzymatic mediation of blood coagulation in *Limulus* is apparently initiated by a protease (Protease N), which activates proactivator in the presence of endotoxin (Figure 1). The activator then produces partial proteolysis of the proclotting enzyme, leading to an active clotting enzyme. Finally, the activated clotting enzyme causes partial proteolysis of the coagulogen molecule and formation of a solid gel (coagulin).

In order to characterize the sequence of the enzymatic activities in the cascade of *Limulus* blood coagulation and identify any previously unrecognized enzymatic activity, the time course of generation of activated enzymatic activities was examined. The study of the time-course of activation of the coagulation enzymes in *Limulus* amebocyte lysate, following incubation with bacterial endotoxin, demonstrated a pattern of three distinct enzymatic activities, based upon the reaction rates with a series of chromogenic substrates (Figure 2). One of these enzymatic activities cleaved chromogenic substrates S-2337, S-2423, S-2222 and S-2422 at a rapid initial rate, prior to visible gelation of endotoxin-treated lysate. The second enzymatic activity for S-2444 developed at a moderate rate, following a lag period. It reached maximum activity during the stage of increased viscosity which preceded formation of a solid gel. The third cleaved S-2160 at a very slow rate. In previous studies of the purified clotting proteins in *Limulus*, Torano

et al [18]. reported the clotting enzyme hydrolyzed chromogenic substrates S-2337, S-2423, S-2222 and S-2422 but not chromogenic substrates S-2160, S-2338, S-2251, S-2444, S-2266 and S-2302. The chromogenic substrate profile of the clotting enzyme in Torano's report was identical to the rapidly increasing enzymatic activity detected by chromogenic substrates S-2337, S-2423, S-2222 and S-2422 in our time-course experiments (Figures 2 and 3) strongly suggesting that this enzymatic activity represents the amidase activity of *Limulus* clotting enzyme. Functionally, the addition of pre-activated enzyme(s), as detected with these four chromogenic substrates (see Figure 3), to partially purified coagulogen or heat-treated lysate produced gel formation. This functional assay further suggested that all four substrates (S-2337, S-2423, S-2222 and S-2422) were measuring the *Limulus* clotting enzyme.

Recently, Nakamura and Levin reported that a highly purified preparation of the *Limulus* activator, the second described enzymatic activity demonstrated negligible amidase activity to a battery of 7 chromogenic substrates [16]; an newly identified LPS-dependent protease Protease N, was capable of hydrolyzing the chromogenic substrates S-2222, S-2160 and S-2338[29]. Although the slow-cleaving enzymatic activity to S-2160 described in Figure 2 corresponded to one of the substrate specificities of Protease N [29], the low level of S-2160 activity throughout the time course of enzymatic activation of *Limulus* lysate made designation of this activity as Protease N inconclusive. Furthermore, persistence of this activity throughout the period of the reaction between endotoxin and amebocyte lysate made it impossible to define the position of the S-2160 activity in the sequence of the coagulation cascade.

Interestingly, the moderate enzymatic activity to S-2444 did not correspond to any previously reported substrate specificity for any of the three *Limulus* clotting proteins. S-2444 activity may define an important, but previously unrecognized clotting factor. The pattern of S-2444 activity suggested the possibility that it played a role in gel formation.

S-2444 activity appeared to parallel the visible changes as gelation proceeded. This observation suggested the possibility that a crosslinking enzyme, detected by S-2444, was contributing to solid gel formation. When studies were performed to investigate the possible presence of factor XIII-like crosslinking activity (see results, section 4), disparate results were defined. A standard urea solubility assay for factor XIII activity, in the presence of 6 M, urea demonstrated that the gel (coagulin) was insoluble. The lack of urea solubility is consistent with crosslinking and/or irreversible aggregation of the coagulin protein[56, 57]. This observation did not confirm the report of Tai and Seid [41] that the gel was soluble in 6 M urea. However, even after a long period of incubation, the coagulin gel was fragile and easily disrupted by mechanical means. Based on this fragility of the coagulin gel, there was no evidence that the S-2444 activity contributed to the physical stability and strengthening of the gel. The fragility of the coagulin gel suggested, rather, that the stability of the solid gel is due to aggregation of coagulin molecules which were greatly susceptible to mechanical disruption (stirring, vortexing). Therefore, the role of S-2444 activity as a crosslinking enzyme (similar to factor XIII) remains unknown.

A major part of this investigation consisted of the partial purification of the coagulation enzymes of the *Limulus* gelation cascade. In several previous studies of purification of *Limulus* coagulation enzymes, unactivated enzymes were described[16, 18, 29]. However, purification of unactivated *Limulus* enzymes requires maintenance of endotoxin-free conditions to isolate the zymogen forms. Contamination by endotoxin from the environment is difficult to prevent and furthermore, could damage the chromatographic columns by the formation of a coagulin clot. Hence, in our attempted purification of enzymatic activities by chromatographic techniques, we determined the optimal conditions for maximal recovery of activated lysate enzymes in the supernatant produced by incubating *Limulus* lysate with excess endotoxin at 37°C for 3 hr. Separation of supernatant from clotted proteins was performed by centrifugation at

20,000 rpm for 20 min at 4°C. Using this supernatant preparation as starting material allowed elimination of the tedious procedures required to maintain endotoxin-free conditions, as well as ensured optimal activation of enzymatic activities. Furthermore, coagulogen was completely removed in one simple step. Thus, simplification of chromatographic separation and prevention of damage to chromatographic columns from the coagulin gel were achieved simultaneously, using the supernatant.

The major problem in using supernatant as starting material for separation of enzymatic activities is the recovery of lysate enzymes from the coagulin gel, which was reported to trap most of the enzymatic activities[18]. In previous papers, Sullivan and Watson[31] and Seid and Liu[43] used the supernatant fraction of lipopolysaccharide-treated *Limulus* amebocyte lysate as a starting material for gel filtration chromatography, in order to purify the *Limulus* clotting enzyme. Some investigators[43] reported a remarkable increase (140-fold) in clotting enzyme activity in the supernatant obtained from centrifugation of endotoxin-treated lysate, after 1 hr incubation with endotoxin. Others[18, 31] recovered much less clotting enzyme activity, and only 7% of the initial clotting enyzme activity was recovered in the preparation of supernatant from centrifugation of endotoxin-treated lysate, after 2 hr incubation with endotoxin[18]. Since the S-2160 activity was much lower throughout the time course experiment than the other two groups of substrate activities, we were especially interested in recovering as much S-2160 activity as possible in the supernatant. In our investigations of the best means of preparation of a pool of activated enzyme (supernatant), either 0.8 M or 4.0 M NaCl extracted a maximum of 20% of the total original S-2160 activity. Because of the low level of S-2160 activity in whole *Limulus* lysate and limited recovery of the activity in supernatant, we investigated the stability of this activity, as compared to various other chromogenic activities, in supernatants stored up to 8 weeks. The activities of the rapid (S-2337, S-2423, S-2422, S-2222) and moderate (S-2444) substrate groups in the supernatant were not affected by 8 weeks of storage. In contrast, storage of supernatants

greater than one week markedly decreased S-2160 enzymatic activity under a variety of conditions (see Figures 6(a)-(c)), suggesting that fresh preparations of supernatant should be used as starting material for subsequent purification of this enzyme.

In order to separate enzymatic activities in *Limulus* by chromatographic techniques, we used traditional gel permeation chromatography G-100 (open column) as well as high performance liquid chromatography (HPLC) and fast protein liquid chromatography (FPLC). The latter methods have not previously been employed in purification of *Limulus* protein fractions and allowed enhanced visualization of minor components in the protein fraction without dilution of protein during the chromatographic separations. Our present data provide estimated molecular weights of the *Limulus* clotting enzymes under a variety of different methods and conditions of separation.

Reported molecular weights of *Limulus* clotting enzyme as determined by gel filtration were 84,000 daltons (*Limulus polyphemus*)[31] and 350,000 daltons (*Tachypleus tridentatus*)[17]. For the unreduced samples by SDS gel electrophoresis, the molecular weights of the clotting enzyme was estimated to be 43,000 daltons (*Limulus polyphemus*)[31] and 42,000 daltons (*Tachypleus tridentatus*)[17]. Similar methods of molecular weight estimation were also utilized for the *Limulus* activator[29]. The activator has an estimated molecular weight of 50,000 daltons, based on gel filtration column as G-100 and based on SDS-gel electrophoresis, a subunit molecular weight of 22,000 daltons [29]. Activator was shown to have no significant enzymatic activity for a battery of chromogenic substrates (i.e., S-2160, S-2222, S-2238, S-2251, S-2266, S-2304, S-2444)[29]. Protease N, the third described enzymatic activity, is poorly characterized so far. The molecular weight has not been determined and its amidase activity hydrolyzed chromogenic substrates S-2222, S-2160 and S-2338[29].

In comparison with previously reported data of the molecular weights of the enzymatic activities our chromatographic data demonstrated some differences. In our

study using a Sephadex G-100 column, most enzymatic activity to S-2423 was eluted close to the calculated void volume (29% of bed volume) (Peak I), indicating the enzyme had a fairly high molecular weight (≥150,000 daltons)[58]. Based on elution volumes, each of the six HPLC fractions, derived from fractions prepared with ion exchange chromatography on Mono Q (all demonstrated amidolytic activity for S-2423 (the rapid activity)), had molecular weight ≥ 160,000-200,000 daltons. However, when each of these fractions was electrophoresed in SDS in the presence of gelatin substrate, there was only one smaller molecular weight band (60-70,000 daltons) which perhaps represented the subunit of the clotting enzyme, as detected in SDS-gel. These differences in molecular weight observed in our study and previous reports are probably due to the tendency for *Limulus* enzymes to aggregate in the aqueous solution present in our HPLC separation system[59]; or alternatively, some anomalous elution behavior occurred in 10 mM tris buffer during the separation by HPLC[60], because of the possible glycoprotein nature of clotting enzyme[17].

The Mono Q fractionation demonstrated the same enzymatic activity band (M.W. 60-70,000 daltons in gelatin substrate SDS-PAGE) throughout the fractions within the gradient range of 0.08 M to 0.21 M NaCl. Each of these Mono Q fractions individually caused gelation of partial purified coagulogen or heat-treated lysate, indicating that these fractions all contain clotting enzyme activities that were eluted at different concentrations of salt. One likely explanation of the anomalous elution behavior observed during Mono Q chromatography is that the clotting enzyme is a glycoprotein, molecules of which are heterogenous with respect to carbohydrate compositions and surface charges[61]. This could result in this enzyme being eluted from the Mono Q column at different concentration of salt present in the gradient. In this regard, our preliminary data from SDS-PAGE have shown that several glycoprotein bands are present in whole supernatant stained with periodic acid-Schiff (PAS). It is not presently certain whether any of these bands are clotting enzyme or other coagulation factors.

Using a similar technique, Nakamura et al. reported that the *Tachypleus* clotting enzyme was a glycoprotein[17]. Thus, our hypothesis is that the *Limulus* clotting enzyme may be a glycoprotein, the microheterogenicity of which accounts for variable elution from an ion-exchange chromatography column and a tendency for aggregation accounts for apparent high molecular weight in certain aqueous solutions.

This study identified a previously unrecognized enzymatic activity detected by S-2444. The pattern of S-2444 activity paralleled the visible changes as gelation occurred, suggesting that it might play a role in gel stabilization or function as a fibrinolytic factor (as human plasmin) after gelation. Levin and Bang had described that the coagulin gel was stable for 10 hr by light scattering assay[13]. Personal communication with Dr. Levin indicated that the coagulin gel can remain visibly intact for several days. In addition, Hawkey reported that fibrinolysis has not yet been found in invertebrate animals[62]. Thus, S-2444 activity is unlikely to contribute as a plasmin-like activity toward the coagulin gel. Since we also were unable to establish that S-2444 activity functioned as a covalently crosslinking enzyme in the stabilization of gelation, the role of this activity in the *Limulus* coagulation system requires further investigation.

The purification of enzymatic activities also explored the employment of high performance chromatography for the separation of *Limulus* clotting proteins. HPLC and FPLC techniques provide rapid separation, isolation and initial purification of *Limulus* proteins in less than 40 minutes, in contrast to the hours required with traditional separation techniques. The systems of HPLC and FPLC also permit preparative separation of protein fractions from 1 ug to 20 mg of protein in a single chromatographic step, and the results are highly reproducible. Utilizing ion-exchange on a FPLC-Mono Q system, we were able to program any degree of salt concentration with various elution times, for the separation of the pertinent *Limulus* proteins. In addition, the application of gelatin substrate SDS-PAGE to characterize the *Limulus* proteases from chromatographic

fractions is another new approach included in our work. The ability of gelatin substrate SDS-PAGE to detect the proteolytic activities of enzymes and estimate the molecular weights simultaneously, is a major improvement in efficient characterization.

Based on our accumulated data, we can suggest future avenues of research. Utilizing supernatant as starting material, we were able to obtain several partially purified protein fractions demonstrating S-2423 activity by anion exchange chromatography (FPLC-Mono Q) following gel permeation chromatography (HPLC). Further subfractionation of each of these fractions can be accomplished by one or more additional chromatographic steps, including cation exchange chromatography (FPLC-Mono S), chromatofocusing (FPLC-Mono P), hydrophobic chromatography (reverse phase HPLC) and/or affinity column chromatography such as heparin-Sepharose (commonly used for clotting factors) or lectin column chromatography for glycoproteins. A battery of chromogenic substrates can then be used to describe the substrate specificities of fractions from these various chromatographic separations. These fractions will also be analyzed by gelatin substrate SDS-PAGE, an approach to define non-specific proteases to gelatin substrate. By eluting out the enzymatic activities from these gelatin substrate SDS-PAGE analyses, specific bands can be analyzed with a battery of chromogenic substrates. Replacement of the gelatin substrate with purified coagulogen or fibrinogen in SDS-PAGE will provide a definite identification of the clotting enzyme.

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Figure 1. Activation of Blood Coagulation in the Horseshoe Crab by Endotoxin. Each of three enzymes that has been identified is a serine protease (indicated by the asterisks). This enzymatic cascade resembles that which has been described in a variety of mammalian coagulation systems[16, 18, 29]. A poorly characterized protease (Protease N) apparently activates the Proactivator in the presence of endotoxin. The Activator results in partial proteolysis of the Proclotting enzyme leading to an active clotting enzyme. The activated clotting enzyme causes partial proteolysis of the coagulogen molecule (the clottable protein) and leads to formation of a solid gel. Coagulogen and Proclotting enzyme have been well-characterized. Proactivator and Protease N have poorly understood enzymatic properties, and the endotoxin sensitive factor has not yet been identified.

Figure 1

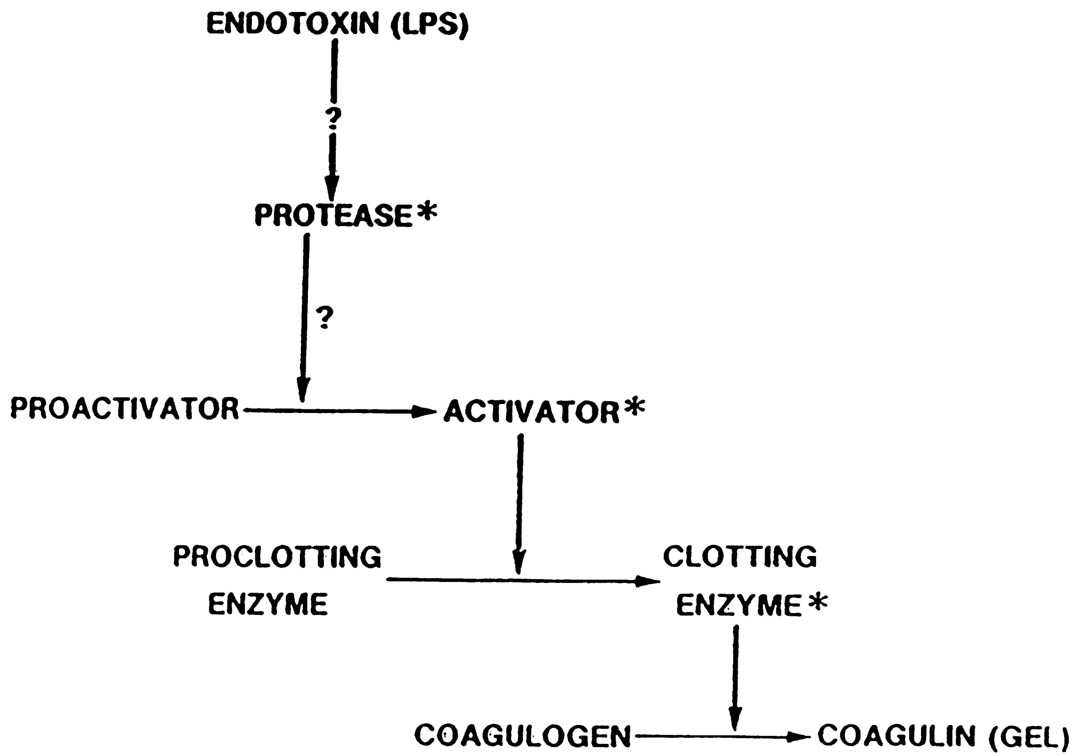


Figure 2. Time Course of Activation of Limulus Lysate Enzymes by Endotoxin. Endotoxin-treated *Limulus* amebocyte lysate was assayed for enzymatic activities with a variety of chromogenic substrates. Sample mixtures, consisting of 100 ul of lysate, 350 ul of 0.1 mM chromogenic peptide substrates S-2222, S-2337, S-2422, S-2423, S-2444, or S-2160, containing 0.1M Tris-HCl (pH 7.8), and 80 ul of 0.0001 ug/ml of lipopolysaccharide (final concentration, 0.000016 ug/ml) were incubated at 37°C. Activated protease enzymes generated by the incubation with endotoxin were detected by their ability to cleave p-nitroaniline groups from the peptide substrates. After incubation for the indicated times, enzymatic activities generated in the reaction mixture were stopped by adding 200 ul of 20% acetic acid and free p-nitroaniline groups were detected spectrophotometrically by absorbance at 405 nm. At an incubation time of 30 min, sample mixtures formed visible flocculation (F); at 45 min there was marked increase in viscosity (V); and at 60 min a solid gel (G) occurred. All sample mixtures became instantly clear after addition of acetic acid. Similar data were generated in three separate experiments.

Figure 2

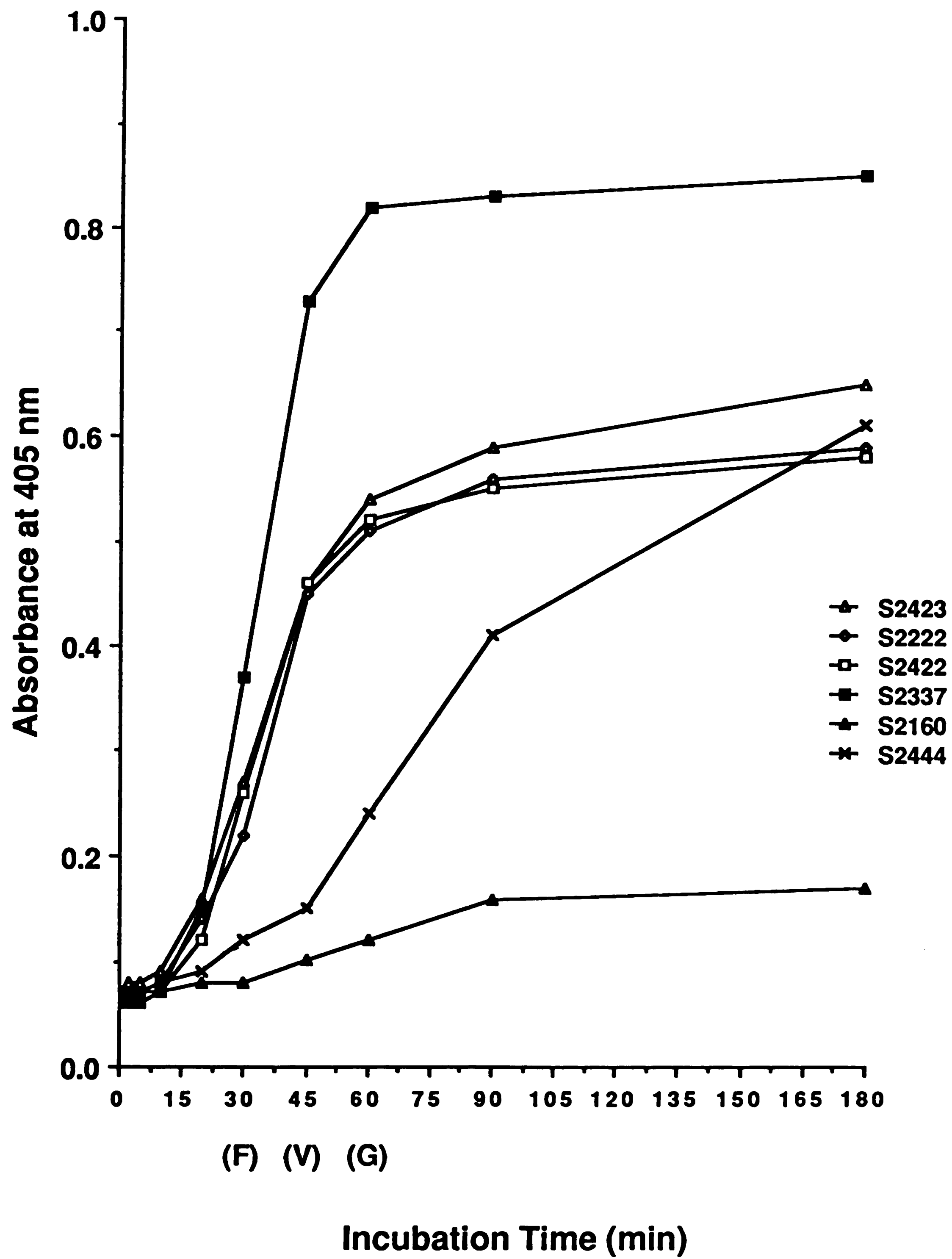


Figure 3. Characterization of Initially Rapid Enzymatic Activity. The initially rapid enzymatic activity in the supernatant of endotoxin-treated *Limulus* amebocyte lysate was examined using approximately 100-fold less enzyme than the previous experiment (Figure 2) by dilution with distilled water. One glass tube, containing 300 ul of lysate and 200 ul of 100 ug/ml of lipopolysaccharide(final concentration, 40 ug/ml), was preincubated at 37°C for 3 hr. After 3 hr preincubation, separation of supernatant and clotted protein was accomplished by centrifugation at 20,000 rpm for 20 min at 4°C. Sample mixtures, consisting of 99 ul of distilled water, 350 ul of 0.1mM chromogenic substrates S-2337, S-2423, S-2222, or S-2422, and 1 ul of supernatant, were incubated at 37°C. After each incubation time indicated, 200 ul of 20% acetic acid was used to stop the reaction. Enzymatic activity was assayed spectrophotometrically by absorbance at 405 nm.

Figure 3

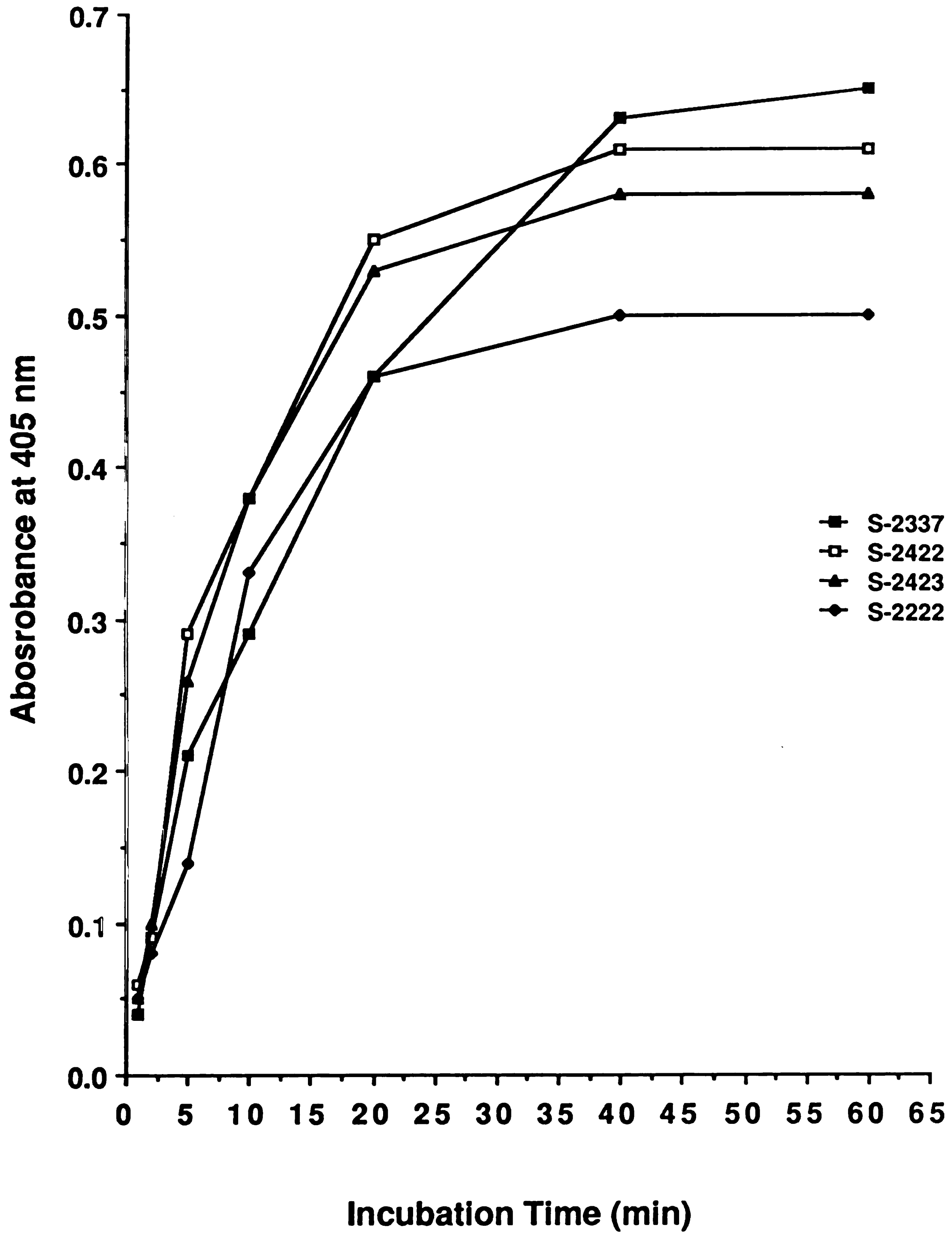


Figure 4. Gelatin Substrate SDS-Polyacrylamide Gel of Supernatant. Activation of *Limulus* enzyme activities was investigated by gelatin substrate SDS-PAGE. Supernatants were prepared from endotoxin-treated *Limulus* lysates following incubation with endotoxin for 5 min (lane 1), 10 min (lane 2), 20 min (lane 3), 1 hr (lane 4) or 3 hr (lane 5). Addition of of 1% SDS and centrifugation at 4°C stopped any subsequent enzyme activation. After electrophoresis, the gelatin gel was incubated at 37°C with glycine and then stained with Coomassie blue. The background of gelatin gel was dark because gelatin (collagen) was stained by Coomassie blue dye. Non-enzymatic protein bands had an intensive dark blue color. There were three clear proteolytic protein bands in each sample lane. Estimated molecular weights for the enzymatic activities were 60-70,000 daltons, 120,000 daltons and 140,000 daltons.

Figure 4

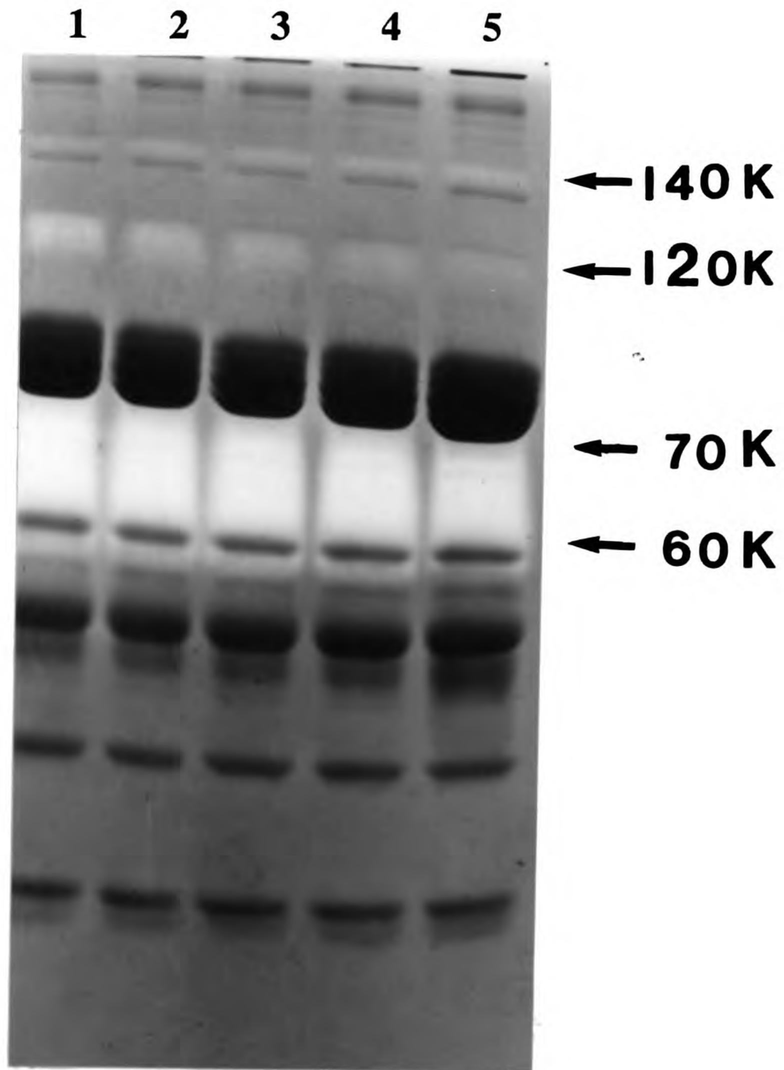


Figure 5. The Effect of Stirring on Recovery of Maximal Enzyme Activities in the Supernatant. Determination of the effect of stirring on the distribution of enzymatic activity between supernatant (Sup) and pelleted coagulin gel (Pellet) after 3 hr preincubation with endotoxin. Two separate tubes, each containing 500 ul of lysate and 333 ul of 100 ug/ml of lipopolysaccharide(final concentration, 40 ug/ml), were incubated in a 37°C water bath for 3 hr. One reaction mixture was stirred at a mild speed with a stirring bar, from the start of the incubation; the other was undisturbed. Temperature was carefully maintained at 37 ± 0.5°C. After 3 hr preincubation, separation of supernatant and clotted protein (coagulin) was performed by centrifugation at 20,000 rpm for 20 min at 4°C, and the coagulin gel was resuspended in 780 ul of 0.1M Tris-HCl (pH 7.8) by stirring. Sample mixtures, containing 350 ul of 0.1mM chromogenic substrate S-2160 with 100 ul of supernatant or 100 ul of gel suspension, respectively, were incubated at 37°C. For each incubation time indicated, 200 ul of 20% acetic acid was used to stop the reaction. Enzymatic activity was assayed spectrophotometrically by absorbance at 405 nm. Similar data were observed in two separate experiments.

Figure 5

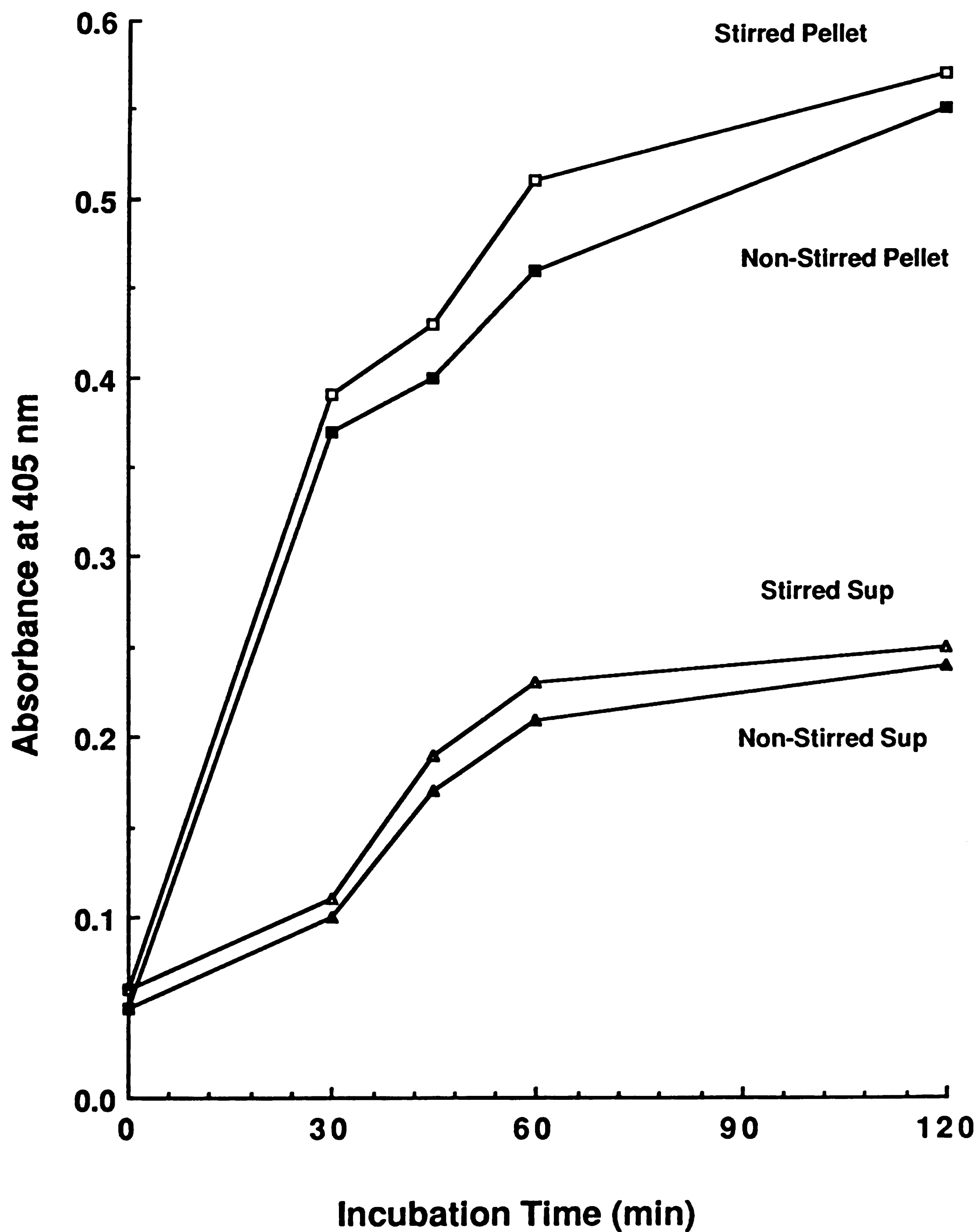


Figure 6(a)-(c). Storage of the Activated Enzyme Pool.

Figure 6 (a). Aliquots of an activated supernatant pool were stored at 4°C. Activated enzyme activities from the supernatant were assayed weekly with chromogenic substrate S-2160 (total of eight determinations). Sample mixtures, consisting of 100 ul of supernatant and 350 ul of chromogenic substrate S-2160 (0.1mM) containing 0.1M Tris-HCl (pH 7.8), were incubated at 37°C. Enzymatic activity was assayed spectrophotometrically by absorbance at 405 nm. S-2160 activity gradually declined during the eight week period of observation.

Figure 6 (c): At -72°C with glycerol

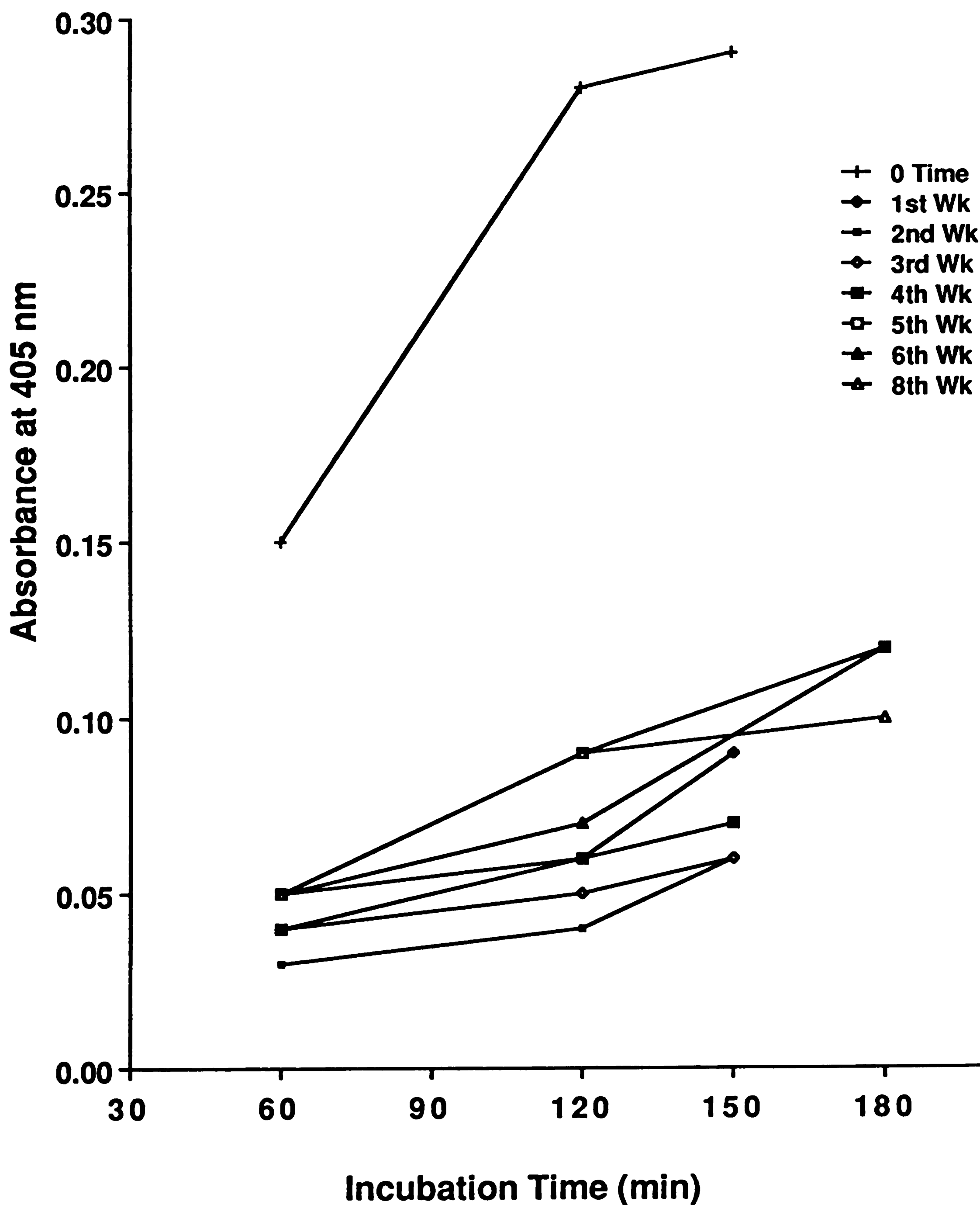


Figure 7(a)-(c). Investigation of the Presence of Factor XIII-Like Cross-Linking Activity.

Figure 7 (a). The relationship between the generation of enzymatic activity for S-2423 and changes in turbidity during gelation. Sample mixtures, consisting of 200 ul of lysate, 10 ul of 0.1 ug/ml lipopolysaccharide (final concentration, 0.0033 ug/ml, moderate concentration of LPS), 10 ul of 5 mM S-2423 and 80 ul of Tris-HCl (pH 7.8), were incubated at 37°C. After incubation for indicated times, the various stages of gelation of endotoxin-treated lysate were assessed visually. 500 ul of 8M urea was added and the mixture subjected to mild agitation with a spatula for 10-20 seconds and assayed spectrophotometrically at 650 nm for turbidity. Then 50 ul of 80% acetic acid was added to the sample mixtures. All sample mixtures became clear following addition of acetic acid and were measured spectrophotometrically at 405 nm for enzymatic activity. Similar data were generated in two separate experiments.

Figure 7 (a)

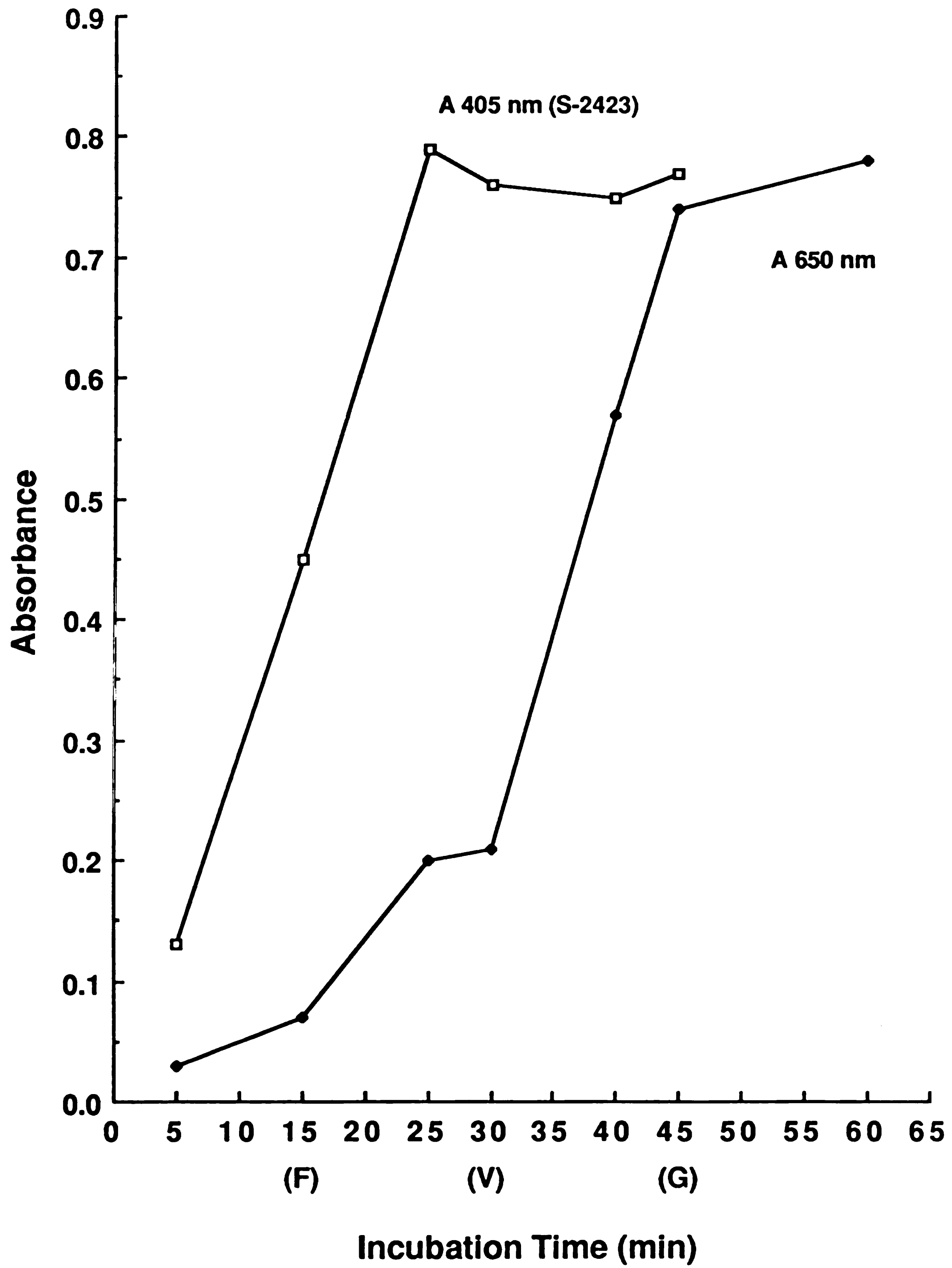


Figure 7 (b). The relationship between generation of enzymatic activity for S-2444 and changes in turbidity during gelation. Sample mixtures, consisting of 200 ul of lysate, 10 ul of 0.01 ug/ml lipopolysaccharide (final concentration, 0.00033 ug/ml, small concentration of LPS), 10 ul of 5mM S-2444 and 80 ul of Tris-HCl (pH 7.8), were incubated at 37°C. After incubation for indicated times, various stages of gelation of endotoxin-treated lysate were assessed visually and 500 ul of 8M urea was added with mild agitation with a spatula for 10-20 seconds and assayed spectrophotometrically at 650 nm for turbidity. Then 50 ul of 80% acetic acid was added to the sample mixtures. All sample mixtures became clear following addition of acetic acid and were measured spectrophotometrically at 405 nm for enzymatic activity.

Figure 7 (b)

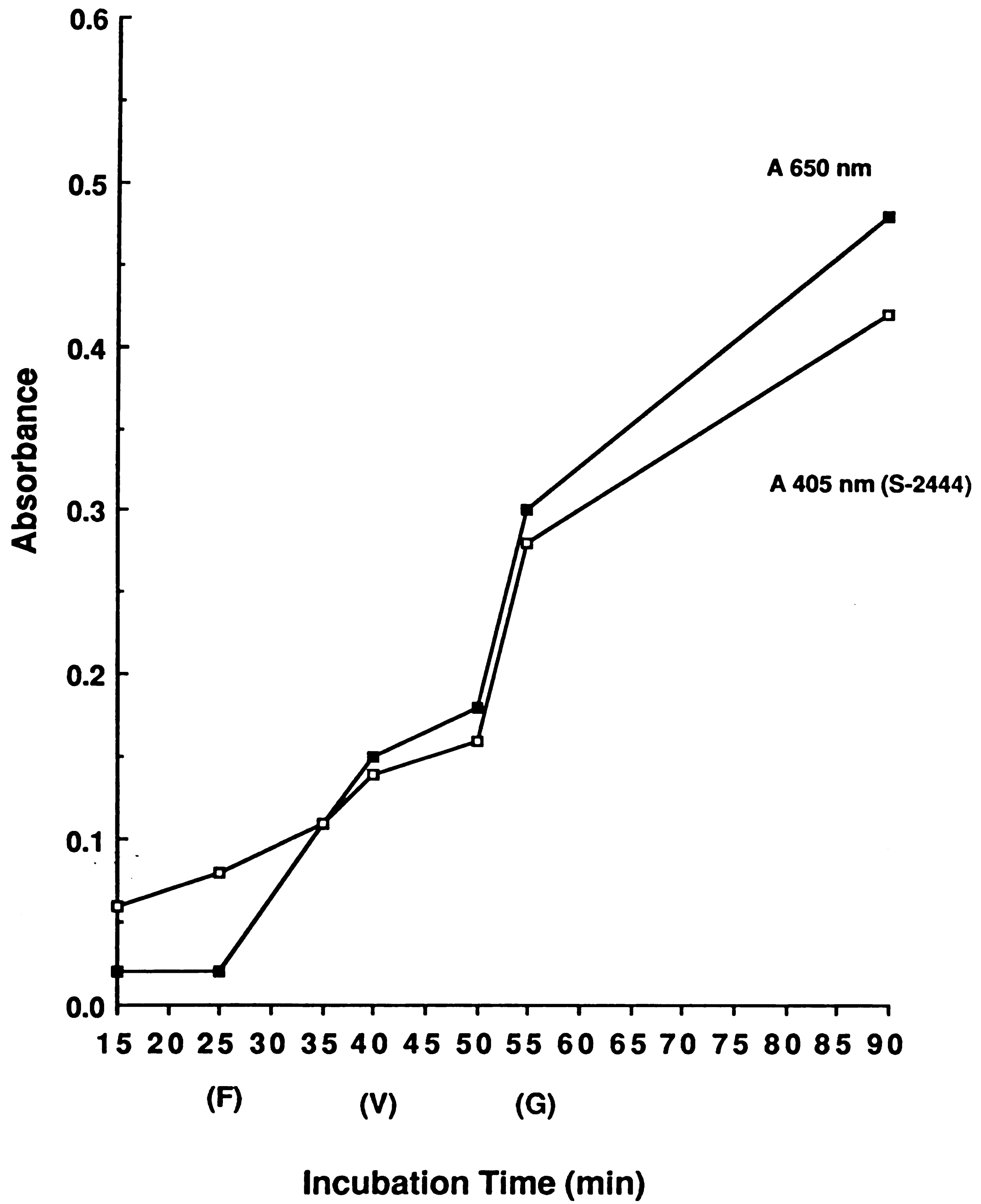


Figure 7 (c). The effect of vigorous mechanical agitation on the turbidity of endotoxin-treated lysate. Sample mixtures, consisting of 200 ul of lysate, 10 ul of 1 ug/ml of lipopolysaccharide (final concentration, 0.033 ug/ml, large concentration of LPS), 10 ul of 5mM S-2444 or S-2423 and 80 ul of Tris-HCl (pH 7.8), were incubated at 37°C. After incubation for indicated times, various stages of gelation were assessed visually, 500 ul of 8M urea was added with vigorous mixing (Vortex for 2 min), and assayed spectrophotometrically at 650 nm for turbidity. Then 50 ul of 80% acetic acid was added to the sample mixtures with urea. All sample mixtures became clear following addition of acetic acid and were measured spectrophotometrically at 405 nm for enzymatic activity. Similar data were observed in two separate experiments.

Figure 7 (c)

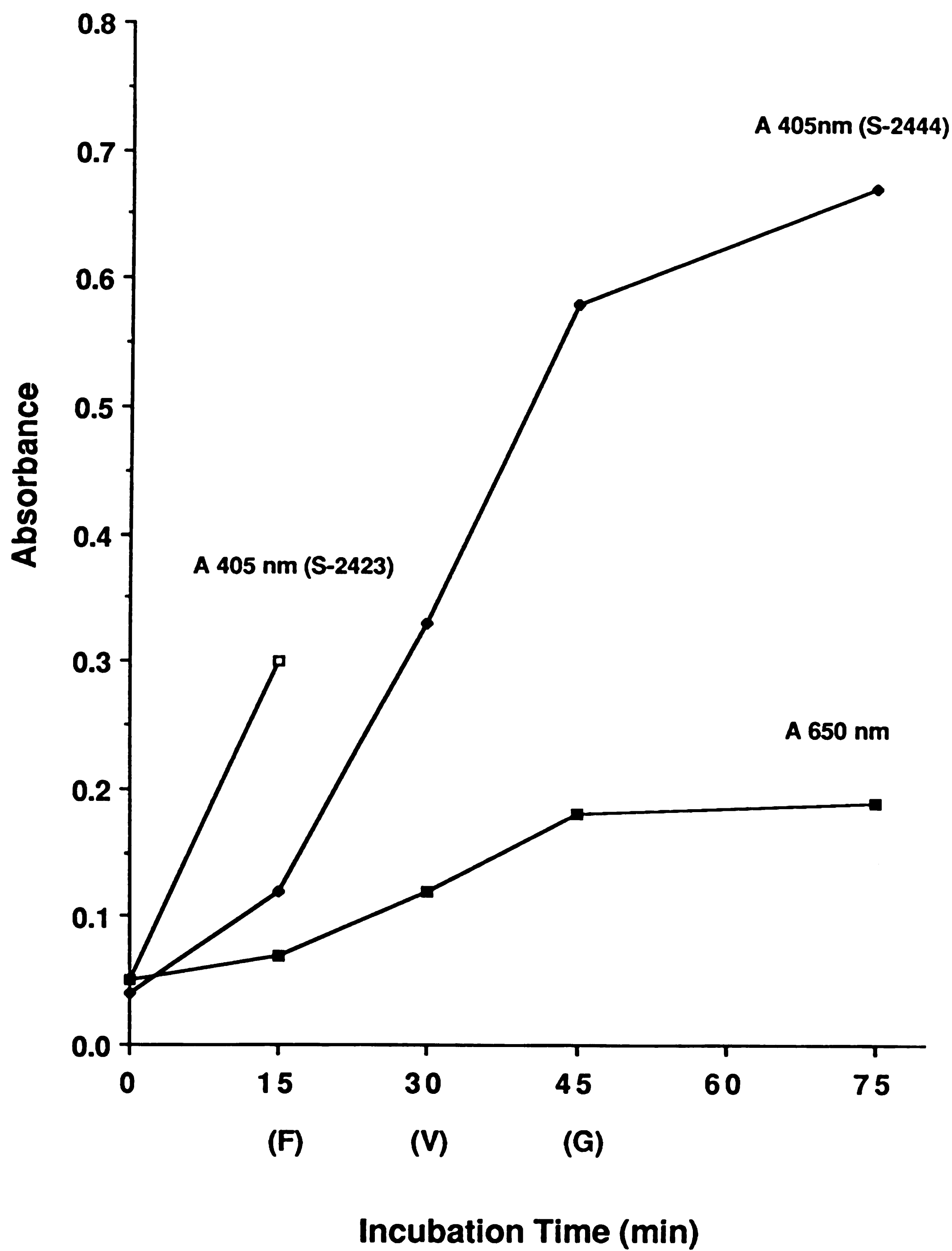


Figure 8. Fractionation of Supernatant of Endotoxin-Treated Limulus Lysate by Gel Permeation Liquid Chromatography G-100. Three ml of supernatant, which was obtained by centrifugation of endotoxin-treated lysate (final endotoxin concentration, 10 ug/ml), was applied to a Sephadex G-100 column (1.6 x 93 cm) preequilibrated with 0.1M Tris (pH 7.7), at a flow rate of 46 ml/hr. Enzymatic activities were detected only in 100 ul aliquots of peaks I (tube 15) and III (tube 28) (data not shown), utilizing S-2423. 100 ul aliquots from peak II (tube 22) and peak IV (tube 38), and from a 5-fold concentrated sample of peak IV, showed no enzymatic activities.

Figure 8

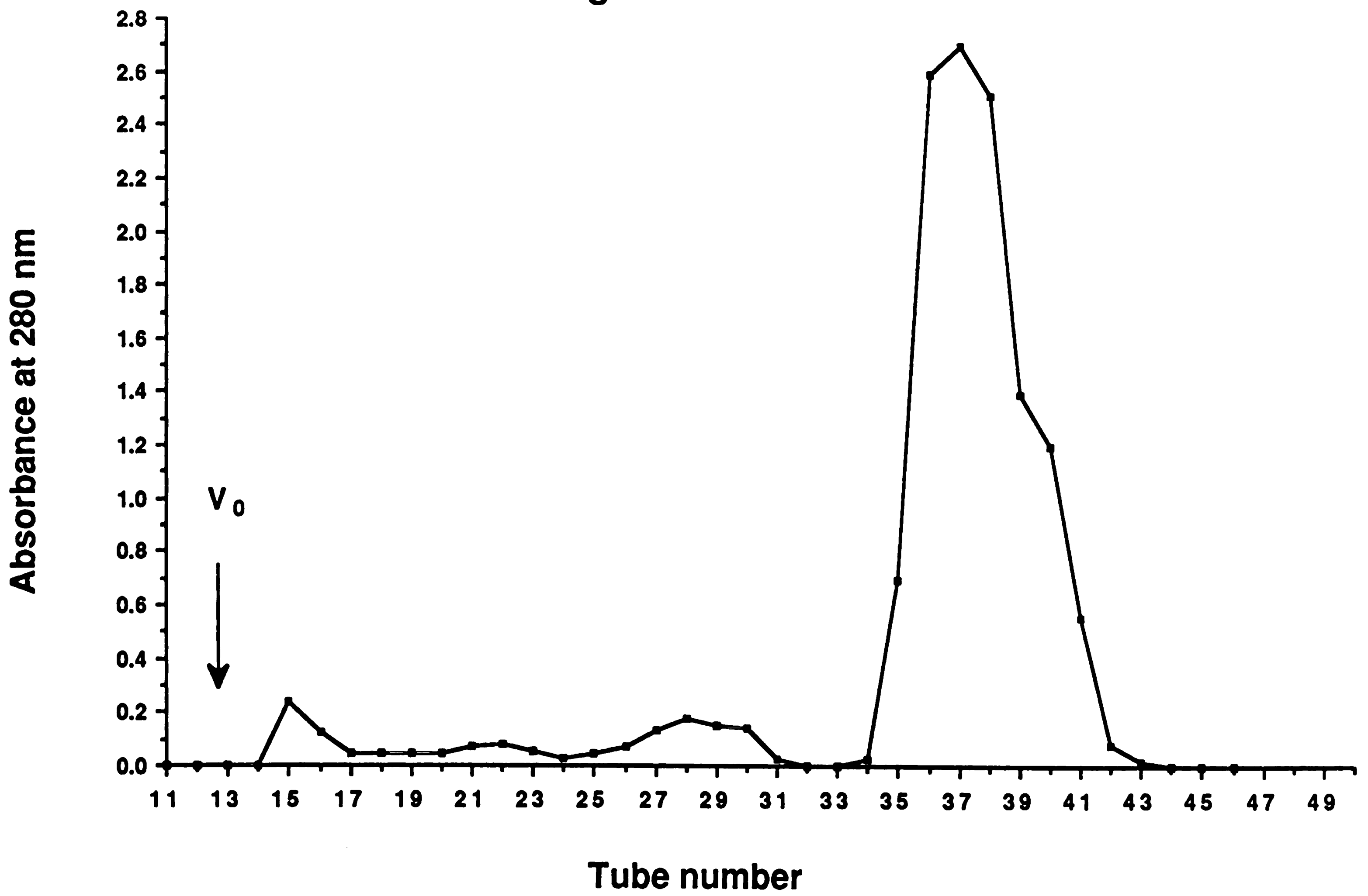


Figure 9. Fractionation of Concentrated Supernatant of Limulus Lysate by Fast Protein Liquid Chromatography Mono Q. 500 ul of 10-fold concentrated supernatant was applied to an anion exchange Mono Q column. Proteins were eluted with a linear 0-0.3 M NaCl gradient, followed by a 1 M NaCl elution. After chromatography, multiple protein peaks were present. Fractions were assayed for enzymatic activity using 0.5 mM S-2423. Enzymatic activities were detected in six of these fractions (F-1, 2, 3, 4, 5 and 6) which eluted between 0.05 M and 0.21 M NaCl.

Figure 9

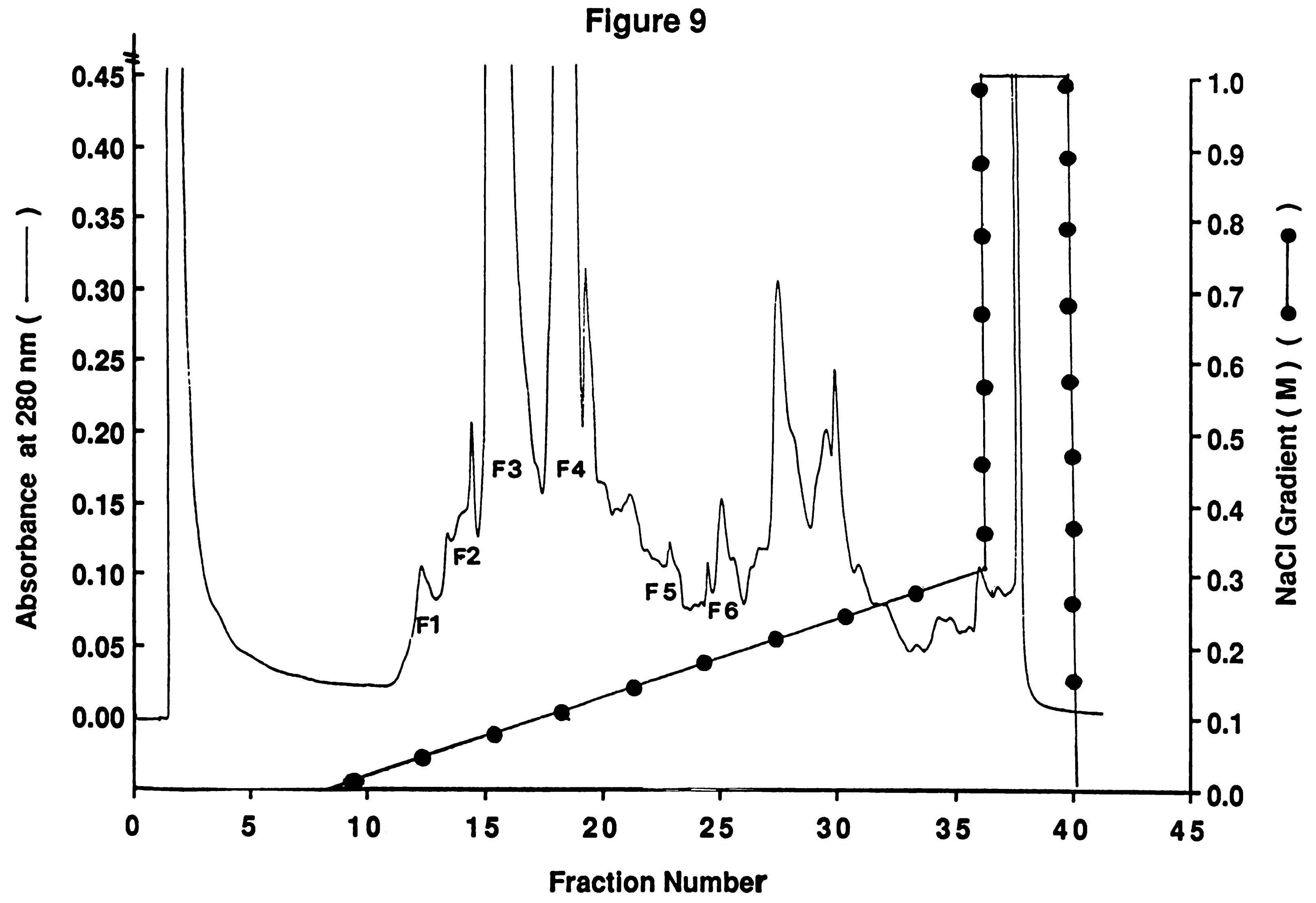


Figure 10 (c). 100 ul concentrated protein fraction from pooled F-3 of Mono Q (Figure 9) was applied to gel permeation-HPLC. Solid line demonstrates the protein absorbance at 280 nm and the dotted line indicates enzymatic activity detected by chromogenic substrate S-2423, with absorbance at 405 nm. The elution volume of maximal protease activity was 8.0-8.8 ml.

Figure 10 (c)

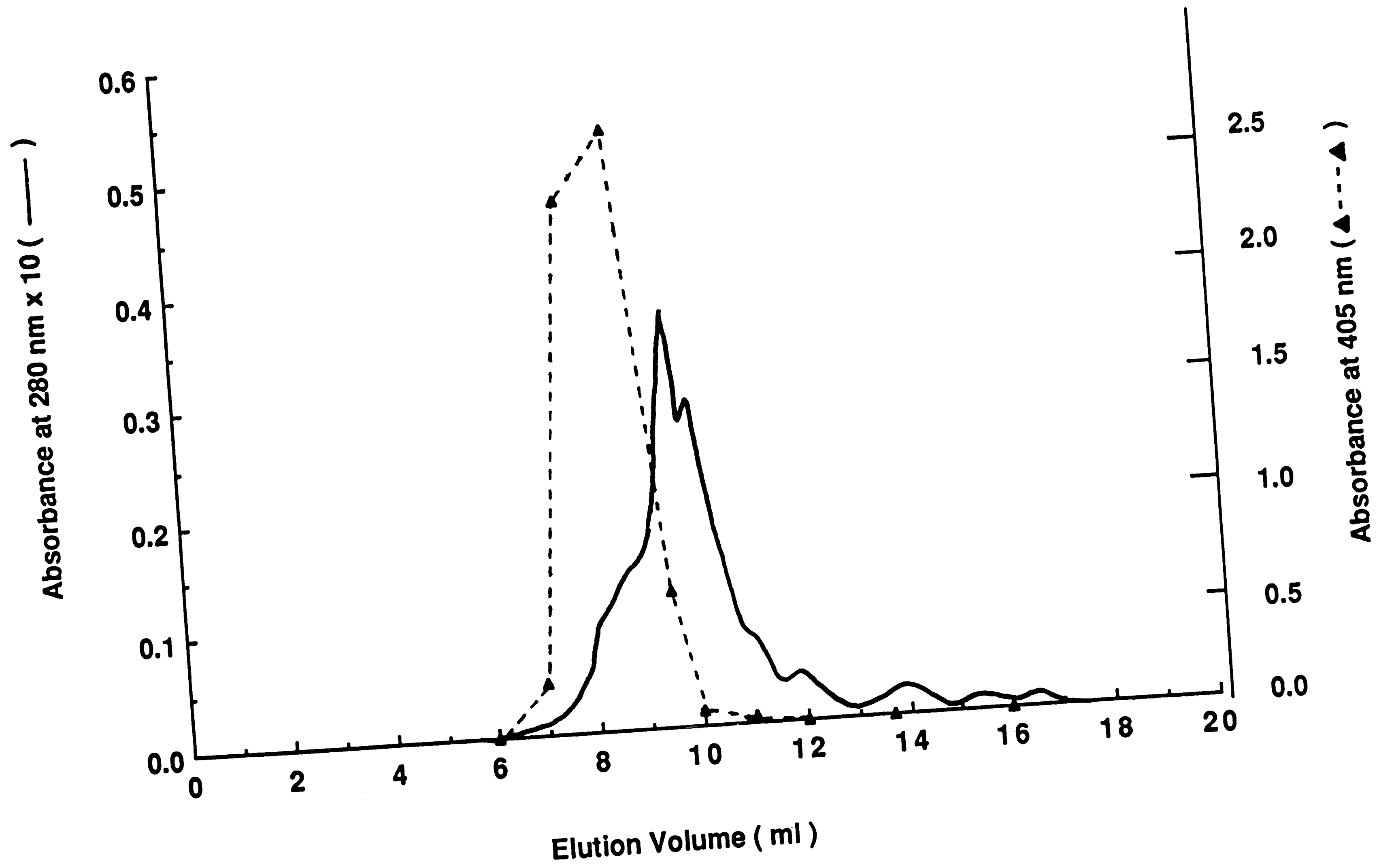


Figure 10 (f). 100 ul concentrated protein fraction from pooled F-6 of Mono Q (Figure 9) was applied to gel permeation-HPLC. Solid line demonstrates the protein absorbance at 280 nm and the dotted line indicates enzymatic activity detected by chromogenic substrate S-2423, with absorbance at 405 nm. The elution volume of maximal protease activity was 6.5-7.0 ml.

Figure 10 (b)

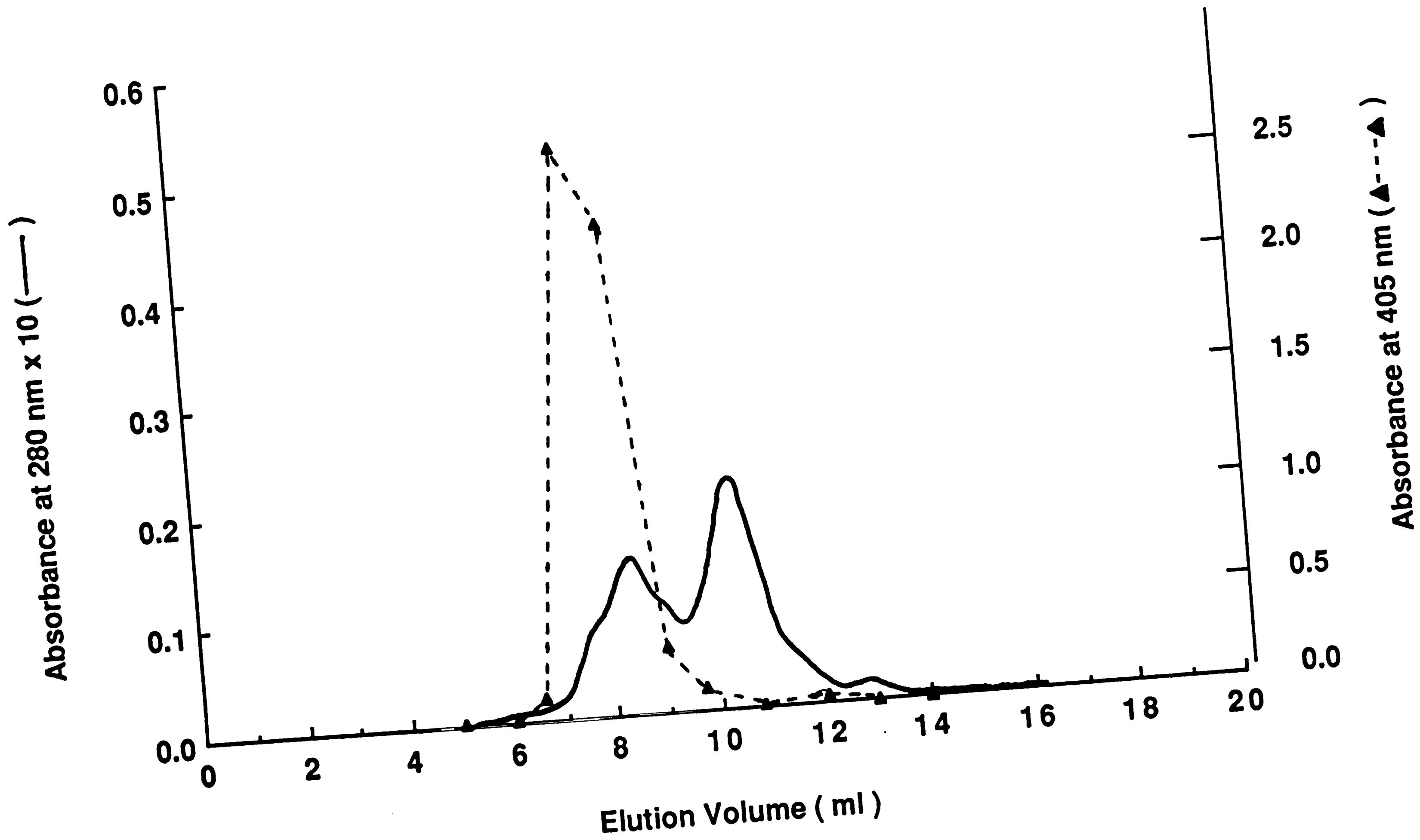


Figure 10 (d)

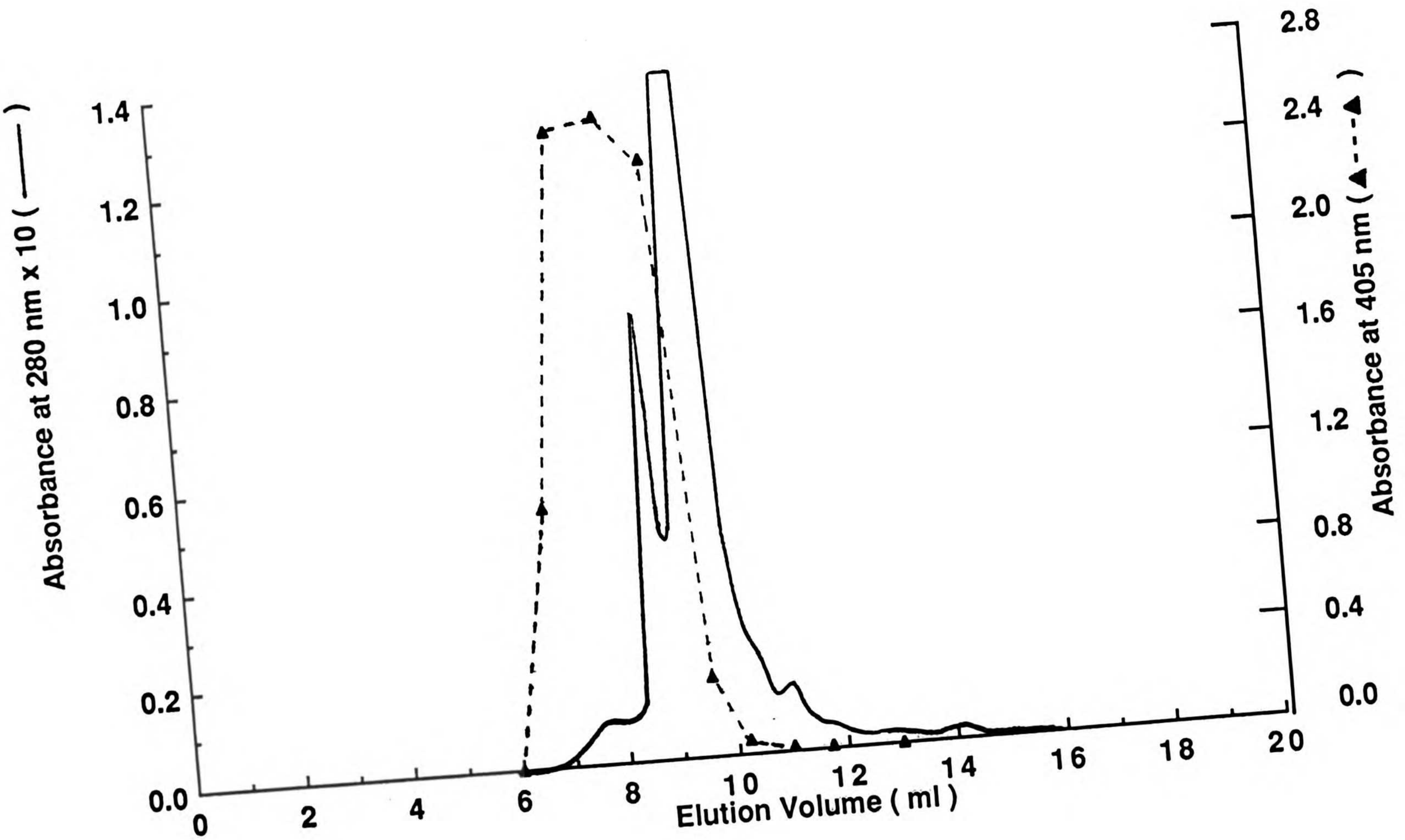


Figure 10 (f)

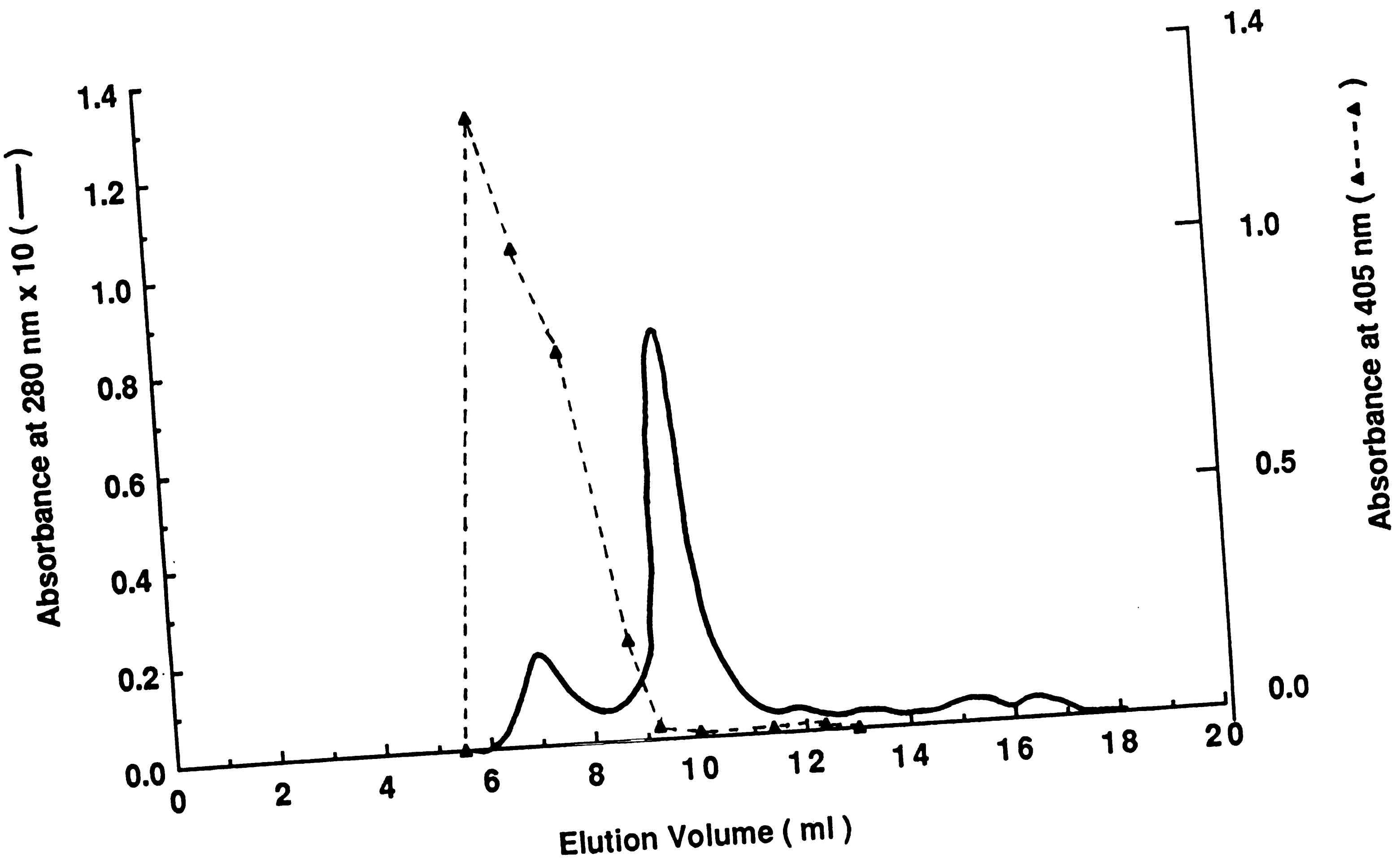


Figure 11. Identification of Limulus Enzymes from Mono Q by Gelatin Substrate SDS-PAGE. Lane (1) shows the protein bands from the supernatant of *Limulus* lysate. Three proteolytic bands are present. These bands had molecular weights of 140,000, 120,000 and 60-70,000 daltons. The clear proteolytic band with M.W. 140,000 daltons was eluted from Mono Q column with 1 M NaCl (lane 6). Lanes 7, 8 and 9 demonstrated one wide proteolytic band with M.W. 60-70,000 daltons which was eluted from Mono Q column with 0.08 M (F2), 0.13 M (F4) and 0.21 M (F6) NaCl, respectively. The remaining lanes (2-5) contained standard molecular weight markers without any proteolytic activity serving as the negative control for enzymatic activity.

Figure 11

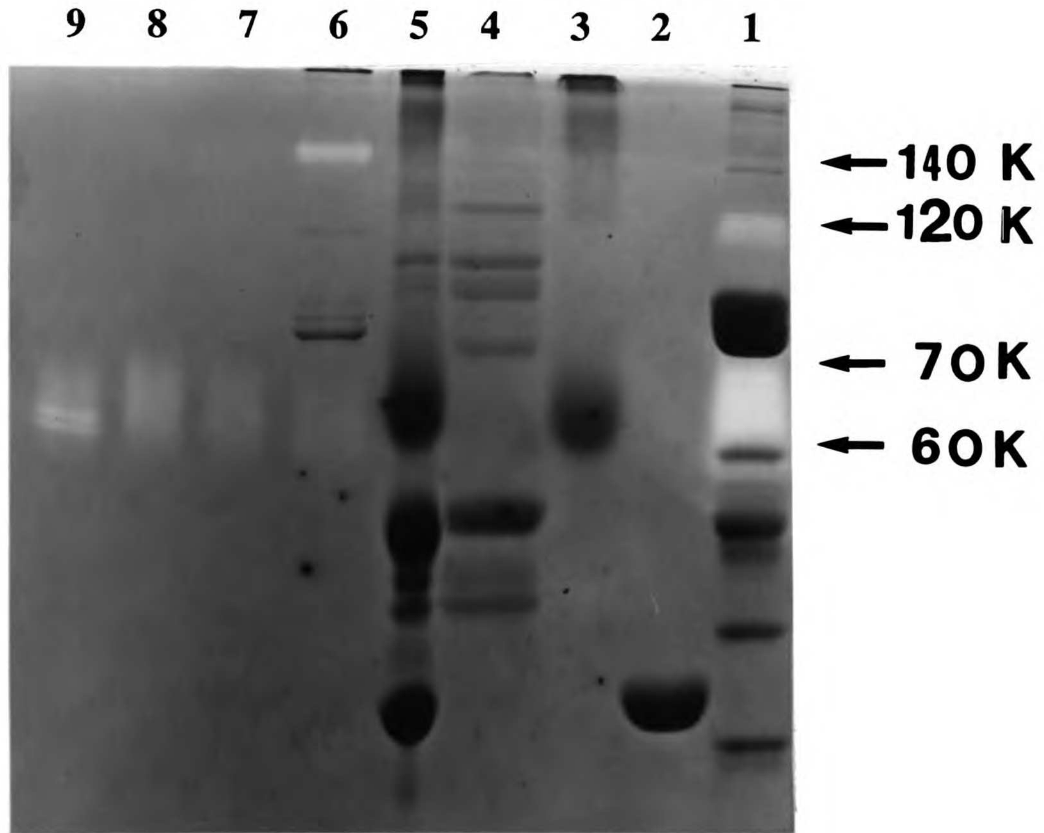


Figure 12. Identification of Limulus Enzymes from GP-HPLC by Gelatin Substrate SDS-PAGE.

(a) Six fractions from gel permeation HPLC were assessed with gelatin substrate SDS-PAGE for enzymatic activity and estimated molecular weight. Lanes 2-6 showed one wide proteolytic band with M.W. 60-70,000 daltons which was fractions with maximal enzymatic activity from gel permeation HPLC (see Figure 10b-10f). The absence of any proteolytic band in lane 1 (sample from Figure 10a) was due to lack of sufficient quantity of sample applied.

(b) Duplicate samples as used in (a) were electrophoresed with SDS-PAGE and then stained with silver stain.

Figure 12 (a)

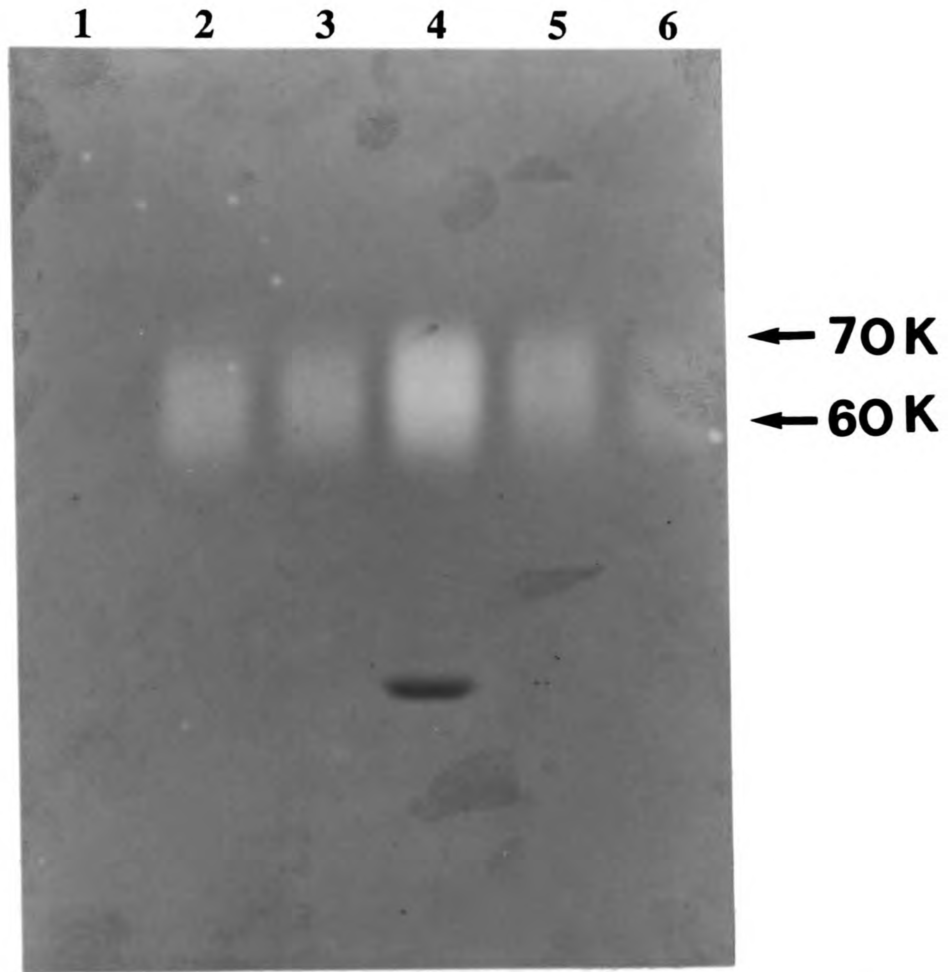


Figure 12 (b)

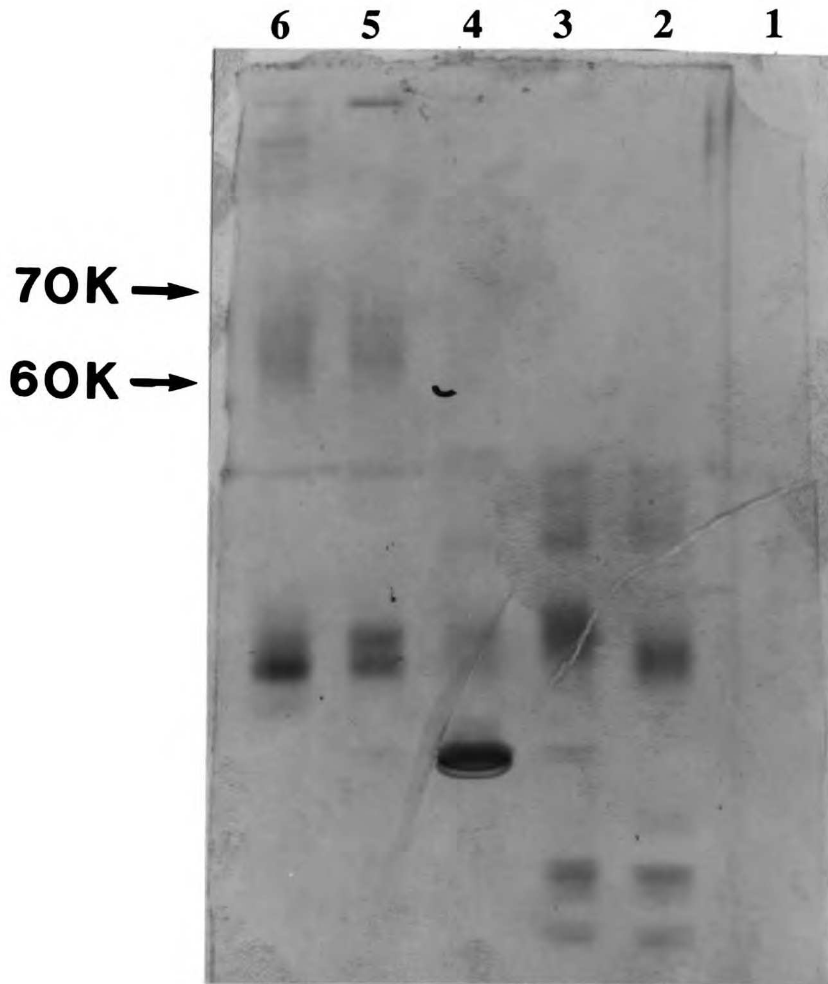


Table 1.

Chromogenic substrates used for detecting the protease activities

Protease activity	
Positive	*Negative*
S-2444 (urokinase)	S-2586 (chymotrypsin)
S-2423 (unspecified)	S-2338 (thrombin)
S-2422 (unspecified)	S-2302 (plasma kallikrein)
S-2337 (unspecified)	S-2251 (plasmin)
S-2222 (Xa)	
S-2160 (thrombin)	

Table 1. A Battery of Chromogenic Substrates for Limulus Protease Activities. Ten chromogenic substrates (KabiVitrum, Mo"lndal, Sweden) were tested for their abilities to detect the protease activities in supernatants of endotoxin-treated *Limulus* lysate. Lysates were gelled by incubation with E. coli endotoxin (final concentrations of 0.1-1.0 ug/ml endotoxin) at 37°C for 4 hr. Separation of supernatant and clotted protein(s) was performed by centrifugation at 20,000 rpm for 20 min at 4°C. Sample mixtures, consisting of 100 ul of supernatant and 350 ul of 0.1 mM chromogenic peptide substrates containing 0.1 M Tris-HCl (pH 7.8), were incubated at 37°C for 90 min. p-Nitroaniline groups, released from the the peptide substrates by protease activities, were assayed spectrophotometrically by absorbance at 405 nm. After incubation, six of ten chromogenic substrates tested were able to detect protease activities.

Table 2.

(A) Agents added at 0 time	Maximum OD after 90 min incubation					
	(1)		(2)		(3)	
	Sup*	Gel	Sup	Gel	Sup	Gel
Tween 20 (0.1%)	0.10	0.50	0.08	0.39		
Triton X-100 (1.0%)	0.09	0.31	0.03	0.33		
Triton X-100 (0.1%)	0.06	0.38	0.08	0.39		
0.8M NaCl	0.32	0.44	0.25	0.48	0.28	0.58
4.0M NaCl	0.38	0.48	0.31	0.44	0.35	0.43
EDTA (0.6%)	0.07	0.21	0.17	0.47		
(B) Agents added after 2 hr incubation						
Tween 20 (0.1%)	0.08	0.50				
4.0M NaCl	0.08	0.48				

*Supernatant

Table 2. Chemical Agents Used to Alter the Recovery of Enzymatic Activity(ies) in Supernatant. (A) Sample mixtures, consisting of 200 ul of lysate, 20 ul of 100 ug/ml of lipopolysaccharide (final concentration, 1.25 ug/ml) and the different concentrations of the above chemical agents in a total volume 400 ul, were preincubated at 37°C for 3 hr. Then, separation of supernatant and clotted protein was accomplished by centrifugation at 20,000 rpm for 20 min at 4°C, and the coagulin gel was resuspended in 350 ul of 0.1M Tris-HCl (pH 7.8) by stirring. Enzymatic activity was measured spectrophotometrically by absorbance at 405 nm, using reaction mixtures containing 350 ul of 0.1mM chromogenic substrate S-2160, and 100 ul of supernatant or gel suspension incubated at 37°C. OD values determined after a 90 min incubation were listed in the table. (B) Conditions were as in (A) except that endotoxin-treated lysate was incubated for 2 hr at 37°C before adding Tween 20 (0.1%) or 4M NaCl. The sample mixtures were then stirred for 1 hr followed by centrifugation, as above, to separate soluble enzymes from the coagulin pellet.

Table 3.

Fraction Number	Estimated M.W. from Elution Volume (HPLC)	Estimated M.W. from Gelatin Substrate SDS-PAGE (HPLC Fractions)	Estimated M.W. from Gelatin Substrate SDS-PAGE (Mono Q Fractions)
1	160-175K	ND*	ND
2	250-300K	60-70K	60-70K
3	≥ 300K	60-70K	ND
4	≥ 300K	60-70K	60-70K
5	≥ 300K	60-70K	ND
6	≥ 300K	60-70K	60-70K

*Not Determined

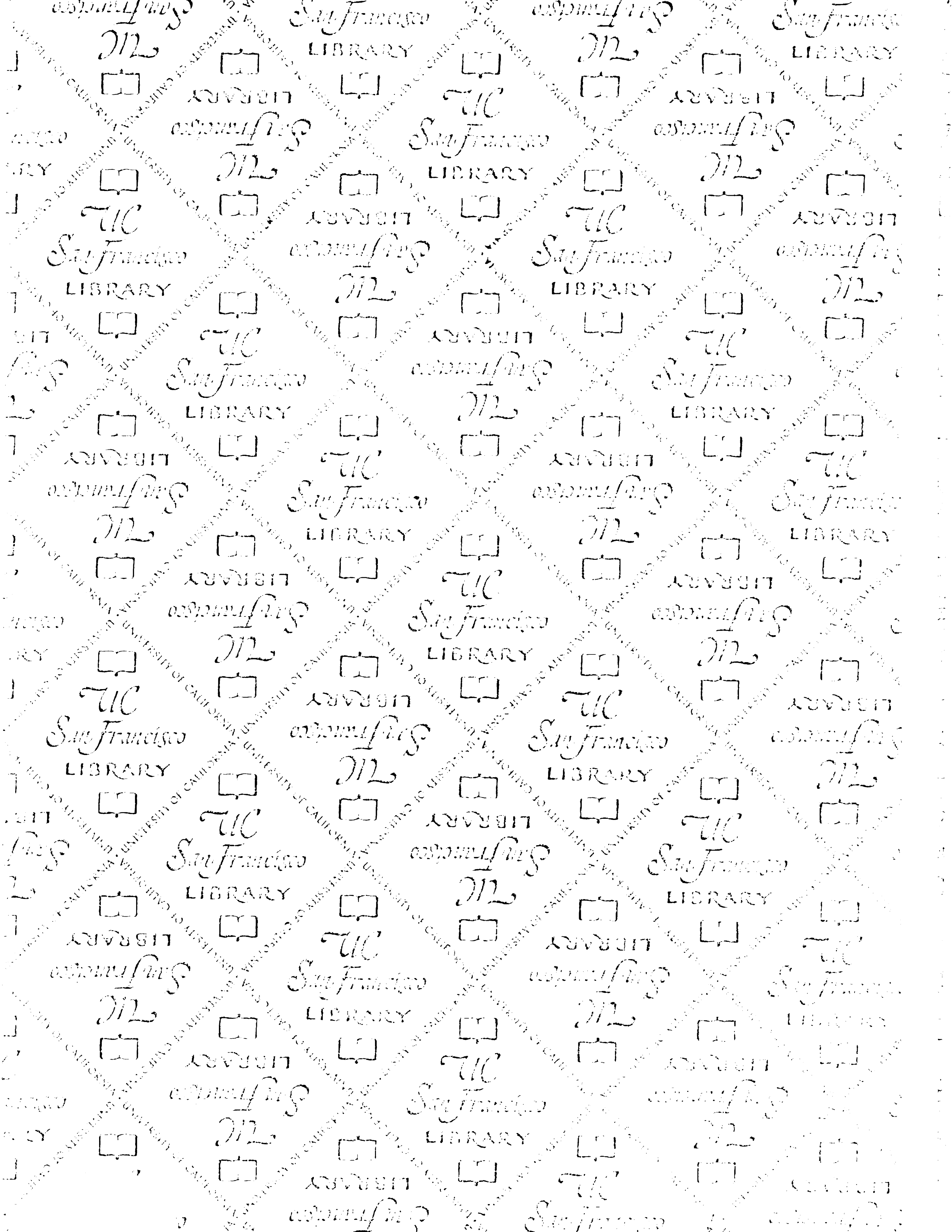
Table 3. Comparison of Estimated Molecular Weights of Clotting Enzymes Based on Elution Volume in HPLC or Migration in Gelatin Substrate SDS-PAGE. In column 1, fraction numbers represent the fractions from HPLC or FPLC-Mono Q. In column 2, samples were obtained from Figures 10(a)-10(f). Calculations of molecular weights were based on the elution volumes of standard molecular weight marker by HPLC. In columns 3 and 4, results are obtained from Figure 12(a) and Figure 11.

Table 4.

Gel piece number (cm from top)	Chromogenic Substrate									
	2423	2422	2222	2337	2444	2160	2238	2302	2251	2586
1	0.40	0.68	0.43	0.44	0.37	-*	-	-	-	-
2	0.36	0.68	0.34	0.47	0.10	-	-	-	-	-
3	0.07	0.37	0.11	0.08	-	-	-	-	-	-
4	0.20	0.12	0.12	0.08	-	-	-	-	-	-
5	-	0.18	0.08	0.20	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-

*Negative (OD value below 0.05)

Table 4. Chromogenic Assays of Gel Slices from Non-Denaturing Polyacrylamide Gel Electrophoresis of Limulus Enzymes. Polyacrylamide disc gels were 10% acrylamide, 100 mM Tris-acetate, pH 8.5, with a 3% stacking gel. Samples were obtained by incubating 1 ml *Limulus* lysate with 10 ul endotoxin (100 ug/ml) at 37°C for three hours, followed by centrifugation at 20,000 rpm to remove the insoluble coagulin gel. The resulting supernatant contained all the endotoxin-activated *Limulus* enzymes. 75 ul of sample was applied to each gel and electrophoresis was performed at 4°C, 1.5 mAmp/tube, for 16 hr. Following electrophoresis, the gels (0.8 x 10 cm) were cut into 1 cm pieces (excluding the stacking gels), and each gel piece was minced and incubated in 0.45 ml of chromogenic substrate (0.1 mM in 0.1 M Tris-HCl, pH 8.0) for 4 hr at 4°C. The incubation mixtures were observed periodically during the four hour assay, and when adequate yellow color had developed, the enzymatic reactions were stopped by the addition of 300 ul 20% acetic acid. Absorbances were determined at 405 nm for each sample as listed in the table. The strongest chromogenic activities were observed in the top two cm gel slices. Within 20-60 min, incubations using S-2423, S-2422 and S-2222 were definitely positive in these two samples. Activity for S-2337 was faint in these samples. Activity for S-2444 was detected after 1 hr primarily in the top gel slice. The remaining chromogenic substrates were not cleaved at all. No activities were present in the lower half of the gels.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