

Review

Biochemical principle of *Limulus* test for detecting bacterial endotoxinsBy Sadaaki IWANAGA^{*1,*2,†}

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Abstract: A hemocyte lysate from horseshoe crab (*Limulus*) produced a gel, when exposed to Gram-negative bacterial endotoxins, lipopolysaccharides (LPS). This gelation reaction of the lysate, so-called *Limulus* test, has been widely employed as a simple and very sensitive assay method for endotoxins. Recent biochemical studies on the principle of *Limulus* test indicate that the hemocytes contain several serine protease zymogens, which constitute a coagulation cascade triggered by endotoxins, and that there is a (1,3)- β -D-glucan-mediated coagulation pathway which also results in the formation of gel. Up to now, six protein components, designated coagulogen, proclotting enzyme, factor B, factor C, and factor G, all of which are closely associated with the endotoxin-mediated coagulation pathway, have been purified and biochemically characterized. The molecular structures of these proteins have also been elucidated. Moreover, the reconstitution experiments using the isolated clotting factors, factor C, factor B, proclotting enzyme and coagulogen in the presence of endotoxin, leads to the formation of coagulin gel. Here, I will focus on the biochemical principle of *Limulus* test for detecting bacterial endotoxins, and its activation and regulation mechanism on the LPS-mediated coagulation cascade.

Keywords: *Limulus* test, bacterial endotoxins, lipopolysaccharides (LPS), horseshoe crab (*Tachypleus tridentatus*), innate immunity, clotting factors

Introduction

As it is well known, a number of enzyme cascade systems have been described which serve to propagate and amplify various biological reactions. These can be generally divided into two major groups: the serine protease zymogen and protein kinase cascades. Well known examples of the serine protease cascade systems include blood coagulation,¹⁾ fibrinolysis,¹⁾ complement systems,²⁾ in mammals, and Toll-like receptor-mediated signaling and prophenoloxidase activating systems^{a)} in arthropod.^{3), 4), 35)} In the serine protease cascade system, the initiator is triggered by an appropriate stimulus, and the reaction

is then propagated by successive activation accompanied usually by limited proteolyses.

Among these cascades, the horseshoe crab (*limulus*) coagulation system is unique, in that the reaction is initiated by pico to nano gram of bacterial endotoxin, lipopolysaccharide (LPS).^{5), 6)} Moreover, several clotting factors constituting this system are stored in granules in hemocytes (amebocytes) and released into hemolymph by degranulation, when the cells are activated.⁷⁾ The clot formed through the activation of the cascade is effective for immobilizing invading microorganisms,^{8), 9)} which are killed by antimicrobial substances secreted simultaneously from the cell.^{10)–12)} The clot formation is also triggered by (1,3)- β -D-glucan, which is mainly present in the cell walls of yeast and other fungi.^{13)–15)} This pathway is initiated by the activation of a serine protease zymogen, named factor G.¹⁶⁾ Thus, the *limulus* clotting system is thought to be critical for both hemostasis and the biological defense of this animal.¹⁷⁾

This review will focus only on biochemical principle of *limulus* clotting reaction widely used for as-

*1 The Chemo-Sero-Therapeutic Research Institute, 1-6-1 Okubo, Kumamoto 812-8581, Japan.

*2 Research adviser.

†) Correspondence should be addressed: S. Iwanaga, 2-34-16 Mizutani, Higashi-ku, Fukuoka 813-0041, Japan.

a) This system has been found mainly in many insects and it links to melanin formation. The system is also activated by LPS and (1,3)- β -D-glucan similar to that of *Limulus* clotting system.

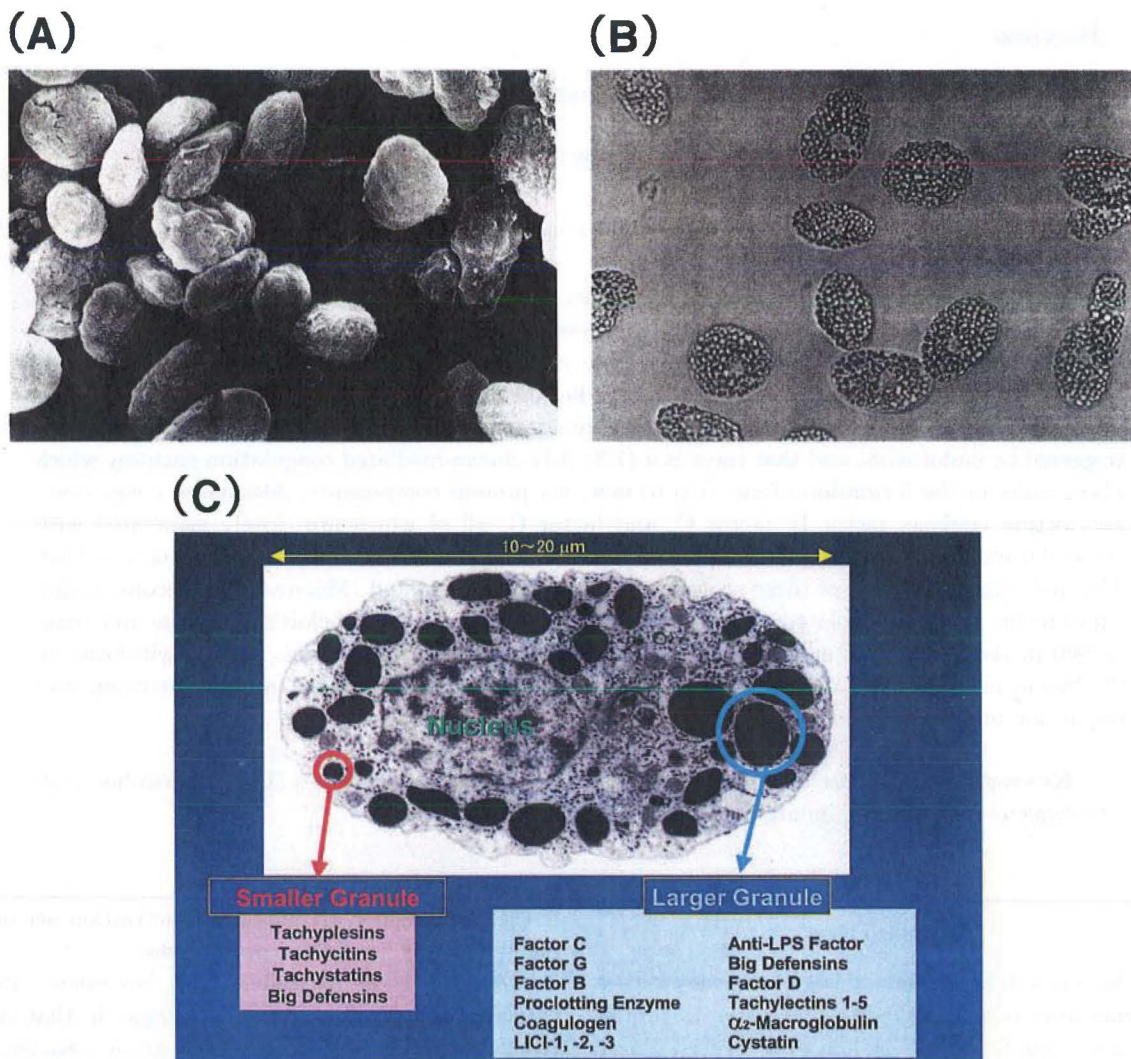


Fig. 1. A light (A and B) and electron (C) micrographs of horseshoe crab (*T. tridentatus*) hemocytes/amebocytes, and major defense molecules (C) that have been identified in large and small cell granules.⁹⁾

say of bacterial endotoxins.

Horseshoe crab/*limulus* hemolymph and hemocytes/amebocytes

The hemolymph is collected by inserting a needle into a joint between the cephalothorax and abdominal region. Fifty to 150 ml of the hemolymph per individual is drawn under sterilized condition, and the hemocytes/amebocytes are collected by centrifuging pooled hemolymph at 2,500 rpm for 10 min. The hemocytes thus prepared are lysed and the lysate is used for endotoxin assay. On the basis of cell morphology, the hemocyte is an oval, and plate

shaped structure, 15~20 μ m in its longest dimension.^{16), 18)} Fig. 1 shows a light and an electron micrographs of the hemocytes separated from Japanese horseshoe crab, *Tachypleus* (*T.*) *tridentatus*. The cell contains numerous dense granules classed into two major types: large(L) and small(S) granules.⁹⁾ The former is larger (up to 1.5 μ m in diameter) and less dense than the latter (< 0.6 μ m in diameter). The L-granules contain more than 25 proteins, the majority of which have molecular masses between 8 and 123 kDa.^{17), 19), 20)} In contrast, the S-granules contain at least 6 proteins with molecular masses of less than 30 kDa, in addition to several antimicrobial peptides

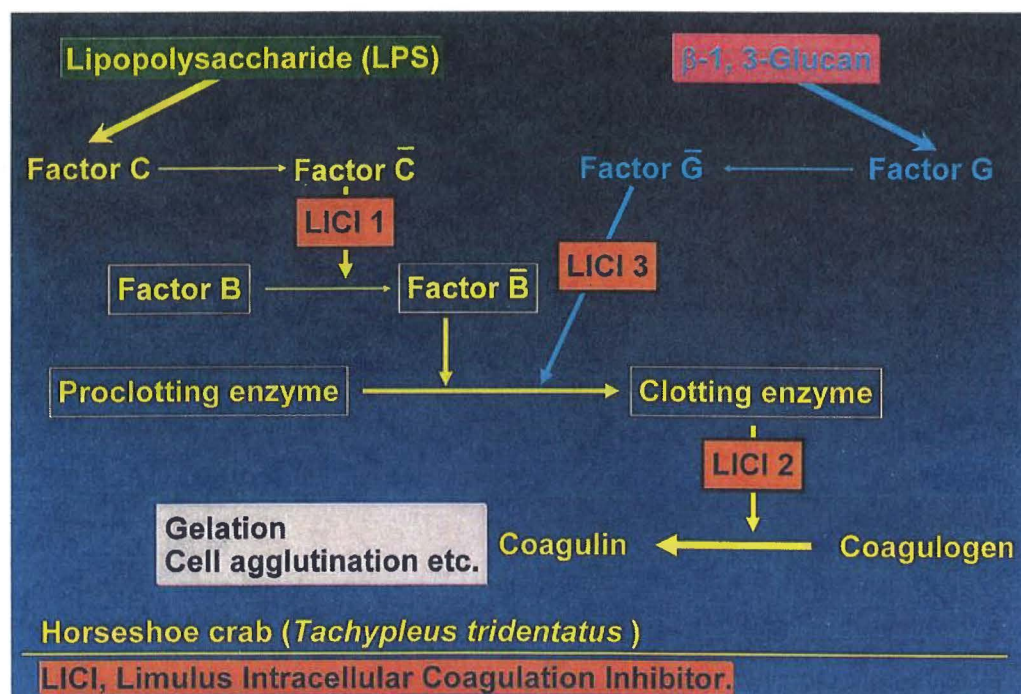


Fig. 2. LPS- and (1→3)- β -D-glucan mediated clotting cascades found in horseshoe crab (*T. tridentatus*) hemocytes.¹³⁾ Recently, Nagai and Kawabata found that the clotting cascade is linked to prophenoloxidase activation with the hemocyanin functioning as a prophenoloxidase substitute.^{36), 37)} LICI, *limulus* intracellular coagulation inhibitor. This figure depicts the biochemical principle of the so called *limulus* test, which is used for detecting bacterial endotoxins. The *limulus* test is dependent on the protease cascade reaction, and is being used extensively in combination with new technology.¹⁴⁾ Three kinds of LICI shown in the figure inhibit or regulate, respectively, the activation reactions of clotting factors (see text).

and their analogues, such as tachyplesins, tachystatins, tachycitins and big defensins, all of which show antimicrobial activities against Gram-negative and -positive bacteria and fungi.^{21)–27)}

When the hemocytes are exposed to LPS, it results in cell adhesion and aggregation associated with rapid degranulation⁴⁷⁾ and clot formation, the process being complete within 90 sec.^{7), 8), 13)} The clot is more soft than mammalian fibrin clot and contains coagulin gel generated from its precursor, named coagulogen,⁷⁾ as described later.

Limulus clotting factors

Since the 1980s, we have directed attention to molecular mechanisms involved in hemolymph coagulation in *limulus*, and we defined the protease cascade, as shown in Fig. 2.^{7), 8), 13)}

The LPS-mediated cascade is based on three kinds of serine protease zymogens, factor C, factor B, and proclotting enzyme, and a gel-forming protein, coagulogen. LPS first activates autocatalytically the

zymogen factor C to the active form of factor $\bar{C}$. Factor $\bar{C}$ then activates factor B to factor $\bar{B}$, which in turn converts the proclotting enzyme to the clotting enzyme.⁷⁾ Each activation proceeds by limited proteolysis. The resulting clotting enzyme cleaves two peptide bonds in coagulogen, a fibrinogen-like molecule in arthropods, to yield an insoluble coagulin gel.¹⁰⁾ 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 enzyme²⁸⁾ (Fig. 2). The biochemical characteristics¹⁰⁾ of these clotting factors are briefly described below.

Fig. 3 summarizes the gross structures of these new clotting factors. All the factors except for coagulogen are typical glycoproteins and differ from each other in molecular mass. The initiation factor, factor C sensitive to LPS, comprises one EGF-like domain, five short consensus repeats (SCR, also called CCP or the sushi domain) found mainly in mammalian complements, one C-type lectin domain, and

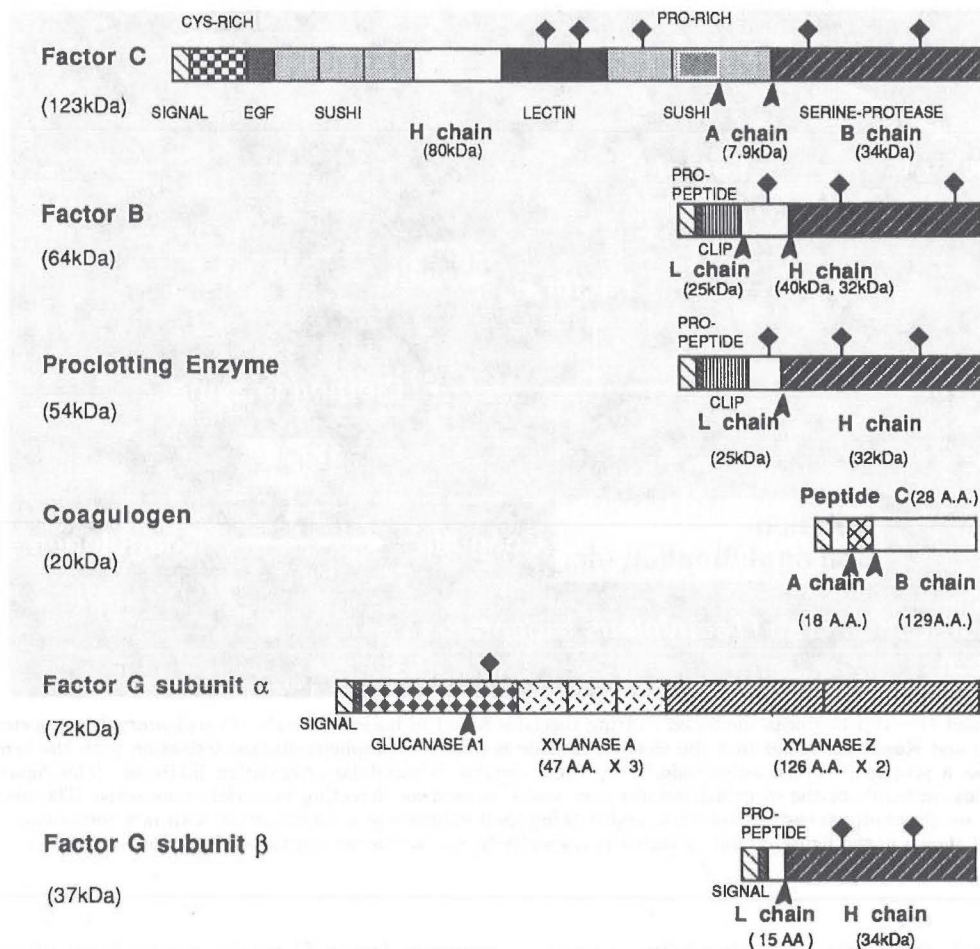


Fig. 3. Domain structures of *limulus* clotting factors. The arrowheads indicate cleavage sites for zymogen activation.¹⁰⁾ The potential oligosaccharide attachment sites are indicated by closed diamonds. Cys-rich: a domain containing a number of cysteine residues, EGF: epidermal growth factor domain, Sushi: this domain corresponds to short consensus repeat (SCR) and also to complement control protein (CCP) often found in mammalian complements, Lectin: structural domain which recognize carbohydrate moiety. Pro-rich: a domain containing many proline residues, Clip: a secondary structure of this domain is similar to feature of clip used as stationary in business work. Recently, a number of the clip domains have been identified in serine-protease originated from invertebrate animals.^{42), 43)}

a serine protease domain, the latter of which is located in the COOH-terminal portion.¹³⁾ The finding of SCR in factor C makes it the first protein in invertebrates that has been discovered to contain this type of domain.^{b)} The fact that this initiator of the clotting cascade contains SCR led us to speculate that coagulation and complement systems may have evolved from a common origin.^{8), 13)} Recently,

^{b)} SCR (short consensus repeat) composing of 40 to 60 amino acid residues is found in many proteins, such as, complements, complement receptors, and transglutaminase-factor XIII subunit B. It is also named as CCP (complement control protein) or sushi domain. SCR is known as functional domain which acts on a protein-protein interaction.

Koshiba *et al.*⁴⁸⁾ reported that the LPS-binding site is present in the NH₂-terminal cysteine-rich region of the zymogen factor C molecule (Fig. 3), and that it contains a tripeptide sequence, such as RWR and KYK, that is conserved in the hemocyte-driven anti-LPS factor¹³⁾ and other mammalian LPS-recognizing proteins.

Both factor B and proclotting enzyme are similar in the domain structure to each other¹⁰⁾ (Fig. 3). In addition to the COOH-terminal protease domain, both clotting factors contain a "clip"-like domain (formerly called the "disulfide-knotted" domain) in the NH₂-terminal light chain, and this portion shows

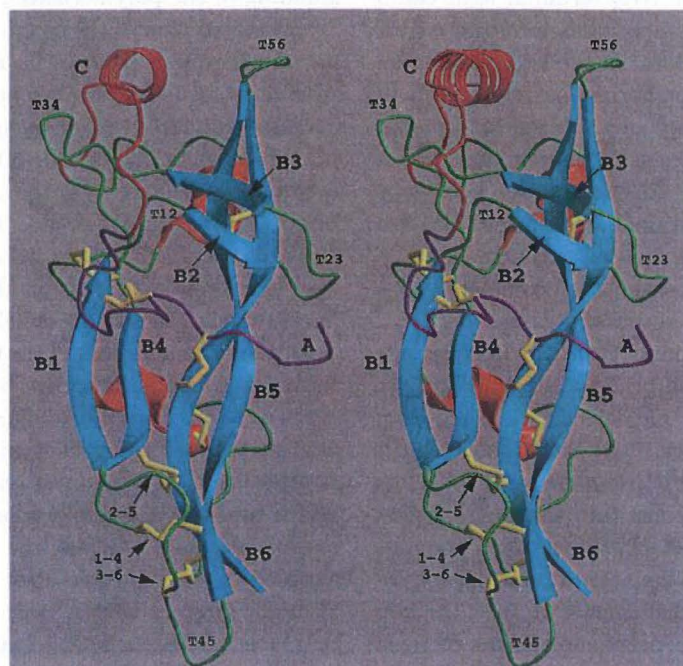


Fig. 4. Stereo view of a coagulogen monomer showing the A chain, peptide C and B chain, and the secondary structure.³⁴⁾ The structure is dominated by the β -strands (blue, labeled sequentially B1 to B6), and multiple coils and turns (green) of the B chain. The main α -helical peptide C (red), which is released upon cleavage, covers a reasonable part of the surface at the top. The NH_2 -terminal A chain is connected to the B chain by two disulfide bridges (yellow). The whole cysteine-rich structure possesses eight disulfide bridges.

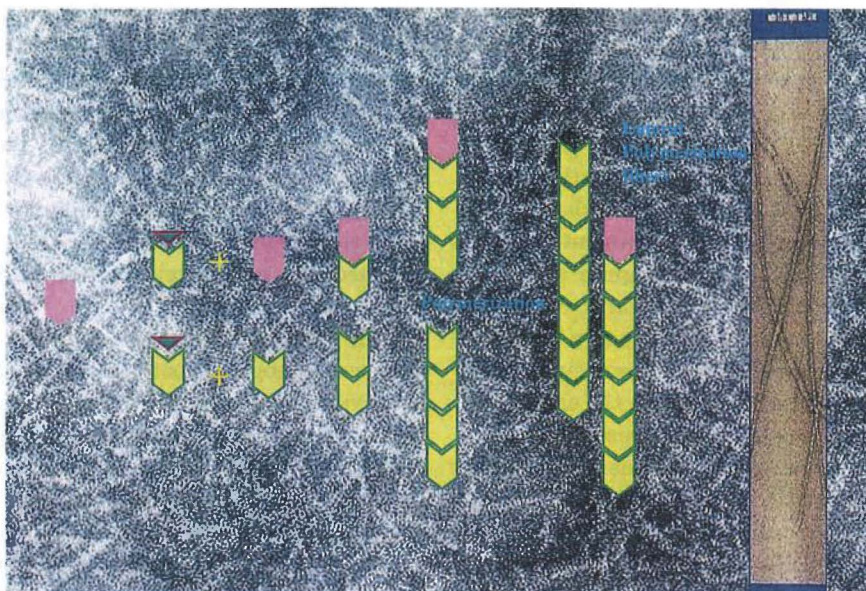


Fig. 5. Hypothetical mechanism of coagulogen gel formation.¹⁰⁾ Upon gelation of coagulogen (pink) by a horseshoe crab clotting enzyme, peptide C (green) is released from the inner portion of the parent molecules. The resulting coagulin (yellow) monomer may selfassemble to form the dimer, trimer, and multimers. The background of this figure shows a fiber-like coagulin gel.

sequence similarity to the NH₂-terminal light chain of *Drosophila* proteins, namely serine protease easter and snake precursors. Both easter and snake proteins are indispensable for normal embryogenesis in flies.^{16), 20), 46)} The presence of this type of domain in *Drosophila* strongly suggests the existence of a protease cascade similar to that in the horseshoe crab.¹⁷⁾ The three dimensional structure of clip domain derived from *Drosophila* has recently been determined,²⁹⁾ and such domain has been found in a number of insect-derived serine proteases.⁴⁴⁾

Recently, we also found that the folding pattern of the three disulfide bridges in the "clip" domain is similar to that of "big defensin" isolated from horseshoe crab hemocytes.^{16), 20)} Since the COOH-terminal ends of the "clip" domain in both factor B and clotting enzyme constitute the hinge region susceptible to protease attack, these "clip" domains may be released through the activation of the zymogens to act as antimicrobial agents. In fact, the clip domain derived from the prophenoloxidase of fresh water crayfish has an antimicrobial activity similar to that of human β -defensin.⁴⁾ Thus, the *limulus* clotting cascade could also produce antimicrobial agent, and, therefore, provide a dual action of clotting and killing systems against invaders.^{4), 16)}

In the final step of the clotting cascade, coagulogen, a 175 amino acid single chain polypeptide, is converted to insoluble coagulin through limited proteolyses at two sites (Arg18-Thr19 and Arg46-Gly47), as shown in Fig. 3. Excision of intermediate peptide C (Thr19-Arg46) results in the formation of the coagulin monomer, AB, consisting of the NH₂-terminal A chain (Ala1-Arg18) and the COOH-terminal B chain (Gly47-Phe175) covalently linked *via* two disulfide bridges. The coagulin monomer self-aggregates to form a gel-like substance composed of polymerized AB monomers. Although a transglutaminase^{c)} has been isolated from hemocytes,^{30), 31)} coagulin itself is not cross-linked as in the case of fibrin clot or clottable proteins (vitellogenin) of insects and crustaceans.³²⁾ The exact mechanism underlying this coagulin gel formation is as yet unknown, but recent X-ray analysis of coagulogen from the Japanese horseshoe crab provided a structural basis for under-

standing of the polymerization mechanism.^{33), 34)}

A stereo view of the coagulogen monomer, showing the A chain, peptide C, and B chain, and the secondary structure is presented in Fig. 4. The coagulogen monomer is an elongated molecule of approximate dimensions, 60×30×20Å. The structure is mainly dominated by the β -strands in blue, labeled sequentially B1 to B6. The multiple coils and turns of the B chain are colored green. Short helical segments in orange, in the B chain are in the background. The mainly α -helical peptide C, in red, covers a reasonable part of the top surface. The NH₂-terminal A chain, in violet, is connected to the B chain *via* two disulfide bridges colored yellow. The whole cysteines comprise 8 disulfide bridges. The peptide C helix covers an extended hydrophobic core, which becomes accessible upon the cleavage and release of peptide C. Thus, the uncovered cove newly exposed in one molecule after the release of peptide C might interact with a hydrophobic edge of a second molecule, like a head to tail, to form a multimer, as shown in Fig. 5.³⁸⁾

During these studies, we found that the COOH-terminal half of the coagulogen molecule exhibits striking topological similarity to the neutrophin nerve growth factor (NGF), providing the first evidence for a neutrophin fold in invertebrates. The unusual large twist of the β -sheet found in coagulogen (Fig. 4) is also found in NGF. The 6 cysteines which link the two β -sheets oriented parallel to the molecular axis are strongly conserved and show the same disulfide linking pattern. Furthermore, sequence alignment based on topological equivalence shows significant sequence similarity of 21% in the topologically identical regions. Thus, coagulogen is assigned as a new member of the TGF β_2 superfamily.³³⁾

In 1981, we found the presence of a possible protease zymogen sensitive to β -glucan in a hemocyte lysate.¹⁶⁾ The purified factor G zymogen is autocatalytically activated in the presence of (1,3)- β -D-glucan.³⁹⁾ The resulting active factor $\bar{G}$ activates proclotting enzyme directly, which is linked with the coagulin gel formation (Fig. 2). As factor G is colocalized in L-granules together with components participating in the LPS-mediated coagulation cascade, it can be released into the hemolymph upon cell activation. This β -glucan-mediated coagulation pathway could be activated on the surface of fungi.^{10), 40)}

Factor G is a heterodimer composed of two

^{c)} It is known that the crayfish hemolymph clotting is based on the direct transglutaminase-mediated cross-linking of a specific plasma protein, which is homologous to vitellogenin. In this case, lysine and glutamine residues contained in vitellogenin are covalently cross-linked to each other by transglutaminase, resulting in the formation of vitellogenin gel.

subunits, α and β , associated through non-covalent bonds.²⁸⁾ As the two subunits are derived from separate genes, they are translated independently and assembled in hemocytes (Fig. 3). Subunit β is a serine protease zymogen with a short 15 amino acid NH_2 -terminal extension. The serine protease domain is most homologous to factor B (40.5% amino acid identity) and proclotting enzyme (37.7% identity), suggesting that these three had a common origin. On the other hand, subunit α shows a unique mosaic structure. The NH_2 -terminal region contains a bacterial β -(1,3) glucanase-like sequence. The COOH-terminal region has two tandem repeats, each of which shows sequence similarity with that found in xylanase Z.²⁰⁾ In the middle part of the molecule, there are three tandem-repeat structures. This type of tandem-repeat has been found in xylanase A, *Rarobacter* protease I, and ricin B chain.¹⁶⁾

In both subunits, an Arg15-Ile16 bond in β and an Arg150-Glu151 bond in α are cleaved in the autocatalytic activation process.²⁰⁾ However, none of horseshoe crab clotting factors with trypsin-like specificity, or trypsin itself, activates zymogen factor G, suggesting that the cleavage site in subunit β is somehow masked by subunit α . Therefore, the binding of β -glucan to subunit α appears to expose the activation site of subunit β , which is hindered in the zymogen form, thus allowing autocatalytic activation through a bimolecular interaction between subunit β s. We suppose that some interaction of subunit α with (1,3)- β -D-glucan may induce a specific conformational change, resulting in the activation of subunit β . The purified factor G is also activated by other various glucans containing (1,3)- β -linkages from different origins, but not by LPS, sulfatides, or cholesterol sulfates.¹⁶⁾ The most effective activators are linear (1,3)- β -D-glucans, such as curdlan and paramylon. As little as 1 ng of curdlan significantly activates zymogen factor G.²⁰⁾ Branching of the linear chain with (1,4)- or (1,6)- β -likages appears to reduce the factor G activating activity. Shorter oligosaccharides containing two to seven glucose units do not activate factor G at all. Kinetic studies on the β -glucan-dose dependency of the factor G activation revealed a bell-shaped curve: activation is inhibited at higher concentrations of β -

glucan. Under the optimum conditions, the molar ratio of factor G and β -glucan is constant, indicating that the activation of factor G occurs through an intermolecular interaction between each factor G molecule bound to β -glucan.

Regulation of the LPS- and (1,3)- β -D-glucan-mediated coagulation cascade

To date, three types of serpins^{d)} have been isolated from the horseshoe crab hemocytes, named limulus intracellular coagulation inhibitor (LICI), LICI-1, LICI-2 and LICI-3. All LICI belong to the mammalian serpin family⁴¹⁾ and form stable complexes with target serine proteinases.²⁵⁾ Of these serpins, LICI-1 specifically inhibits active factor $\bar{C}$, LPS-sensitive serine proteinase, while both LICI-2 and -3 not only inhibit factor $\bar{C}$ but also factor $\bar{G}$ and the clotting enzyme activities. LICI-2 inhibits the clotting enzyme more strongly, and LICI-3 favors factor $\bar{G}$ more than other enzymes. All the serpins are stored in L-granules and exocytosed upon activation of the hemocytes. Thus, these serpins are likely to act to prevent diffusion of the active clotting factors, which may cause unnecessary clot formation. They may also function as scavengers of the proteinases that have escaped into the hemolymph from the site of injury.^{42), 43)} It is of interest to note that one of the antimicrobial substances, big defensin,¹⁷⁾ is copurified during the purification of LICI-1 and that it interacts specifically only with LICI-1, but not with LICI-2 and -3. Thus, such specific interaction may be of physiological importance in the neutralization and intercellular sorting of big defensin.

Principle of *Limulus* test⁴⁴⁾

Limulus test, a test for detecting nano gram of bacterial endotoxins, was invented by Levin and Bang based on their finding that a trace amount of endotoxin coagulates hemocyte lysate of the horseshoe crab, *Limulus polyphemus*.⁵⁾ This gelation reaction has been widely employed as a simple and very sensitive assay method for bacterial endotoxins. The original method is qualitative or semiquantitative; the presence of endotoxin is determined by reading the formation of gel clot after incubation of a sample with the hemocyte lysate at 37° for 1 hour (*Limulus* gelation test).

During the past decade, we studied the molecular mechanism of hemolymph coagulation in horse-

d) Serpin is abbreviation of serine protease inhibitor. For example, mammalian clotting system is regulated by several plasma serpins, such as, antithrombin III, protein C inhibitor and α_2 -plasmin inhibitor.

shoe crab and established a protease cascade described above. Because the *Limulus* lysate contains all the enzymes described above, the *Limulus* test reacts with (1,3)- β -D-glucan as well as endotoxin. The latter activates factor C, whereas the former activates factor G; both pathways converge on proclotting enzyme, ensuing its activation and hydrolysis of a chromogenic peptide substrate.¹²⁾ The chromogenic substrate used for specific assay of bacterial endotoxins is Boc-Leu-Gly-Arg-*p*-nitroanilide (*p*NA). The sequence of this substrate originates from the sequences located close to the site cleaved during the gelation of coagulogen by *limulus* clotting enzyme (Fig. 3). The chromogenic substrate is hydrolyzed by clotting enzyme to release *p*NA. By measuring the absorbance of released *p*NA at 405 nm, endotoxin concentration in the samples can be determined. Endotoxin concentration can also be determined by measuring the absorbance at 545 nm after the diazo coupling of *p*NA, when a yellowish color in samples interferes with the measurement at 405 nm.⁴⁴⁾ The methods described above are a 100 times more sensitive than the *limulus* gelation test and are very reproducible. If this technique is to be applied to blood samples, however, the activities of *limulus* test-interfering factors in the samples, such as thrombin, blood coagulation factor Xa, and α_1 -antitrypsin, need to be abolished. To remove such interferences, various methods have been studied and applied to blood samples, such as pretreatment with chloroform, ether, acid, or alkali and heating.^{45), 49)}

Conclusion

The hemolymph clotting system in the horseshoe crab is similar to the blood coagulation system found in 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. The sensitive assay of pico to nano gram of bacterial endotoxins depends on the amplification system consisting of the sequential activation of the *limulus* clotting factors. This biochemical principle appears to be a basis for the *Limulus* test.

Acknowledgments

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Profile

Sadaaki Iwanaga was born in 1933. He graduated from doctor course of Pharmaceutical Science in Kyoto University and earned his Ph.D. at the same university in 1960. He started his career working on protein chemistry at the Institute for Protein Research, Osaka University. In 1965–68, he conducted blood coagulation research as a visiting scientist at Royal Karolinska Institute, Stockholm. Afterwards, he was promoted to Associate Professor at the Institute for Protein Research, Osaka University, in 1968 and continued his studies on molecular mechanism of blood clotting. In 1978, he was promoted to Professor at the Faculty of Science, Kyushu University, where he educated many students in the field of biochemistry. After he moved to the Kyushu University, he focused on the molecular basis of innate immunity in invertebrate animals, mainly using horseshoe crab. He was awarded the Biannual Awards for Contribution to Haemostasis from the International Society of Thrombosis and Haemostasis, in 1991, and also the Naito Foundation Research Prize in 1993. He was elected an honorary member of the American Society for Biochemistry and Molecular Biology in 1989, and the Japanese Biochemical Society in 1997, an honorary member of the E.K. Frey and E. Werle Foundation of the Henning L. Vogt Family, Germany, in 1998, and an honorary member of the Japanese Society of Thrombosis and Hemostasis in 1998. In 2000, he was awarded Medal with Purple Ribbon.

