Purification of *Limulus polyphemus* Proclotting Enzyme*

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Horseshoe crabs (*Limulus polyphemus* and *Tachypleus tridentatus*) possess a proteolytic blood coagulation system within their amebocytes that, after release and endotoxin activation, generates a polymerized insoluble coagulin clot. Clotting enzyme from horseshoe crab amebocyte lysate is the protease that activates the clottable protein (coagulogen) which then forms the coagulin clot. Comparison of the previously published descriptions of this enzyme has revealed significantly discordant biochemical characteristics. We purified a 60-kDa proclotting enzyme from *L. polyphemus* amebocyte lysate to a single band on polyacrylamide gel electrophoresis. After electrophoresis, evaluation of enzymatic activity of this protein within gels demonstrated that the band of purified protein corresponded to enzymatic activity, as detected by amidolytic activity for chromogenic substrates and by gelation of coagulogen applied to the gel. The enzymatic activity was inhibited by serine protease inhibitors. The purified proclotting enzyme had a molecular weight and amino acid composition different from the previously published characterizations of proclotting enzymes from both *L. polyphemus* and *T. tridentatus*.

Amebocytes, the only type of circulating blood cell of the North American horseshoe crab (*Limulus polyphemus*) and the Japanese horseshoe crab (*Tachypleus tridentatus*), contain a blood coagulation mechanism that is activated by bacterial endotoxin (LPS). This coagulation system contributes importantly to hemostasis in the horseshoe crab and is critical for the organism's defense against aquatic gram-negative bacteria. Endotoxin-activated coagulation has been studied by us and others (1-3), using lysates of washed plasma-free amebocytes. LPS activates a proteolytic cascade, ultimately resulting in the partial proteolysis of coagulogen (the clottable protein), with release of peptide C. The result of enzymatic activity responsible for the limited proteolysis of coagulogen has been termed clotting enzyme (6).

Although the enzymatic properties indicated above pertain to the clotting enzyme of both *Limulus* and *Tachypleus*, no such process has been described in Limulus lysate; however, although it is possible that removal of an inhibitor could result in the activation of proclotting enzyme, no such process has been described.

Clotting enzymes isolated from lysates of the amebocytes of *L. polyphemus* and *T. tridentatus* are known to be serine proteases on the basis of their activities for synthetic substrates and their inhibition by typical proteinase inhibitors such as diisopropyl fluorophosphate, benzamidine, p-chloromercuri phenylsulfonate, antithrombin III, a2-antiplasmin, and soybean trypsin inhibitor (1, 7, 8). Clotting enzyme exists within amebocytes as the zymogen protease, proclotting enzyme, and is inactive until the coagulation cascade is initiated by LPS. Conversion of proclotting enzyme in amebocyte lysate to the active protease is accomplished by a proteolytic activity designated as activator (9) or Factor B (10) and can also be produced by trypsin (9, 11). Protease inhibitors also have been described in Limulus lysate; however, although it is possible that removal of an inhibitor could result in the activation of proclotting enzyme, no such process has been described.

Although the enzymatic properties indicated above pertain to the clotting enzymes of both *Limulus* and *Tachypleus*, review of the published literature on the characterization of horseshoe crab clotting enzyme discloses many areas of inconsistency. As shown in Table I, unresolved issues concern molecular weight, presence or absence of γ-carboxyglutamic acid residues of protein, coagulin, polymerizes into a solid gel (2, 4, 5). The enzymatic activity responsible for the limited proteolysis of coagulogen has been termed clotting enzyme (6).

### Table I

<table>
<thead>
<tr>
<th>Proclotting enzyme</th>
<th>Molecular mass</th>
<th>Requires calcium</th>
<th>Endotoxin-activated</th>
<th>Limulus (L) or Tachypleus (T)</th>
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<td>Yes</td>
<td>L</td>
</tr>
<tr>
<td>Tai (8)</td>
<td>&gt;150 kDa</td>
<td>No</td>
<td>No*</td>
<td>L</td>
</tr>
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<td>Muta (16)</td>
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<th>Clotting enzyme</th>
<th>Molecular mass</th>
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<th>Limulus (L) or Tachypleus (T)</th>
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<td>Yes</td>
<td>T</td>
</tr>
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<td>Nakamura (15)</td>
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<td>Yes</td>
<td>L</td>
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<td>Seid (19)</td>
<td>40/80 kDa</td>
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<td>No</td>
<td>T</td>
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<td>No</td>
<td>ND</td>
</tr>
</tbody>
</table>

*From sequence data analysis; the cloned cDNA does not code for the sequence of amino acids that result in γ-carboxylation of glutamic acid in Vitamin K-dependent coagulation factors.

*ND, not determined.

*Two proteins identified, with molecular sizes of 40 and 80 kDa. Amino acid compositions were consistent with a monomer/dimer relationship.

*Molecular mass estimated by gel filtration; clotting enzyme consists of two identical subunits (45 kDa) by SDS-polyacrylamide gel electrophoresis.
dues, requirement for Ca²⁺, and mode of activation. In this work, we purified to homogeneity and characterized L. polyphemus proclotting enzyme and used detection of enzymatic activity within SDS-polyacrylamide gels to definitively assign clotting enzyme activity to the isolated protein.

** MATERIALS AND METHODS **

** Chromogenic Substrate—** Chromogenic substrate S-2222 (N-benzoyl-Ille-Gly-Arg-p-nitroaniline; substrate for mammalian coagulation factor Xa) (AB Kabi Vitrum, Molndal, Sweden) was the generous gift of Dr. Peter Friberger (KabiVitrum Ltd.) and was reconstituted using pyrogen-free water (Travenol Laboratories, Deerfield, IL).

**Reagents—** Sterile endotoxin-free water and saline solutions were purchased from Travenol Laboratories. TLCK, TIPC, diisopropyl fluorophosphate, benzamidine, soybean trypsin inhibitor, chicken egg trypsin inhibitor, phenylmethylsulfonyl fluoride, aprotinin, p-hydroxymercuribenzoate, iodoacetamide, N-acetylglucosamine, and p-di-(0)

**FPLC Mono P chromatofocusing, and HPLC TSK**

**Isolation factor Xa** (AB Kabi Vitrum, Molndal, Sweden) was the reconstituted using pyrogen-free water (Travenol Laboratories, Deerfield, IL).

**Endotoxins used were Escherichia coli lipopolysaccharide B,**

**reagents were from Bio-Rad. Heparin-agarose gel was purchased from**

**was determined with a sensitive modified chromogenic Limulus test**

**brane (Millipore Corp., Bedford, MA).**

**activity was detected within the gel by applying 20 µl of enzyme and 100% D-2222 (0.25 mM, pH 8), and the reaction rate of Limulus clotting enzyme was compared with a wide range of trypsin concentrations (1 mg/ml to 100 ng/ml) in order to approximate match chromogenic activities of the two enzymes. It was determined that the pool of Limulus clotting enzyme had amidolytic activity for S-2222 which was equivalent to 10 µg/ml trypsin. A source of Limulus coagulogen was prepared by heating Limulus lysate at 65 °C for 15 min, which has been shown to destroy all enzymatic activity. 20 µl of Limulus clotting enzyme or trypsin was incubated in microtiter plate wells with 45 µg of coagulogen pool, pH 6, at 37 °C, and absorbances at 340 nm were determined at 5-min intervals. Concomitantly, control wells containing S-2222 (0.25 mM, pH 8) to provide a direct simultaneous comparison between the chromogenic activities and gelation activities of Limulus clotting enzyme and trypsin.

**RESULTS **

**Isolation of Limulus Proclotting Enzyme—** Purification of proclotting enzyme was accomplished with a series of chromatographic steps. Limulus amebocyte lysate (200 ml) was initially chromatographed on heparin-agarose (70-ml bed volume) under pyrogen-free conditions at 4 °C, in the absence of buffer. Proclotting enzyme did not bind to the column, whereas coagulogen was the predominant protein eluted with 0.15 M NaCl, and activator(s) present in the proteins which eluted with 0.52 M NaCl (Fig. 1). Unbound material from heparin-agarose was then applied to wheat germ lectin sepharose (10 ml) at 4 °C. Proclotting enzyme bound to the lectin column and was eluted with 0.4 M N-acetylglucosamine, pH 7. Wheat germ lectin-binding material, including proclotting enzyme, was then dialyzed against 0.25 M bis-(tris, pH 6.3, in M, 12,000 cut-off dialysis membranes (Spectrum Medical Industries, Inc., Houston, TX) which had been rendered endotoxin-free by extensive rinsing with pyrogen-free water. The dialized material then was applied to a Mono P chromatofocusing column, and a linear pH gradient from pH 6.3 to 4.0 was developed with Polybuffer 74 (Pharmacia)
FIG. 1. **Chromatography of Limulus amebocyte lysate on heparin-agarose.** 200 ml of lysate in sterile pyrogen-free water was applied to endotoxin-free heparin-agarose. Bound material was sequentially eluted with 0.15, 0.32, 0.57, and 0.83 M NaCl. Proclotting enzyme was in the unbound fraction, and coagulogen was eluted with 0.15 M NaCl. Fraction size was 6 ml.

FIG. 2. **Mono P chromatofocusing.** Partially purified proclotting enzyme (material unbound on heparin-agarose and bound on wheat germ lectin-Sepharose) was applied to Mono P at pH 6.3. A gradient from pH 6.3 to pH 4.0 was developed, during which the majority of applied proteins eluted, followed by a high salt wash with 2 M NaCl, pH 4.0 (arrow). Proclotting enzyme eluted with the high salt (bar). Fraction size was 2 ml.

diluted 10-fold (Fig. 2). Proclotting enzyme remained bound on the Mono P column during the gradient, but subsequently was eluted with 2 M NaCl, pH 4.0, after completion of the gradient. Material containing proclotting enzyme was then dialyzed against 0.15 M NaCl, 10 mM sodium phosphate, pH 6.8, and additionally chromatographed by gel permeation HPLC (TSK 3000; 0.75 x 60 cm, with a 0.75 x 15-cm guard column). Proclotting enzyme eluted from the gel permeation column with an approximate molecular mass of 55–65 kDa (Fig. 3). Three sequential gel permeation fractionations of proclotting enzyme activity ultimately yielded a protein of 60 kDa which was homogeneous on SDS-polyacrylamide gel electrophoresis with either Coomassie Blue or silver staining (Fig. 4).

**Identification of Purified Proclotting Enzyme**—Using the protein fraction that did not bind to heparin-agarose as a source of partially purified proclotting enzyme, the zymogen protease was activated to clotting enzyme on an immobilized trypsin column (see “Materials and Methods”). Clotting enzyme then was electrophoresed in SDS under reversibly de-naturing conditions and subsequently renatured within the gel, as described under “Materials and Methods.” Clotting enzyme activity within the gel was localized by visualization of a chromogenic band (Fig. 5) and by focal deposition of gelled coagulogen (Fig. 6). Both protease assays (for amidolytic and procoagulant activities, respectively) identified a single proteolytic activity which coincided in migration with the 60-kDa band detected by protein staining of the purified proclotting enzyme (Fig. 4). In the absence of activation of proclotting enzyme by immobilized trypsin, this proteolytic activity was not detected (data not shown).

**Characterization of Proclotting Enzyme**—From 200 ml of Limulus amebocyte lysate, approximately 8 μg of proclotting enzyme (homogeneous by SDS-polyacrylamide gel electrophoresis) was isolated as described above and activated on the
immobilized trypsin column. Clotting enzyme activities (amidolytic activity for chromogenic substrate S-2222 and gelation of coagulogen) were maximal in calcium- and magnesium-free buffer. Addition of calcium (concentrations 1–100 mM) did not alter enzymatic activity, and there was no inhibition by EDTA (10 mM). Purified proclotting enzyme was not activated by incubation with either E. coli endotoxin (100 μg/ml) or S. minnesota Lipid A (1 mg/ml). Comparisons of inhibition of partially purified clotting enzyme (approximately 90-fold purified) by a battery of protease inhibitors (Table II) demonstrated sensitivities characteristic of a serine protease.

Kinetic analysis of clotting enzyme, activated on immobilized trypsin, demonstrated a $K_m$ for chromogenic substrate S-2222 at pH 8 of 67 μM and a $V_{max}$ of $4.6 \times 10^3$ μmol/min/μmol clotting enzyme (Table III). The affinity and catalytic constants of clotting enzyme for S-2222 were each approximately one-fourth that of porcine trypsin (Table III; trypsin data obtained from AB Kabi Vitrum). When preparations of Limulus clotting enzyme and porcine trypsin were prepared with approximately equal amidolytic activities for S-2222 (see "Materials and Methods") (Fig. 7A), the abilities of the two enzymes to gel coagulogen were dramatically different (Fig. 7B). During a 40-min incubation at 37 °C, absorbance at 340 nm (a measure of coagulation) increased dramatically for Limulus clotting enzyme, whereas no absorbance change was detectable with trypsin. In addition, after 1 h, the incubation mixture containing Limulus enzyme was a solid gel, whereas the incubation mixture with trypsin was transparent, with no visible evidence of flocculation. Therefore, Limulus clotting enzyme demonstrated a greater affinity for its natural substrate, coagulogen, than did trypsin.

Amino acid analysis of purified proclotting enzyme (Table IV) demonstrated an amino acid composition that showed substantial differences from the compositions reported pre-
Polyphemus but not quantified. Attempts at NH2-terminal amino acid developed for the assay of mammalian coagulation factor Xa. There was even less resemblance to the previously reported consistent with a blocked amino terminus.

Amino sugar was detected, as reported previously (8, 15), amino acid composition of Limulus clotting enzyme (Table IV). Amino sugar was detected, as reported previously (8, 15), amino acid composition of Limulus clotting enzyme (Table IV)

DISCUSSION

Proclotting enzyme was purified to homogeneity from L. polyphemus amebocyte lysate by a series of chromatographic steps. The enzyme, when activated by immobilized trypsin, had activity for the chromogenic substrate S-2222, a substrate developed for the assay of mammalian coagulation factor Xa. Previous reports have described substrate specificities, inhibitor profiles, and amino acid sequence data for clotting enzyme of L. polyphemus (7) and Tachypleus (15, 16) that demonstrate the factor Xa-like nature of clotting enzyme. The substrate specificities and inhibitor sensitivities of the unpurified Limulus clotting enzyme activity described previously (1, 7) are in generally good agreement with the characteristics of the purified enzyme in this report. We also were able to establish that the 60-kDa electrophoretic band of the activated coagulation protein had clotting enzyme activity demonstrable both by an in situ chromogenic assay that detected amidolytic activity and an in situ clotting assay using coagulogen, the native substrate for the protease. The in situ chromogenic assay was extremely useful, because the low concentrations of proteases in lysate could lead to erroneous attribution of enzymatic activities to non-enzyme electrophoretic bands. In addition, the in situ protease assay using coagulogen, the native substrate of the clotting enzyme, was critically definitive, because more than one coagulation protease exists in amebocyte lysate (3, 17), and other noncoagulation proteases probably also are present. The total number of proteases in Limulus amebocyte lysate is not known. However, in preliminary experiments (data not shown) we have detected a minimum of four protease activities in gels of Limulus lysate, using immobilized collagen as a nonspecific protease substrate (18).

The molecular mass of the previously described proclotting enzyme of Limulus (8) is high (>150 kDa), and its amino acid composition is substantially dissimilar to ours. The molecular mass and amino acid composition of Limulus clotting enzyme (the activated protease) (19) is also substantially different from ours. It is unlikely that the high molecular weight protein reported by Tai and Liu (8) is a multimer of the 60-kDa enzyme we describe, since we have not observed multimers during detection of clotting enzyme in electrophoretic gels by analysis for enzymatic activity. Furthermore, the proclotting enzyme we have isolated is not directly activated by endotoxin and does not require calcium for activity, whereas the enzyme isolated by Tai and Liu (8) required calcium and was reported

<table>
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<tr>
<th>L. polyphemus (L.P.)</th>
<th>T. t. (Ref. 16)</th>
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<td>Trp ND</td>
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<td>6 (1.5)</td>
<td>20 (1.5)</td>
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*a Present work.

Numbers in parentheses indicate percent of total residues.

ND, not determined.

Fig. 7. Comparison of Limulus clotting enzyme and trypsin: chromogenic activity versus ability to gel coagulogen. Incubation mixture contained 20 μl of Limulus clotting enzyme or trypsin (20 μg/ml), 5 μl of Tris, pH 8, and either 150 μl of S-2222 (A) or 45 μl of coagulogen (B), as described under "Materials and Methods." Chromogenic activity (A) was measured at 405 nm and turbidity (B) was measured at 340 nm.
to be directly activated by endotoxin. The clotting enzyme we have identified is only minimally inhibited (5%) by phenylmethylsulfonyl fluoride (1 or 0.1 mM), whereas the clotting enzyme reported by Tai and Liu (8) and Seid and Liu (19) was totally inhibited by 1 mM phenylmethylsulfonyl fluoride. Furthermore, the $K_m$ for chromogenic substrate S-2222 of the enzyme we have characterized (67 μM) is substantially different from that of the enzyme described by Seid and Liu (2 μM) (19), whereas, in contrast, it is similar to that reported previously (80 μM) for unpurified Limulus clotting enzyme (7).

Therefore, based on the differences in molecular masses, activation by endotoxin, calcium requirements, inhibitor sensitivity, and kinetic analyses, we have concluded that the proclotting enzyme described in this report is different from the proclotting enzyme reported by Tai and Liu (8) and Seid and Liu (19). It is different from the enzyme described previously (8, 19).

We believe it also is likely that the 60-kDa proclotting enzyme isolated from T. tridentatus (11, 15, 16). The Tachypleus enzyme has a molecular mass of 38 kDa calculated from DNA sequence analysis (16), whereas the molecular weight of the isolated Tachypleus clotting enzyme has been reported to be 54 (11) or 42 kDa (15). Although the 38-, 42-, and 54-kDa Tachypleus enzymes had similar reported amino acid compositions, their respective amino acid contents differ substantially from the Limulus enzyme we purified. The geographic separation of the different species of North American (L. polyphemus) and Asiatic (T. tridentatus, Tachypleus gigas, and Carcinoscorpius rotundicauda) horseshoe crabs during their evolution over hundreds of millions of years provides a basis for potential major differences between their coagulation proteins. Coagulation factor differences between the species might also explain the presence of a β-glucan-sensitive coagulation pathway in T. tridentatus via Factor G (20), which in one study was not detected in L. polyphemus using native β-glucans, despite careful investigation (21). In another study (22), Limulus amebocyte lysate was reactive with glucans, although with at least 1,000-fold less sensitivity than to bacterial endotoxin. Independent demonstration of evolutionary differences between Limulus and the three Asiatic species also has been provided by the inability of Limulus gametes to support cross-fertilization of gametes of the other three species (23).

REFERENCES