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Primitive Coagulation Systems and their Message to Modern Biology

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Introduction

Blood circulating in all living organisms carries with it the ability to maintain a constant flow but to thicken and cease to flow when a lesion occurs in the tissue. This phenomenon, known as blood coagulation, is a primary event in one of the biological defense systems of an organism. No animal species has yet been described in which plasma coagulates without some contribution from cells, including platelets, thrombocytes and amebocytes. Endothelial cells also participate in regulating the rate of coagulation reaction and in lysing the fibrin clot.

Hemostatic reactions can be roughly divided into two stages (Fig. 1): one (primary hemostasis) in which the cellular components take part, and the other (secondary hemostasis), in which plasma factors participate. Cell adhesion and agglutination and release of cellular components are biological reactions indispensable for primary hemostasis as well as for triggering secondary hemostasis (1). In mammals, numerous plasma factors and tissue-derived protein factors, cofactors, serine proteases, phospholipids and calcium ions participate in the process of secondary hemostasis (2-5).

In 1961, Heilbrunn suggested that plasma clotting in higher animals is the result of a progressive evolutionary extension into the plasma of the cytoplasmic sol-gel mechanism which is present in cells of all the living organisms and underlies such basic reactions as protoplasmic streaming, amoeboid movement, phagocytosis, muscle contraction and cell division (6). However, since knowledge of mechanisms of blood clotting and wound sealing in primitive animals is mostly morphological (7-10), little is known of the biochemistry. Nevertheless, currently available knowledge permits selection of specific examples which support Heilbrunn's idea (6) in Limulidae (11) and Crustacea (12). For instance, in the horseshoe crab, *Limulus* (L.) *polyphemus* and *Tachypleus* (T.) *tridentatus*, the clottable protein exists in a specific granule of amebocytes (11,13,14). When these cells contact an activating surface, the protein is released into the surrounding fluid where it forms an insoluble clot. Thus, a hemolymph plasma clot is formed without the participation of plasma factors, unlike events in the mammalian clotting system. In contrast, in Crustacea such as lobsters, crabs and crayfish, a clottable protein known as vitrogenin-like substance is present in the plasma (14); it is converted to its insoluble form by a cellular transglutaminase released from certain hemocytes, when they are activated by a foreign surface (12,15). Therefore, hemocytes in circulating blood always have a role in plasma coagulation and there is an apparent evolutionary increase in the number of plasma factors involved in the hemostatic reaction. These extra clotting factors amplify the reaction, thereby increasing its potential activity to meet the demand imposed by the increased circulatory efficiency and higher blood pressure in the more advanced vertebrates. Evidence of a direct evolutionary pathway from invertebrates to vertebrates would mean an intracellular origin for clottable protein and its coagulants and indeed, biochemical studies on limulus clotting reactions support this thesis (16).

This short paper will focus on recent advances in our knowledge of limulus clotting factors, especially in comparison with mammalian clotting factors, and of antimicrobial substances participating in biological defense systems.

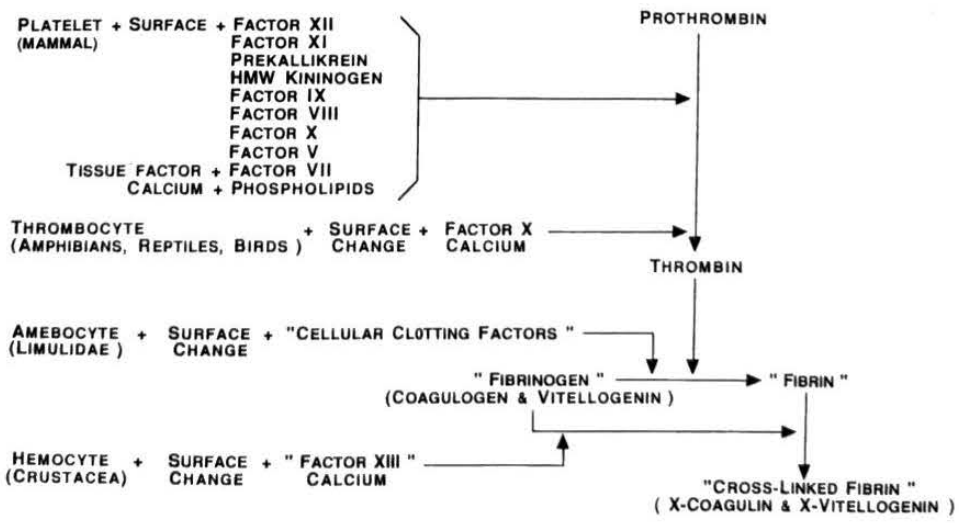


Figure 1. Mechanisms of hemostasis in various species.

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Limulus hemocytes

The hemolymph circulating in limulus contains two types of cells, granular hemocytes and cyanocytes. Based on cell morphology, there appears to be only one type of hemocyte in the systemic circulation of the adult intermolt animal, the so-called granulocyte or amebocyte (17-20). This cell is a nucleated, oval, plate-shaped structure, 15 to 20 μm in its longest dimension, with numerous dense granules of two major types: large (L) and small (S). The L-granules contain at least 20 protein components including five clotting factors and one antimicrobial factor (anti-lipopolysaccharide (LPS) factor), whereas the S-granules contain a unique antimicrobial substance, tachyplesin peptide, in addition to six major protein components. After treatment with bacterial endotoxin (LPS), the L-granules are released more rapidly than the S-granules, although almost all granules are finally exocytosed. This exocytosis is associated with clot formation, the process being complete within 90 seconds (16).

Limulus clotting reaction

The protease cascade in hemolymph coagulation in limulus is shown in Figure 2 (16). This cascade is based on three kinds of serine protease zymogens—factor C, factor B, proclotting enzyme—and a clottable protein, coagulogen. LPS activates the zymogen factor C to the active factor $\overline{C}$. Factor $\overline{C}$ then activates factor B to factor $\overline{B}$, which in turn converts the proclotting enzyme to the clotting enzyme. Each activation proceeds by limited proteolysis. The resulting clotting enzyme cleaves two bonds in coagulogen, a fibrinogen-like molecule

Table 1: Hemocyte components closely associated with biological defense systems in limulus (Tachypleus tridentatus)

Component	Mol. Wt. (residues)	Carbohydrate content (%)	Active Form	Function	Intracellular localization	References
Coagulogen	19,700 (175)	none	Coagulin (two chains)	Gelaton	*L-granule	16, 21, 22
Factor C	123,000 (994)	9%	Factor $\overline{C}$ (three chains)	Serine protease (LPS-sensitive)	L-granule	27-31
Factor B	64,000 (400)	Presence	Factor $\overline{B}$ (two chains)	Serine protease	unknown	26
Proclotting enzyme	54,000 (346)	11%	Clotting enzyme (two chains)	Serine protease	L-granule	24
Factor G	110,000 (a=673) (b=278)	Presence	Factor $\overline{G}$ (two subunits)	Serine protease	unknown	32, 33
Factor D	42,000 (386)		unknown	Pseudo serine protease	unknown	unpublished
Transglutaminase (TGase)	86,000 (764)	none	TGase (Ca^{2+}-dependent)	Cross-link	Cytosol	37, 38
Anti-LPS factor	11,600 (102)	none		Antimicrobial	L-granule	16, 39-41
Tachyplesin	2,263 (17)	none		Antimicrobial	*S-granule	42-45
Polyphemusin	2,453 (18)	none		Antimicrobial	S-granule	43
Factor C inhibitor	50,000 (394)	Presence		Anticoagulant	L-granule	unpublished
Trypsin inhibitor	6,800 (61)	none		Protease inhibitor (Aprotinin-type)	unknown	46
L-6	27,000 (221)	none		LPS-binding	L-granule	unpublished
8.6 kDa protein	8,671 (81)	none		TGase substrate	L-granule	37
Pro-rich protein	80,000	Presence		TGase substrate	L-granule	37

*L= large; S= small

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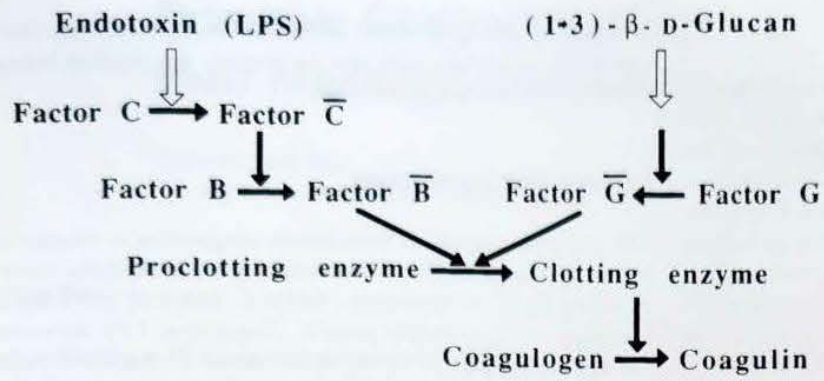


Figure 2. Hemolymph coagulation cascade system in Limulus (Tachypleus tridentatus) *(16).*

in arthropods, to yield an insoluble coagulin gel. The coagulation cascade is also activated by (1, 3)-β-D-glucan; the serine protease zymogen, factor G, which is initially activated leads to activation of the proclotting enzyme.

Limulus clotting factors (Table 1)

Coagulogen and coagulin Coagulogen plays a central role in the limulus clotting system (Fig. 2). In the intact animal, this protein is absent from the plasma but is sequestered in the cytoplasmic L-granules of hemocytes. Exocytosis from these granules releases coagulogen into the external milieu. Coagulogen consists of a single basic polypeptide chain with a calculated molecular weight of 19,700 (±50) (16,21). The clotting enzyme cleaves the Arg-Gly and Arg-Thr linkages located at the amino-terminal region. The Arg-Gly linkage is the same type as that cleaved by α-thrombin in the transformation of mammalian fibrinogen to fibrin. The amino acid sequences of four coagulogens isolated from the hemocytes of American (*L. polyphemus*), Japanese (*T. tridentatus*), and two Southeast Asian (*T. gigas* and *Carcinoscorpius* (C) *rotundicauda*) horseshoe crabs have now been elucidated (16,21). In addition, the cDNAs for two of them (*L. polyphemus* and *T. tridentatus*) have been cloned (22,23). Upon gelation of all the coagulogens by a limulus clotting enzyme, a large peptide, named peptide C, is released in common from the inner portion of the parent molecules. The gel, coagulin, consists of two chains—A (18 residues) and B (129 residues)—bridged by two disulfide linkages. The overall sequences of the four coagulogens have considerable homology (73-92% identity), particularly in the A and B chain regions. In addition, 16 half-cystine residues of the four are in the same positions in the sequences, suggesting that they are paired in the same fashion so that the molecules have similar conformations. In contrast, the sequence homology of the peptide C region is less than that of the A and B chain regions. Homologies of the peptide C region in the coagulogen of four species take up approximately 43-57% of the structure.

Proclotting enzyme and clotting enzyme A clotting enzyme which catalyzes the transformation of coagulogen to coagulin exists in hemocytes in its zymogen form (24). This proclotting enzyme is activated by either factor $\overline{B}$ or factor $\overline{G}$ (Fig. 2) and is a single chain glycoprotein with an apparent molecular mass of 54 kDa. Upon activation by factor $\overline{B}$, it is converted to a two chain active form, clotting enzyme, composed of light (L, 25 kDa) and heavy (H, 31 kDa) chains. Thus, the proclotting enzyme/clotting enzyme seems to be a prothrombin/α-thrombin counterpart in invertebrates, since both active enzymes catalyze the final step for gelation. The proclotting enzyme consists of 346 amino acid residues, and the cleavage site associated with the zymogen activation is Arg^{98}-Ile^{99}, the sequence of which is cleaved by factor $\overline{B}$. The H chain of the proclotting enzyme shows the typical structure of a serine protease. The locations of four disulfide linkages in the H chain are the same as those in prothrombin and factor X (24). The entire sequence of the H chain corresponding to the serine protease region shows close similarity to that of human factor Xa (34.1%). It also contains the His^{143}-Asp^{199}-Ser^{297} triad known to be the catalytic triad of serine proteases. The substrate binding site corresponding to Ser^{189} in chymotrypsin numbering is aspartic acid, indicating that this clotting enzyme has a typical trypsin-like specificity. The amino-terminal portion of the L chain consists of a novel disulfide-knotted structure. This structure forms a "clip-like" shape consisting of a discrete domain, which has a sequence similarity in its amino-terminal light chain portion with *Drosophila* protein named serine protease easter precursor (25). Although the significance of these results is unknown, they suggest that the "clip-like" domain found in limulus proclotting enzyme and in factor B (described below) is one of the common structural elements in the serine protease zymogen of invertebrate animals.

Factor B and factor $\overline{B}$ The zymogen factor B is activated by factor $\overline{C}$ and it in turn activates proclotting enzyme (Fig. 2). Factor B is a single chain glycoprotein with a molecular mass of 64 kDa (Table 1). Upon activation by active factor $\overline{C}$, zymogen factor B is converted into factor $\overline{B}$ with heavy (32 kDa) and light chains (25 kDa), releasing an activation peptide (26) (Fig. 4). We have determined the cDNA sequence for factor B (Muta, T. *et al.*, manuscript in preparation). It consists of 400 amino acid residues with 23 residues of signal sequence. The mature protein has 377 amino acids and its entire sequence is similar to that of the limulus proclotting enzyme (24). The sequence identity of the carboxyl-terminal serine protease domains between factor B and proclotting enzyme is 43.9% (Fig. 3). Moreover, the amino-terminal regions up to the 60th residue of both proteins

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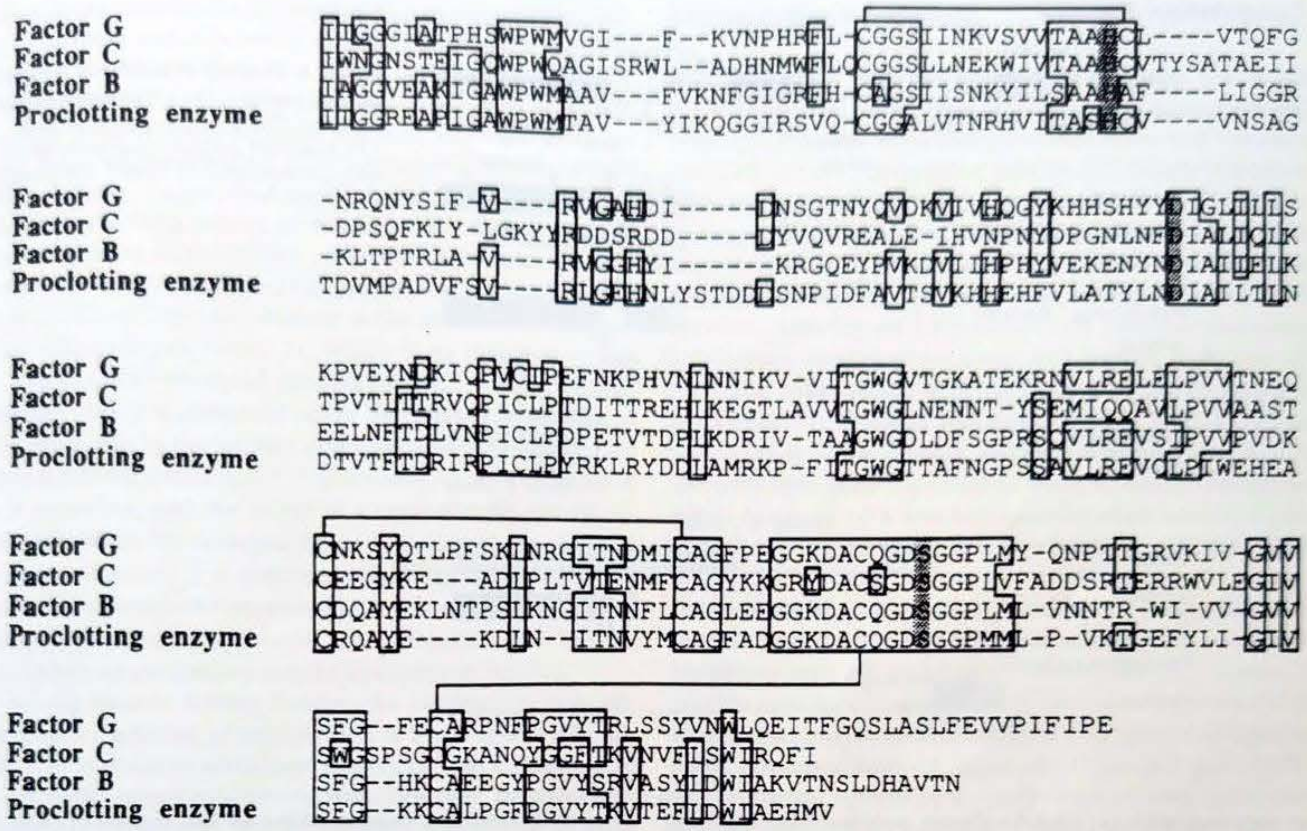


Figure 3. Sequence alignment of serine protease domains in limulus clotting factors. The disulfide locations are taken from data on the limulus proclotting enzyme (24). A serine active site triad, Asp-102, His-57, Ser-195, in chymotrypsin numbering is shadowed.

share a similar structure with six cysteines, suggesting that these proteins arose from gene duplication. Factor B also shows the closest sequence similarity with human plasma prekallikrein (37.1% identity).

Factor C and factor $\overline{C}$ Factor C is an initial activator of the clotting cascade triggered by LPS (16,27,28) (Fig. 2). The zymogen factor C is a 123 kDa glycoprotein consisting of a heavy chain (80 kDa) and a light chain (43 kDa) (27,29). Factor C is converted autocatalytically to an activated form, designated factor $\overline{C}$ (123 kDa), in the presence of LPS or lipid A. Activation results from cleavage of a Phe-Ile bond in the light chain (27), producing a B chain (34 kDa) and an A chain (8.5 kDa) (Fig. 4). The zymogen factor C consists of 994 amino acid residues (30) (Table 1). There are several interesting amino acid sequences in the H and A chains (30). First, factor C has five repeating units ("Sushi" domains or short consensus repeats) of about 60 amino acids each, which are found in many proteins participating in the mammalian complement system. Factor C is the first example of a protein that has the complement-like structure in invertebrates. Second, an epidermal growth factor (EGF)-like domain is present in the H chain. Several blood coagulation factors, complement factors, and some receptors contain this type of domain. Third, a lectin-like domain is present between the third and fourth "Sushi" domains. This region is composed of about 120 amino acids and shows homology with the so-called C-type lectins. Two other characteristic sequences are also found in the H chain. One is a Cys-rich region located in the amino-terminal portion of the H chain. Eleven of 56 cysteine residues are clustered in this region. The other is in the carboxyl-terminal portion of the H chain, in which 13 of 48 residues are proline. This region has sequence homology with the connecting region of mammalian factor XII. The B chain portion of factor C is composed of a typical serine protease with an active site triad (30,31), which shows the strongest homology with human α-thrombin (36.7% identity).

Factor G and factor $\overline{G}$ In 1981, we and others obtained evidence for a (1, 3)-β-D-glucan-mediated clotting pathway in the hemocyte lysate of *T. tridentatus* (32, 33). An initiator of this pathway was named factor G. This (1, 3)-β-D-glucan-sensitive factor G consists of two subunits, a (72 kDa) and b (34 kDa). In the presence of (1, 3)-β-D-glucan, factor G is autocatalytically converted to active factor $\overline{G}$ (Fig. 2). We have just cloned and sequenced cDNA encoding for the a and b subunits of factor G, respectively (Table 1). Subunit b is a typical serine protease zymogen with a calculated molecular mass of 30.8 kDa (Fig. 3) and shows the strongest sequence similarity with the serine protease domain of limulus factor B (40.5% identity). Subunit a is a protein with intriguing features. Its amino-terminal sequence shows similarity with the carboxyl-terminal sequence of β-1, 3-glucanase A1 (EC 3.2.1.39) from *Bacillus circulans*, suggesting that this region

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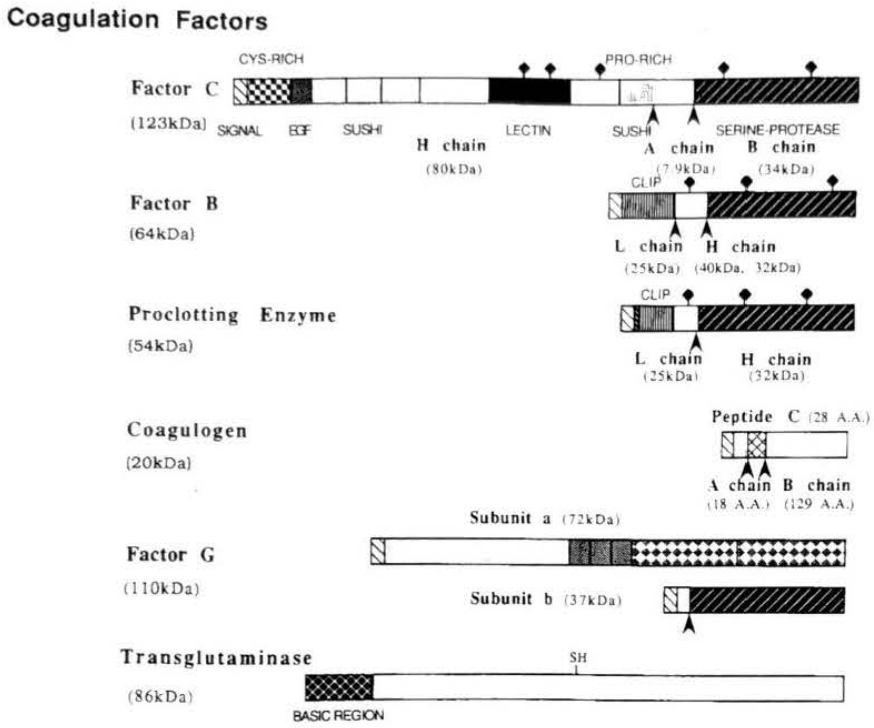


Figure 4. Domain structures of limulus clotting factors. The arrowheads indicate cleavage sites for zymogen activation. The potential carbohydrate attachment sites are indicated by closed diamonds.

may interact with (1, 3)-β-D-glucan polymer and implying glucanase activity. Furthermore, the carboxyl-terminal sequence of subunit a has a long direct repeat (126 amino acids in length), which shows a sequence similarity to the amino-terminal portion of xylanase Z (EC 3.2.1.8) from *Clostridium thermocellum*. Between the amino-terminal glucanase and the carboxyl-terminal xylanase homologous regions, there are five "QQWSY"-like motifs, the function of which is unknown. Therefore, factor G is a newly identified type of serine protease zymogen (subunit b) non-covalently associated with the (1, 3)-β-D-glucan recognition subunit a, which plays an important role in the (1, 3)-β-D-glucan-mediated clotting pathway.

Transglutaminase (TGase) In the mammalian clotting system, the fibrin clot generated via the cascade reaction is cross-linked to form a huge fibrin network as well as being cross-linked to other plasma proteins, such as soluble fibronectin, α2-macroglobulin, and α2-plasmin inhibitor (34, 35). This final step is catalyzed by plasma TGase (factor XIIIa), and the cross-linking of fibrin with itself and with other proteins is essential for normal hemostasis and wound healing. It is therefore expected that in the limulus clotting system, a TGase might participate not only in cross-linking of the coagulin gel, but also in immobilization against invading microorganisms. Indeed, in 1973, Lorand *et al.* discovered the existence of a Ca^{2+}-dependent TGase in *L. polyphemus* amebocytes and showed the TGase-catalyzed incorporation of [^{14}C]putrescine into β-lactoglobulin and α-casein (36). Recently, we purified and characterized a cellular TGase from the hemocytes of *T. tridentatus* and examined the cDNA sequence for TGase mRNA (37,38). The limulus TGase has a molecular mass of 86 kDa and properties of the mammalian type II TGase-like enzyme. One of the cloned cDNAs for TGase consists of 2,884 base pairs. An open reading frame of 2,292 base pairs encodes a sequence comprising 764 residues of the mature protein with a molecular mass of 87,021 Da. Limulus TGase shows significant sequence similarity with the mammalian TGase family, as follows: guinea pig liver TGase (32.7%), human factor XIIIa subunit (34.7%), human keratinocyte TGase (37.6%), and human erythrocyte band 4.2 (23.0%). Limulus TGase has a unique amino-terminal cationic extension of 60 residues with no homology to the amino-termini of mammalian TGases (38). The TGase activity is Ca^{2+}-dependent and is inhibited by primary amines, EDTA, and SH-reagents. Moreover, two major potential substrates for TGase have been identified in the hemocyte lysate, using dansylcadaverine (DCA) incorporation in the presence of 10 mM $CaCl_2$ and 10 mM dithiothreitol. Of these protein substrates, an 80-kDa protein contains a large number of proline residues, amounting to about 22% of the total amino acids (Table 1). On the other hand, an 8.6-kDa protein abundantly present in hemocytes is a Cys-rich protein consisting of 81 amino acid residues and a calculated molecular mass of 8,671. This protein is readily crosslinked intermolecularly by TGase, forming multimers as large as pentamers (37). We speculate that, like plasma factor XIIIa, limulus TGase and its two protein substrates in hemocytes may play an important role in the defense of the animal against invading microbes.

Relationship between limulus and mammalian clotting factors

Figure 4 summarizes the gross structures of limulus clotting

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factors so far described. Like mammalian clotting factors, factor C, factor B and proclotting enzyme constituting the LPS-mediated clotting pathway (Fig. 2) are typical serine protease zymogens related to the trypsin family. They have structural domains in their amino-terminal portions which are different from those of mammalian clotting factors. There are no γ-Gla, kringle, finger, and apple domains (5). Moreover, mammalian clotting factors do not have the "Sushi" domain, whereas limulus factor C, like complement factors, contains several "Sushi" domains, and the proclotting enzyme and factor B contain a "clip-like" domain in the amino-terminal portions. The zymogen factor G, which is an initiator of the (1, 3)-β-D-glucan-mediated clotting pathway, is an intriguing molecule (32). It is composed of the two physically associated subunits a and b; the former contains a so-called cellulose binding domain found in β-1, 3-glucanase A1 and xylanase Z from microbes, and the latter is a typical serine protease zymogen. When the zymogen factor G is exposed to (1, 3)-β-D-glucan polymer, it is autocatalytically activated to active factor $\overline{G}$. Although the structural changes of subunits a and b during β-glucan-mediated activation are unknown, a heretofore undefined mechanism may be operative in limulus.

Among limulus clotting factors, the coagulogen molecule essential for gelation of hemolymph is the smallest (Fig. 4). This is in contrast to vertebrate fibrinogen which is known to be one of the largest macromolecules. However, on activation, coagulogen molecules, like fibrinogen, polymerize to form coagulin gel, and convert to a protofibril observed electron microscopically (16). Limulus TGase may also participate in formation of the coagulin gel network and lead to development of covalently crosslinked heterooligomers with other proteins, such as the 8.6 kDa and Pro-rich proteins that coexist in L-granules in hemocytes (37). These processes seem important for immobilization of invading microbes. At present, however, the biochemical mechanisms related to polymerization and gelation of coagulogen are poorly understood.

Antimicrobial substances

Anti-LPS factor In 1982, a protein component that inhibits the coagulation cascade was detected in the hemocyte lysate from Japanese and American horseshoe crabs and was named anti-LPS factor. The purified protein specifically inhibits the LPS-mediated activation of zymogen factor C and has a strong antibacterial effect, especially on the growth of Gram-negative R-type bacteria (39,40). Furthermore, it has hemolytic activity on red blood cells sensitized with LPS and cytolytic activity on LPS-sensitized polymorphonuclear leukocytes, mononuclear cells, and human leukemia cells (41). The entire amino acid sequence of anti-LPS factor isolated from *T. tridentatus* was determined in 1986. In our continued study of the limulus clotting system, we also found in the hemocyte debris a cationic peptide named tachyplesin which binds to LPS and has antimicrobial activity against both Gram-negative and -positive bacteria, and also against fungi (42).

Tachyplesin family and its precursors Tachyplesin I is a cationic peptide first isolated from acid extracts of Japanese horseshoe crab (*T. tridentatus*) hemocytes (42). Later, two peptide analogs of tachyplesin I, named tachyplesins II and III were isolated from hemocytes of two species of Southeast Asian horseshoe crabs, *T. gigas* and *C. rotundicauda* (43). In the hemocyte debris of *L. polyphemus*, two tachyplesin analogs, named polyphemusins I and II were also found (43). The primary structures of the tachyplesin family are shown in Figure 5. They are highly basic polypeptides composed of 17 or 18 residues with arginine α-amide at the carboxyl-terminal end. Tachyplesin has a characteristic structure with three tandem repeats of a tetrapeptide sequence: hydrophobic amino acid-Cys-hydrophobic amino acid-Arg (42). The ^{1}H-NMR studies indicate that tachyplesin I takes on a fairly rigid conformation constrained by two disulfide bridges and adopts a conformation consisting of an anti-parallel β-sheet (residues

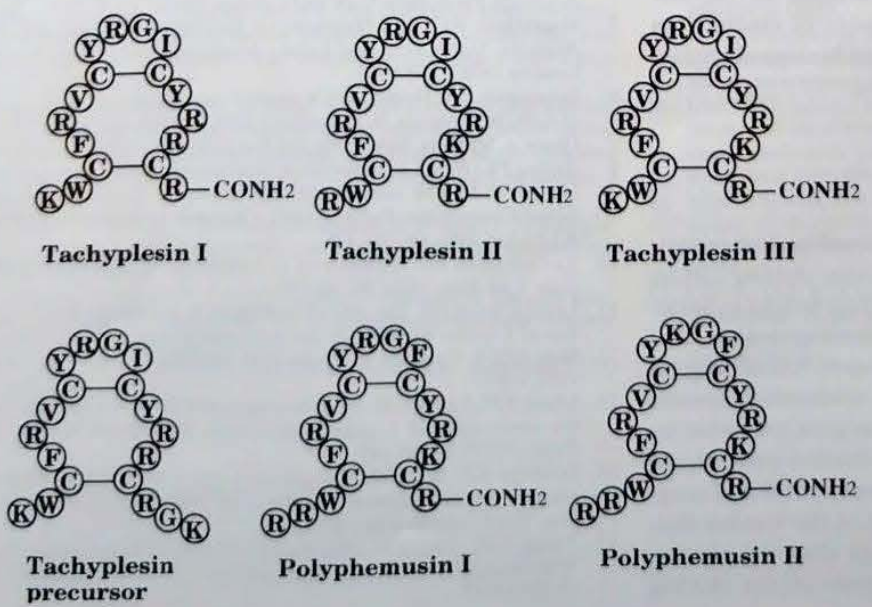


Figure 5. Primary structures of six kinds of tachyplesins and its analogs isolated from four species of horseshoe crabs. Tachyplesin III was isolated from T. gigas, *I from* T. tridentatus, T. gigas *and* C. rotundicauda, *and II from* T. tridentatus, *respectively. Polyphemusins I and II from* L. polyphemus.

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3-8 and 11-16) connected by a β-turn (residues 8-11) (44). In this planar conformation, five bulky hydrophobic side groups are localized on one side of the plane and six cationic side groups are distributed at the "tail" part of the molecule (residues 1-5 and 14-17). This conformation may be closely associated with the bactericidal activity [33].

The cDNA encoding for tachyplesin precursors has been cloned and sequenced (45). The precursors consist of 77 amino acids with 23 residues in a presegment, and there are two types of mRNAs corresponding to the isopeptides of tachyplesins I and II. Both precursors contain a putative signal peptide, a processing peptide sequence and a carboxyl-terminal amidation signal "Gly-Lys-Arg" connected to the mature tachyplesin peptide. Moreover, an unusual acidic amino acid cluster with Asp-Glu-Asp-Glu-Asp-Asp-Asp-Glu-Glu-COOH is present in the carboxyl-terminal portions of both precursors. This acidic region might interact with a cationic part of the tachyplesin peptide to stabilize a conformational structure of the precursor for proteolytic degradation. Immunohistochemical analysis showed that mature tachyplesin was localized in the S-granules of the hemocytes (20,45). Tachyplesin displays potent antimicrobial activity against several strains of microorganisms. *Salmonella* strains irreversibly lose viability in the presence of 3.5 μg/ml tachyplesin. Therefore, tachyplesin seems likely to act as an arthropod antibiotic for the defense of horseshoe crabs against microbial infections (43).

Humoral factors in hemolymph plasma

There are also many humoral factors, such as proteinase inhibitors (46), α2-macroglobulin (47), lectins (17), C-reactive protein (48) and polyphemin (17), which are important for antimicrobial defense. Liu and colleagues found several endotoxin-binding proteins (49-51) and a cell adhesion protein in the limulus hemocytes (52). Although the exact functions of these substances are unknown, they may act in concert with other components to provide biological defense for the animal. However, compared to our knowledge of mammalian blood cells, much remains to be learned of biological/physiological events in horseshoe crab hemocytes.

Conclusion

The hemolymph clotting system in the horseshoe crab is similar to that of mammals although the limulus clotting factors seem to be separate entities from the clotting factors in vertebrates. The sequential activation of the clotting cascade consists of at least four serine protease zymogens linked to gelation, which is comparable to that of the mammalian system. Furthermore, each activation of the zymogens mediated by LPS or (1, 3)-β-D-glucan proceeds by limited proteolysis, releasing an activation peptide in zymogen factor B and coagulogen. The clear difference is that most of the limulus clotting factors exist in the hemocytes rather than the plasma. Specifically, the L-granules seem to contain all the clotting factors essential for hemolymph coagulation. On the other hand, antimicrobial tachyplesin peptides are stored exclusively in the S-granules. In response to LPS, the contents of both granule populations are released into the hemolymph via exocytosis, where they work together to immobilize and kill invaders. Therefore, this clotting system may have a crucial role in host defence. In addition, the structural similarity of limulus LPS-sensitive factor C to mammalian complement Clr and Cls suggests that the limulus clotting system and the complement system may have evolved from a common origin.

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