Proclotting Enzyme from Horseshoe Crab Hemocytes

Proclotting enzyme is an intracellular serine protease zymogen closely associated with an endotoxin-sensitive hemolymph coagulation system in limulus. Its active form, clotting enzyme, catalyzes conversion of coagulogen to insoluble coagulin gel. We present here the cDNA and amino acid sequences, disulfide locations, and subcellular localization of proclotting enzyme. The isolated cDNA for proclotting enzyme consists of 1,501 base pairs. The open reading frame of 1,125 base pairs encodes a sequence comprising 29 amino acid residues of prepro-sequence and 346 residues of the mature protein with a molecular mass of 38,194 Da. Three potential glycosylation sites for N-linked carbohydrate chains were confirmed to be glycosylated. Moreover, the zymogen contains six O-linked carbohydrate chains in the amino-terminal light chain generated after activation. The cleavage site that accompanies activation catalyzed by trypsin-like active factor B, proved to be an Arg-Ile bond. The released carboxyl-terminal heavy chain is composed of a single chain glycoprotein with molecular mass of 54,125 kDa and a heavy (H, 31 kDa) chain (7). The clotting enzyme has a substrate specificity similar to that of human coagulation factor XIa (34.5%) or factor Xa (34.1%). The light chain has a unique disulfide-knotted domain which shows no significant homology with any other known proteins. Thus, this proclotting enzyme has a mammalian serine protease domain and a structural domain not heretofore identified in coagulation and complement factors. Immuno-histochemical studies showed that the proclotting enzyme is localized in large granules of hemocytes.

Limulus has a unique endotoxin (lipopolysaccharide, LPS)-sensitive hemolymph coagulation system thought to be one defense system against invading microorganisms (1). The Gram-negative bacteria contain in the outer membrane various LPS analogues. When limulus is infected by Gram-negative bacteria, such as Vibrio cholerae, at the time of injury, hemocytes circulating in the hemolymph recognize them and responses rapidly occur, including cell adhesion, aggregation, and degranulation (2). Following degranulation of the large granules in hemocytes, hemolymph coagulation is initiated by the granular components (3). Several components associated with this coagulation reaction have been purified, and reconstitution experiments using the purified clotting factors suggested to us a cascade-type of reaction (4).

In the presence of LPS, a LPS-sensitive serine protease zymogen, factor C, is autocatalytically activated (5). The active factor C then activates zymogen factor B (6) to factor B, which subsequently activates proclotting enzyme (7) to clotting enzyme. The resulting clotting enzyme converts soluble coagulogen (8), an invertebrate fibrinogen-like substance, to an insoluble coagulin gel. Like the mammalian coagulation and complement systems, this cascade reaction is propagated by limited proteolysis. On the other hand, the hemocyte lysate is known to respond to (1,3)-β-D-glucan as well as to LPS (9). This (1,3)-β-D-glucan-sensitive system also activates the proclotting enzyme through a glucan-sensitive factor(s), factor G(s), resulting in the formation of coagulin gel. Because (1,3)-β-D-glucan is a major cell wall constituent of yeast and other fungi, this cascade reaction also seems to play an important role in biological defense mechanisms.

In 1985, the proclotting enzyme from the Japanese horseshoe crab Tachypleus tridentatus was purified (7). This protein is a single chain glycoprotein with molecular mass of 54 kDa. Upon activation by factor B, it is converted to a two-chain active form clotting enzyme composed of a light (L, 25 kDa) and a heavy (H, 31 kDa) chain (7). The clotting enzyme cleaves two sites in coagulogen and forms a coagulin gel. Thus, proclotting enzyme/clotting enzyme seems to be a prothrombin/α-thrombin counterpart to invertebrates, since both catalyze a final step for gelation. Interestingly, the clotting enzyme has a substrate specificity similar to that of mammalian coagulation factor Xa and can catalyze the conversion of bovine prothrombin to α-thrombin at a reasonable rate (10).

We cloned a cDNA for the serine protease zymogen, factor C, an initiator of the coagulation cascade. The structure of this protein revealed it to be a novel type of mosaic protein, hence a possible evolutionary relationship between the limulus coagulation and mammalian complement systems would have to be considered. We then investigated the structure of the proclotting enzyme in an attempt to acquire further information concerning the evolutionary origin of this protein and its biological role in the horseshoe crab coagulation system.

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The abbreviations used are: LPS, lipopolysaccharide; bp, base pairs; HPLC, high performance liquid chromatography; L, light; H, heavy; TPCK, L-(tosylamido 2-phenyl)ethyl chloromethyl ketone; PTH, phenylthiohydantoin.
EXPERIMENTAL PROCEDURES

RESULTS

Partial Amino Acid Sequence—To determine a partial amino acid sequence, the S-pyridylethylated H and L chains derived from the clotting enzyme were separated by gel filtration followed by reverse-phase HPLC, as described under "Experimental Procedures." The isolated H chain was subjected to cyanogen bromide digestion, but the L chain was digested with trypsin, since it did not contain a methionine residue (7). The sequences of purified peptides derived from both chains were analyzed, in addition to the amino-terminal sequences of both chains. The sequences of these peptides are summarized in Table M I.

When the purified proclotting enzyme was subjected to amino acid sequence analysis, the recovery of the amino-terminal PTH-derivative was very low, although the partial amino acid sequence analysis, the recovery of the amino-terminal sequences of both chains were analyzed, in addition to the amino-terminal sequences of both chains. The sequences of these peptides are summarized in Table M I.

To confirm these observations, we examined a masked amino-terminal peptide in a trypsin digest of S-pyridylethylated proclotting enzyme (Fig. M I A). Among the 19 tryptic peptides obtained, the peptide Pe-PC-T21 was thought to be derived from the amino-terminal region, as its amino-terminal residue did not react with phenylisothiocyanate. As the amino acid composition (Table MII) indicated a 1 mol excess of glutamic acid, it was further digested with pyroglutamylpeptidase and the digest was fractionated by HPLC (Fig. M I B). The amino acid composition of the resulting peptide showed 1 mol less of glutamic acid (Table MII), and its amino-terminal sequence proved to be the amino terminus of the intact protein (Table MIII). Based on these results, we conclude that the amino terminus of the intact protein is blocked by pyroglutamic acid.

cDNA Cloning—On screening 1.5 million clones of the hemocyte cDNA libraries with anti-clotting enzyme IgG, we found 23 positive clones. After sequence analyses, one was found to encode a partial amino acid sequence of the clotting enzyme and was named XPC 17 (Fig. M2). Since this clone seemed to be derived from an EcoRI fragment of the cDNA, another preparation of the library was screened using XPC 17 as a probe. The longest clone composed of 1,501 bp, named XPC 11, was thus obtained (Fig. M2) and was then sequenced as a probe. The longest clone composed of 1,501 bp, named XPC 11, was thus obtained (Fig. M2) and was then sequenced as a probe. XPC 17 contains almost the full length of the proclotting enzyme cDNA, apart from the poly(A) tail, which was missing. Northern blot analysis of the hemocyte poly(A)⁺ RNA showed one band of about 1.5 kilobases that strongly hybridized with XPC 17 as a probe. The longest clone composed of 1,501 bp, named XPC 11, was thus obtained (Fig. M2) and was then sequenced (Fig. 1). Following 216 bp of 5'-noncoding region, there is a long open reading frame encoding 375 amino acid residues, followed by a TAG termination codon and 157 bp of 3'-noncoding region, although both the poly(A) signal and the poly(A) tail were missing. The amino-terminal initiation codon of the mature protein proved to be the 30th glutamine residue of the deduced sequence, in the form of pyroglutamic acid. Thus, the mature protein is composed of 346 amino acids, with calculated molecular mass of the protein portion 38,194 Da. From the amino-terminal sequence of the H chain (Table M I), the cleavage site for zymogen activation was identified to be an Arg⁹'-Ile⁹. Therefore, the L chain is composed of 98 residues and the H chain of 248 residues. The amino acid composition of the proclotting enzyme, in addition to those of the L and H chains of clotting enzyme, agreed well with the sum of the total residues deduced from the cDNA sequence (Table M IV).
chains attached to these three asparagine residues will be described later.

**Disulfide Locations**—There are 14 cysteine residues in the sequence of the mature protein. When this protein was S-pyridylethylated, without reduction, S-pyridylethylcysteine was not detected in the amino acid analysis (data not shown). Thus, all these cysteines are linked by intramolecular disulfide bridges. Although the locations of disulfide linkages in the H chain could be assigned by analogy with those in trypsin, the linkages of 7 cysteine residues in the L chain could not be presumed, since there was no homologous protein. Thus, we decided to determine the complete disulfide linkage locations of this protein.

The purified proclotting enzyme and clotting enzyme were first digested, respectively, with lysyl endopeptidase, without reduction. All the procedures for the determination of disulfide locations, including enzyme digestions and separations of peptides, were carried out below pH 6.5, to avoid disulfide exchange reactions. The digests were separated by reverse-phase HPLC (Fig. 3, A–C), and the isolated peptides were assigned based on the amino acid compositions (Table IV) and the sequence data (Table VI), referring to the entire amino acid sequence predicted from the cDNA sequence for proclotting enzyme. These peptides were termed K1 to K18 from the amino terminus of the entire protein, as shown in Fig. 3.

Peptide K9 contains 2 cysteines, indicating a linkage between Cys/sub>29 and Cys/sub>44. The disulfide containing peptides K7–K10 and K14–K17 were also obtained, respectively, as a single peak, as shown in Fig. 3, A and B. Three individual amino-terminal sequences of K2, K4, and K5 could be detected in this peptide, as based on the sequence data, since K1 has a masked amino-terminal residue. This cysteine-containing peptide was further digested with trypsin, and the digest was separated by HPLC, and sequenced. Through these procedures, K4 was identified to be linked with the carboxyl-terminal part of K1 (K1–K2–K4–K5–T1) (data not shown). Thus, a linkage between Cys/sub>29 and Cys/sub>44 was demonstrated. Among the remaining 4 cysteine residues to be determined, two exist in peptide K5, sequence -Cys-Cys-. A possible combination for the disulfide bridges is Cys/sub>11-Cys/sub>24 and Cys/sub>27-Cys/sub>55 or Cys/sub>11-Cys/sub>55 and Cys/sub>27-Cys/sub>44.

**Carbohydrate Chains**—Amino acid analyses of the lysyl endopeptidase peptides indicated that three peptides, K6, K7–K10, and K11–K12–K13, contain hexosamines (Table IV). K6 was rich in galactosamine and had 1 serine and 5 threonine residues that appeared to be modified by O-linked carbohydrate chains, since these PTH-derivatives could not be detected in the sequence analyses (Tables I and IV). K11–K12–K13 had one potential N-linked glycosylation site, whereas K7–K10 had two. In the sequence analyses, all cycles corresponding to these 3 asparagines yielded no PTH derivative (Tables I and IV), thereby indicating that these residues are indeed glycosylated. K7–K10, which contained two glycosylated asparagines, was digested with trypsin and the resulting carbohydrate containing peptides were separated by HPLC (data not shown). Two peptides, K7–K10–T4 and K7–K10–T8, were isolated and both contained N-acetylglucosamine and N-acetylgalactosamine (data not shown). Four carbohydrate-containing peptides, K6, K7–K10–T4, K7–K10–T8, and K11–K12–K13, were obtained. In the HPLC profile, K6 and K7–K10–T4 were seen to have an apparent heterogeneity, presumably due to the carbohydrate chains, although it has been reported that the proclotting enzyme contains no sialic acid (7). The component sugar compositions of the four peptides were analyzed by derivatization with 2-aminoypyridine. Under the conditions used (in 4 M trifluoroacetic acid at 100 °C for 3 h), hexosamines could not be quantitatively determined. However, all the peptides were confirmed to contain both N-acetylgalactosamine and N-acetylglucosamine (data not shown), in accord with the result of amino acid analysis. Monosaccharides derived from the N-linked carbohydrate chains in K7–K10–T4, K7–K10–T8, and K11–K12–K13 showed similar compositions rich in mannose and fucose. Galactose was also detected in K7–K10–T4 and K7–K10–T8. On the other hand, monosaccharides in K6 were distinct. Galactose and fucose are the major monosaccharides and K6 contains no mannose. A total of at least six O-linked carbohydrate chains could be found in the entire protein.

**Subcellular Localization**—To investigate the subcellular localization of proclotting enzyme in the hemocytes, an immunohistochemical technique was used. The hemocytes were first fixed with a mixture of paraformaldehyde and glutaraldehyde and then sections of these cells were stained with the purified anti-clotting enzyme IgG and gold particle-conjugated second antibody. When these preparations were analyzed by electron microscopy, numerous gold particles were observed in the granules (Fig. 4). Although two types of granules were apparent, large but less dense granules and smaller but dense ones, the gold particles were exclusively present in the former. This type of large granule is secreted from cells by stimulation with LPS (2). Thus, the proclotting enzyme is localized in the large granules and is apparently released through exocytosis on LPS stimulation.
bars are 50 nm (A) and 1 μm (B), respectively.

**FIG. 4.** Localization of proclotting enzyme in the cell. Hemocytes were fixed, stained with anti-clotting enzyme IgG and gold-particle-conjugated second antibody, and then scanned. Numerous gold particles are present in the larger but less dense granules. The bars are 50 nm (A) and 1 μm (B), respectively.

**DISCUSSION**

From the above cDNA and protein-based studies, the nucleotide sequence and primary structure of proclotting enzyme was deduced, as shown in Figs. 1 and 3. The isolated cDNA consists of 1,501 bp, which is almost the full-length cDNA based on the results of Northern blotting. Although there is no termination codon and two ATG codons exist in the same frame of the upstream 5′-noncoding region, the first ATG codon is presumed to be a translation initiation codon, based on the following criteria: first, this clone has been shown to involve a full-length cDNA sequence; and, second, the amino acid sequence from the first ATG codon shows characteristics of a signal sequence. Thus, the 29-amino acid residues (−29 to −1) preceding the amino-terminal glutamine residue of the mature protein seem to constitute a propeptide region. Using a method for predicting a signal sequence cleavage site (20), the most likely site cleaved by a signal peptidase is between Ser−13 and Val−12 and the second possibility is between Ser−8 and Arg−4. The hydrophy profile calculated by the method of Kyte and Doolittle (21) indicates that this presequence region is highly hydrophobic (Fig. M5). The sequence between the mature protein and the signal peptide is assumed to consist of 12 (or 5) residues and appears to represent a propeptide region of the immature protein. In fact, the carboxyl-terminal sequence in this region shows the sequence -Arg-X-Arg-Arg (amino acid −4 to −1), similar to that found in mammalian serine protease zymogens, such as prothrombin (22), factors VII (23), IX (24), X (25), protein C (26), and complement factors C3 (27), C4 (28), and C5 (29). The presence of this sequence strongly suggests that an enzyme which proteolytically processes the dibasic cleavage site may also exist in the invertebrate hemocyte. The propeptide region mentioned above may function to prevent unexpected activation of the proclotting enzyme, the result being clot formation within the cell.

The mature protein is composed of 346 amino acid residues with a molecular mass of 38,194. 85.5% of the total sequence was confirmed by protein sequencing, in which all the residues of the L chain were identified at the protein level, except for the carbohydrate-linked amino acid residues. The amino-terminal residue of the intact proclotting enzyme is masked by the pyroglutamyl residue. The purified material, however, contains a small amount of a molecular species with a second phenylalanine at the amino terminus. This suggests that the amino-terminal pyroglutamyl residue is partially removed during purification procedures and that a pyroglutamylpeptidase-like enzyme is present in horseshoe crab hemocytes.

The cleavage site associated with zymogen activation is Arg89-Ile90, the sequence of which connects the L and H chains. The sequence preceding this site has a unique structure, -Thr-Thr-Thr-Thr-Arg. This sequence agrees well with the fact that the horseshoe crab factor B shows an apparent affinity for the hydroxy amino acid at the P1 site and efficiently hydrolyzes the synthetic peptide substrates N-tert-butoxycarbonyl-Met-Thr-Arg-4-methylcoumaryl-7-amide and benzoyl-Thr-Thr-Arg-4-methylcoumaryl-7-amide (6). The hydrophaty profile shown in Fig. M5 indicates that the sequence around the cleavage site is relatively hydrophobic. Furthermore, the asparagine residue at the P2 position found in the carboxyl-terminal portion of the L chain is glycosylated, which renders the region more hydrophilic (Fig. 1). Thus, the proclotting enzyme can be readily activated by trypsin (7).

The H chain of the proclotting enzyme is composed of a typical serine protease structure. To our knowledge, this is the first example of an invertebrate serine protease for which the disulfide locations have been determined (Fig. 3). The locations of four disulfide linkages in the H chain are the same as those in prothrombin (30) and factor IX (24). This clotting enzyme shows a substrate specificity similar to that of factor Xa (10). In fact, the entire sequence of the H chain corresponding to the serine protease region shows a close similarity to that of human factor Xa (34.1%), although the closest identity with the H chain is found in factor XIa (34.5%). It contains the His42-Asp92-Ser97 triad known to be the catalytic triad of serine proteases (Fig. M6). The substrate binding site corresponding to Ser189, in chymotrypsin numbering, is aspartic acid, thereby indicating that this clotting enzyme has a typical trypsin-like specificity. Interestingly, the residue before His185 has serine replacing alanine. The sequence around this histidine is strongly conserved in various serine proteases. However, some of the serine proteases from Streptomyces griseus are known to possess glycine at this position (Fig. M6). It is noteworthy that this enzyme does not contain the Ser-Trp-Gly sequence that is well conserved in the serine proteases and stabilizes the backbone of the substrate binding site. This sequence is replaced by Ser187-Phe186-Gly185 in the clotting enzyme.

The L chain has no significant homology with any other protein thus far examined. The amino-terminal portion of the L chain consists of a novel disulfide-knotted structure, as shown in Fig. 5. This structure forms a clip-like shape consisting of a discrete domain. Actually, the clotting enzyme prepared by trypsin activation does not contain this portion and has Ser8 as its amino terminus (Table M1). This would suggest that the carboxyl-terminal portion of the clip-like structure forms a hinge region susceptible to trypsin. The resulting smaller L chain may correspond to the L chain derivative (7). It seems likely that the disulfide-knotted do-
main acts as a recognition site for factor B, factor G or for coagulogen. Following this domain, a serine and threonine-rich sequence is highly glycosylated with O-linked carbohydrate chains. Furthermore, a proline-proline dimer sequence is conspicuously evident three times in the following region, a finding which suggests that the region forms an anomalous structure. The molecular mass of the L chain shows a large discrepancy in comparison with that estimated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and is stained less strongly with Coomassie Brilliant Blue (7). We presume that these properties relate to the presence of carbohydrate chain clusters in the L chain.

As shown in Fig. 4, the proclotting enzyme is present in large granules in hemocytes, an observation in good agreement with findings that all the components required for coagulation are found in the hemocyte lysate are present in the granules (3). Various serine proteases in granules of mammalian mast cell and cytotoxic T cells have been noted but their functions remain to be clarified (31-33). These enzymes differ from the proclotting enzyme described here in molecular mass and primary structure.

Tai and Liu (34) reported the isolation of proclotting enzyme from the American horseshoe crab (Limulus polyphemus). This zymogen has a molecular mass of 150,000 and contains 43 γ-carboxyglutamic acids. It is activated directly by LPS, in a Ca2+-dependent manner. However, our proclotting enzyme differs in terms of the apparent molecular mass, the cytotoxic T cells have been noted but their functions remain to be clarified (31-33). These enzymes differ from the proclotting enzyme described here in molecular mass and primary structure.

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REFERENCES
Invertebrate Procoenzymic From Limulus

S U P P L E M E N T A L M A T E R I A L

**EXPERIMENTAL PROCEDURES**

**Materials**—All DNA-staining enzymes were obtained from Nippon Gens-Do Co., Ltd. Tokyo, Ltd. (Tokyo, Japan). Sigma Chemical Co. (St. Louis, MO). All reagents were of analytical grade unless otherwise indicated. All solutions were made with distilled water.

**Activity Assay**—The activity of the purified enzyme was determined by its ability to cleave DNA under conditions described previously (14). Experiments were performed in a final volume of 0.1 ml containing 0.05 M sodium phosphate buffer, pH 7.0, 0.01 M potassium chloride, 0.005 M magnesium chloride, 0.01% sodium metaperiodate, and 0.01% bovine serum albumin to a final concentration of 20 pg/ml. The incubation was carried out at 37°C for 30 min. The reaction was stopped by the addition of 20 µl of 10% trichloroacetic acid. The resulting precipitate was removed by centrifugation and the amount of DNA synthesized by the enzyme was determined by the method of Burton (15) using calf thymus DNA as a standard.

**Protein Determination**—Protein was determined by the method of Lowry et al. (16) using bovine serum albumin as a standard.

**RESULTS**

The purified enzyme was shown to be an endonuclease that catalyzes the cleavage of DNA at specific sites. The enzyme activity was inhibited by high concentrations of magnesium ions and was activated by low concentrations of potassium ions. The enzyme was stable at pH 7.0 for 24 h and was active at temperatures between 30°C and 50°C.

**DISCUSSION**

The results presented here indicate that the enzyme from Limulus polyphemus is a DNA-staining enzyme that cleaves DNA at specific sites. The enzyme is active over a wide range of pH values and temperatures and is not inhibited by high concentrations of magnesium ions. The enzyme is a valuable tool for the study of DNA cleavage and the mechanisms of DNA damage.

**ACKNOWLEDGMENTS**

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**REFERENCES**


### Table II

Amino acid sequence of T2I-H

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Yield (mol% of total amino acid)

- Phe 137
- Val 118
- Leu 144
- Ser 14
- Thr 106
- Arg 60
- Glu 107
- Asp 51
- Tyr 16
- Ile 25
- Met 28
- Ala 28
- X 13

Not identified.

### Table III

Amino acid compositions of heavy and light chains of clotting enzyme and proclotting enzyme

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Total residues: 570, 480 (248) 345.9 (349)

Glutamine: 3.6
Glutamic acid: 5.1
Cysteic acid: 5.1
Cystine: 2.4

### Table IV

Amino acid compositions of the heavy and light chains of clotting enzyme and proclotting enzyme

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Position: 1-14, 1-16

- Analysed with a PDQ-TRC system on hydrolysis at 6 M HCl vapor at 110°C for 20 h.
- Values in parentheses are from the hydrolysis data on the peptide.
- Not determined.