Reference (7) - Appendix B

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Further Studies on Lipopolysaccharide-Sensitive Serine Protease Zymogen (Factor C): Its Isolation from *Limulus polyphemus* Hemocytes and Identification as an Intracellular Zymogen Activated by α-Chymotrypsin, Not by Trypsin[1]

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An intracellular serine protease zymogen, factor C, is an initiator in the hemolymph coagulation system of horseshoe crab. We purified this zymogen from the hemocytes of the American horseshoe crab, *Limulus* (*L.*) *polyphemus*, the objective being to compare its properties with those of the Japanese horseshoe crab, *Tachypleus* (*T.*) *tridentatus*, factor C. The purified zymogen *L.*-factor C showed similar properties to those of *T.*-factor C, in terms of molecular mass (123,000), amino acid composition (1,011 residues), subunit structure (two chains), and antigenicity. Like the zymogen *T.*-factor C, this zymogen was also activated autocatalytically in the presence of bacterial lipopolysaccharide (LPS) and its synthetic lipid A analogue. A most interesting finding is that both protease zymogens are rapidly activated by α-chymotrypsin or rat mast cell chymase, but not by trypsin. The active enzyme factor C̄ showed α-thrombin-like specificity toward synthetic tripeptide substrates. This factor C̄ was also strongly inhibited by an α-thrombin inhibitor, D-Phe-Pro-Arg-chloromethyl ketone. Thus, the enzymatic properties of factor C̄ are similar to those of mammalian α-thrombin. On the other hand, the coagulation cascade system present in the hemocyte lysate was activated when chymotrypsin, free from LPS, was added to the lysate used to detect the endotoxins. The implication of our findings is that the chymotrypsin-catalyzed initiation of the horseshoe crab coagulation system is unique, since all known mammalian coagulation, fibrinolysis and complement systems are initiated by trypsin-like enzymes.

Horseshoe crab hemocytes are highly sensitive to Gram-negative bacterial endotoxins, lipopolysaccharides (LPS) (*1-3*), exposure to LPS resulting in activation of intracellular coagulation systems. In the Japanese horseshoe crab, *Tachypleus* (*T.*) *tridentatus*, the coagulation system consists of at least three intracellular serine protease zymogens, named proclotting enzyme (*4*), factor B (*5*), and factor C (*6-9*), in addition to the fibrinogen-like substance, coagulogen (*10, 11*). These zymogens and coagulogen constitute the cascade pathway responsible for the LPS-mediated activation, as shown in Fig. 1 (*6*). Factor C is an LPS-sensitive protease zymogen consisting of a single chain glycoprotein with a molecular mass of 123,000. During the LPS-mediated activation of factor C, its single chain form is converted to a two chain intermediate form comprising an 80 kDa heavy chain and a 43 kDa light chain, and the latter is subsequently transformed to a 7.9 kDa A chain and a 34 kDa B chain on cleavage of a unique Phe-Ile bond (*6, 7*). This three chain-factor C̄ has the potential to activate zymogen factor B (*5*) and to hydrolyze a synthetic tripeptide substrate, such as Boc-Val-Pro-Arg-NH-Np (*6*). The entire cDNA sequence of this factor C has been determined (manuscript submitted). The deduced amino acid sequence data suggest that the zymogen factor C is a mosaic protein composed of a Cys-rich, an EGF-like, a lectin-like, five β_2GP-I-like, and serine protease domains.

Although much progress has been made on the zymogen, factor C, isolated from *T. tridentatus* hemocytes, little is known of coagulation factors in hemocytes of the American horseshoe crab, *Limulus* (*L.*) *polyphemus*. In 1977, Liu and coworkers (*12, 13*) reported the existence of a proclotting enzyme in *L. polyphemus* hemocytes, and postulated that it is an initiation factor sensitive to LPS and closely associated with the limulus coagulation system. However, Nakamura and Levin (*14, 15*) examined *L. polyphemus* hemocytes and suggested that the lysate contains another LPS-sensitive factor differing from the known proclotting enzyme.

We attempted to shed light on these conflicting reports, and we purified and characterized a factor C-type zymogen from *L. polyphemus* hemocytes and compared its properties with those of the LPS-sensitive factor C from *T. tridentatus* hemocytes. We obtained evidence that the

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Abbreviations: LPS, lipopolysaccharide; factor C̄, activated factor C; *T.*, *Tachypleus*; *L.*, *Limulus*; Boc, *N-tert*-butoxycarbonyl; NH-Np, *p*-nitroanilide; NH-Mec, 4-methylcoumaryl-7-amide; OBzl, *O*-benzyl; Z, benzyloxycarbonyl; Tris/HCl/SA buffer, 0.1 M Tris/HCl buffer, pH 8.0, containing 0.5 mg/ml of human serum albumin; MUGB, 4-methylumbelliferyl *p*-guanidinobenzoate; MUTMAC, 4-methylumbelliferyl *p*-(*N,N,N*-trimethylammonium)cinnamate.

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zymogen factor C sensitive to LPS does exist in *Limulus* hemocytes, and that the immunological and biochemical properties of the purified factor C are indistinguishable from those of factor C isolated from *Tachypleus* hemocytes. Moreover, this serine protease zymogen is unique in that it is activated by α-chymotrypsin, not by trypsin.

RESULTS

Characterization of L. polyphemus Factor C—The purity of the final preparation of *L.*-factor C was examined by SDS-PAGE. The purified zymogen factor C gave a single band of 123 kDa under nonreduced conditions, and two bands corresponding to an 80 kDa heavy chain and a 43 kDa light chain after reduction with β-mercaptoethanol, as shown in Supplementary Fig. 4. After activation with LPS, the zymogen factor C was converted to its active form, *i.e.*, factor $\bar{C}$, consisting of three chains of 80, 34, and 8 kDa, respectively, in the same manner as in the case of *T.*-factor C (data not shown). Western blotting performed with rabbit antiserum raised against *T.*-factor $\bar{C}$ indicated that the zymogen *L.*-factor C reacts with anti-*T.*-factor $\bar{C}$ antiserum, three bands corresponding to 123, 80, and 43 kDa being detected under reduced conditions (Supplementary Fig. 4, lane 3). The faint 123 kDa band seems to represent a single chain factor C (*7*). On Ouchterlony analysis using anti-*T.*-factor $\bar{C}$ antiserum, both preparations, *i.e.*, *T.*- and *L.*-factor Cs, formed a fusion type precipitin line (Supplementary Fig. 5).

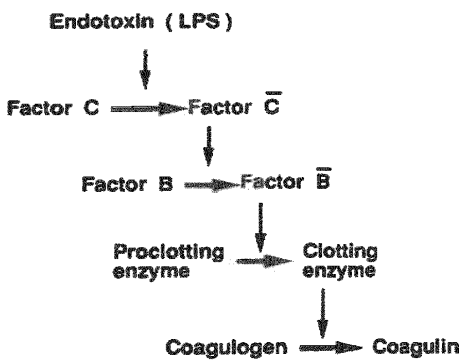

Fig. 1. Putative mechanism for the coagulation cascade of the horseshoe crab hemocyte lysate.

Activation of the zymogen *L.*-factor C by LPS and a synthetic *E. coli*- type lipid A analogue was examined by monitoring the generation of amidase activity of factor $\bar{C}$, using Boc-Val-Pro-Arg-NH-Np as a substrate. As shown in Supplementary Fig. 6, both LPS and the lipid A analogue activated the zymogen factor C, the activation reaction being completed within 30 min. Amidase activity did not appear to be generated in the absence of LPS.

Amino Acid Compositions and NH_2-Terminal Sequences of L.-Factor C and Its Chain Subunits—To compare the polypeptide chain structure of *L.*-factor C with that of *T.*-factor C, the purified zymogen factor C and its activated form were reduced and *S*-alkylated, and then the resulting chain subunits were separated by means of the high performance liquid chromatography used for the zymogen *T.*-factor C (*6, 7*). The materials thus obtained were subjected to amino acid and sequence analyses. Supplementary Table II shows the amino acid compositions of the intact *L.*-factor C and its chain subunits. *L.*-factor C was calculated to comprise a total of 1,011 residues per molecule, this number being much the same as that for *T.*-factor C (*6*). Furthermore, the individual amino acid compositions of the two proteins and of their chain subunits were indistinguishable. Like *T.*-factor C (*6*), *L.*-factor C also contained hexosamines, which were present in the acid hydrolyzate.

Supplementary Fig. 7 shows the partial NH_2-terminal

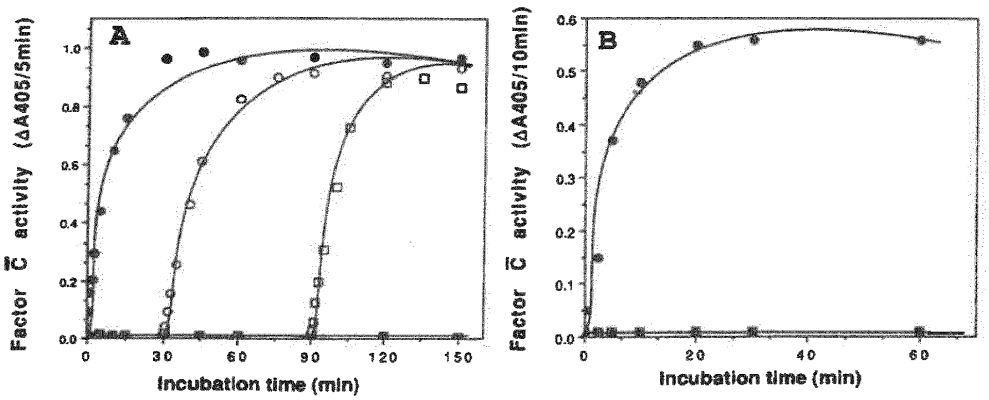

Fig. 2. α-Chymotrypsin-mediated activation of factor C. A: The purified *T.*-factor C was incubated, respectively, with α-chymotrypsin (●) and trypsin (■) at an enzyme to substrate weight ratio of 1 : 200. At the indicated times, a sample of the reaction mixture was withdrawn and factor $\bar{C}$ activity was measured as described under "MATERIALS AND METHODS." In other experiments, the reaction mixture containing factor C and trypsin was preincubated, respectively, for 30 (○) and 90 min (□), and then α-chymotrypsin was added after abolition of the trypsin activity with *N*-tosyl-L-lysyl chloromethyl ketone, followed by further incubation, was allowed to continue. The factor $\bar{C}$ activity generated was measured at the indicated times. B: The purified *L.*-factor C (95.6 μg/ml) was treated in the same manner as in A.

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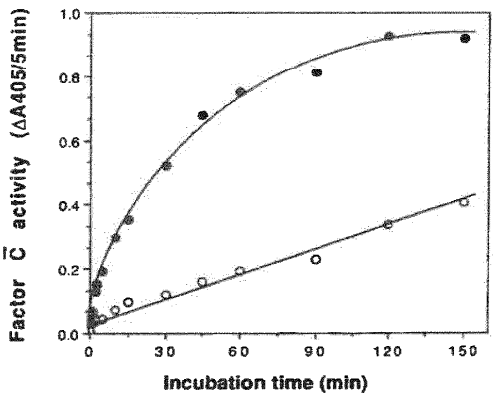

Fig. 3. Chymase-mediated activation of factor C. *T.*-factor C was incubated with chymase at enzyme to substrate weight ratios of 1 : 50 (●) and 1 : 200 (○), and then the factor C activity generated was measured as described under "MATERIALS AND METHODS."

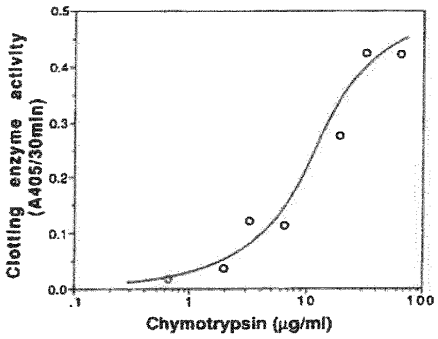

Fig. 4. Activation of the Limulus coagulation system by α-chymotrypsin. The toxicolor® reagent containing the hemocyte lysate of *T. tridentatus* was incubated with α-chymotrypsin for 30 min at 37°C and the clotting enzyme activity generated was measured using Boc-Leu-Gly-Arg-NH-Np as substrate. For details, see "MATERIALS AND METHODS."

sequences of the heavy, light and B chains isolated from *L.*-factor C and C̄, in comparison with those of *T.*-factor C. These sequences showed a close resemblance, thus the gross structure of *L.*-factor C must resemble that of known *T.*-factor C, as shown in Supplementary Fig. 8. These results indicate that the zymogen factor C's isolated from hemocytes of American and Japanese horseshoe crabs, respectively, are identical protein entities, both immunologically and biochemically. Thus, the following kinetic studies on zymogen activation and substrate specificities were performed mainly using the purified *T.*-factor C, since it was more readily available.

Time Course for Activation of the Zymogen Factor C with α-Chymotrypsin—Figure 2A shows the time course for activation of *T.*-factor C in the presence of trypsin and α-chymotrypsin. The factor C was rapidly activated at 37°C in the presence of α-chymotrypsin. However, trypsin

TABLE I. Kinetic parameters of *T.*-factor C. The active site-titrated factor C̄ (2.7 nM) was used for kinetic analyses. Kinetic parameters were determined by direct linear plotting. The concentration of *p*-nitroaniline released was estimated by assuming $A^{1\%}_{405nm}$ = 10,000.

Substrates				k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)
P4	P3	P2	P1			
Boc-	Val	-Pro	-Arg-NH-Np	391	54	7,240,000
Boc-Asp(OBzl)		-Pro	-Arg-NH-Mec	75	26	2,880,000
Boc-	Gln	-Pro	-Arg-NH-Mec	34	66	514,000
Boc-Asp(OBzl)		-Ala	-Arg-NH-Mec	18	39	489,000
Boc-	Val	-Pro	-Arg-NH-Mec	48	114	418,000
Boc-	Ala	-Pro	-Arg-NH-Mec	72	190	379,000
Boc-	Met	-Ala	-Arg-NH-Mec	27	88	212,000
Boc-	Lys(Z)	-Gly	-Arg-NH-Mec	10	45	227,000
Boc-	Gly	-Ala	-Arg-NH-Mec	29	155	190,000
Boc-Ser(OBzl)		-Ala	-Arg-NH-Mec	26	152	172,000
Boc-	Lys	-Gly	-Arg-NH-Mec	19	126	149,000
Boc-Glu(OBzl)		-Gly	-Arg-NH-Mec	34	275	122,000
Boc-	Val	-Gly	-Arg-NH-Mec	26	250	104,000
Boc-	Ile	-Gly	-Arg-NH-Mec	15	189	80,000
Boc-	Asp	-Pro	-Arg-NH-Mec	17	221	76,000
Boc-	Gly	-Pro	-Arg-NH-Mec	8.5	167	51,000

did not activate factor C at an enzyme to substrate weight ratio of 1 : 200 or even of 1 : 20 (w/w) (data not shown). Such α-chymotrypsin-mediated activation of the *T.*-factor C was also observed for that of the purified *L.*-factor C (Fig. 2B). To determine whether or not trypsin would destroy the zymogen factor C, it was incubated with trypsin at 37°C for 30 and 90 min, and then 20 μg of *N*-tosyl-L-lysyl chloromethyl ketone was added to abolish the trypsin activity. α-Chymotrypsin, at 1 : 200 (w/w), was then added to the reaction mixture. As shown by open circles and squares in Fig. 2A, the zymogen factor C was activated by α-chymotrypsin in the same manner as with α-chymotrypsin alone, confirming again that it is not activated by trypsin. The activation of the factor C by α-chymotrypsin was also examined by SDS-PAGE under reduced conditions (Supplementary Fig. 9). After 30 and 60 min incubation, the factor C-light chain (43 kDa) was converted to a 34-kDa B chain. The factor C-heavy chain was also partially cleaved, because some bands appeared between those of the heavy and B chains upon α-chymotrypsin treatment. As rat mast cell chymase shows a similar specificity to α-chymotrypsin (*17*), we examined the chymase-mediated activation of factor C. As shown in Fig. 3, the zymogen factor C was activated by chymase at enzyme to substrate weight ratios of 1 : 200 and 1 : 50, and a plateau was reached as the same extent as in the case of α-chymotrypsin. The K_m and k_{cat} values of α-chymotrypsin toward the zymogen *T.*-factor C were estimated to be 7.4×10^{-8} M and 5.6×10^{-7} $M^{-1}\cdot s^{-1}$, respectively (data not shown). Other proteases, such as porcine elastase, lysyl endopeptidase from *Achromobacter lyticus* M497-1, *Staphylococcus aureus* V8 protease, subtilisin, and papain, did not activate the zymogen factor C (data not shown).

Activation of the Horseshoe Crab Coagulation System by α-Chymotrypsin—Figure 4 shows the α-chymotrypsin-mediated activation of horseshoe crab coagulation with the hemocyte lysate. As expected, the amidase activity generated due to the activation of proclotting enzyme (*4*) increased in a dose-dependent manner. When α-chymo-

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trypsin treated with Z-Gly-Gly-Phe-chloromethyl ketone for 15 min at 37°C or the enzyme treated at 70°C for 1 h was used, the amidase activity generated in the hemocyte lysate decreased to 73.4 and 89.5%, respectively (data not shown). These results indicate that activation of the coagulation system was not induced by LPS contaminating the α-chymotrypsin sample, but rather that it was catalyzed by α-chymotrypsin.

Substrate Specificity of Factor $\bar{C}$—Since the purified factor C was activated autocatalytically in the presence of LPS through the cleavage of a Phe-Ile linkage located in the NH_2-terminal portion of the light chain (*7*), the possibility that factor C itself might have intrinsic α-chymotrypsin-like activity would have to be considered. We examined in detail the substrate specificity of factor $\bar{C}$ toward various synthetic peptidyl substrates, including substrates for α-chymotrypsin. The concentration of factor $\bar{C}$ was first determined by active-site titration with 4-methylumbelliferyl *p*-guanidinobenzoate (MUGB), which is used as a titrant for trypsin-type proteases. However, the rate of deacylation of the reagent was too rapid to estimate the enzyme concentration, as shown in Supplementary Fig. 10A. Therefore, 4-methylumbelliferyl *p*-(*N,N,N*-trimethylammonium)cinnamate (MUTMAC), synthesized as a suitable titrant for α-chymotrypsin (*18*), was used for the titration of factor $\bar{C}$. MUTMAC proved to be an appropriate titrant for factor $\bar{C}$, as deduced from the burst and stable saturation curve for the titration, as shown in Supplementary Fig. 10B. As this means that factor C probably has an intrinsic chymotrypsin-like activity, this titrant was used to determine the concentration of active factor $\bar{C}$.

Table I shows the kinetic constants of factor $\bar{C}$ toward various peptidyl substrates. The most effective substrate for factor $\bar{C}$ was Boc-Val-Pro-Arg-NH-Np. It has a high k_{cat}/K_m value, 7,240,000, which is 17-fold higher than that of Boc-Val-Pro-Arg-NH-Mec. Among 75 peptidyl-NH-Mec substrates (*14*), Boc-Asp(OBzl)-Pro-Arg-NH-Mec was also a good substrate factor $\bar{C}$, thereby indicating that the enzyme effectively cleaves substrates with a Pro residue at the P2 site. The NH-Mec substrates for α-chymotrypsin, such as succinyl-Ala-Ala-Pro-Phe-NH-Mec and succinyl-Leu-Leu-Val-Tyr-NH-Mec, were not hydrolyzed by factor $\bar{C}$ (data not shown).

Inhibition of Factor $\bar{C}$ by Natural and Synthetic Inhibitors—To further elucidate the enzymatic properties of factor C, the inhibitory effects of some natural or synthetic inhibitors on factor $\bar{C}$ activity were examined. Natural inhibitors, such as antithrombin III-heparin complex, and synthetic inhibitors, such as di*iso*propylfluorophosphate, benzamidine, and leupeptin, are known to inhibit factor $\bar{C}$ activity (*6*). Other natural inhibitors tested were aprotinin, soybean trypsin inhibitor, potato II inhibitor, C1 inhibitor, corn inhibitor, *Streptomyces* subtilisin inhibitor, hirudin, ovomucoid, and ovoinhibitor. However, these inhibitors had little effect on the factor $\bar{C}$ activity, even in the presence of an excess molar ratio to the enzyme. Among the synthetic inhibitors, *N*-tosyl-L-lysyl chloromethyl ketone, chymostatin, Z-Gly-Gly-Phe-chloromethyl ketone, and H-Ala-Ala-Phe-chloromethyl ketone also did not inhibit factor $\bar{C}$ activity, at a final concentration of 1 mM. On the other hand, D-Phe-Pro-Arg-chloromethyl ketone, an α-thrombin inhibitor, had a potent inhibitor effect on the factor $\bar{C}$ activity. The inhibition constant of this inhibitor towards factor $\bar{C}$ was determined by the method of Walker *et al.* (*19*), and the K_i value was estimated to be 7.4×10^{-6} M and k_i to be 0.69 min^{-1}(data not shown).

DISCUSSION

A factor C, which is indistinguishable from those of the known *T.*-factor C in terms of immunological and biochemical properties, was proved to be present in the hemocytes of *L. polyphemus*. Both factor C's contain two molecular species with, *i.e.*, single chain and a two-chain forms. Both molecules have the same molecular mass of 123,000, but the latter consists of a heavy chain of $M_r = 80{,}000$ and a light chain of $M_r = 43{,}000$ held together by a disulfide linkage(s). The major nicked form may be produced by a trace amount of contaminating of LPS during the purification procedures, as experienced in the study on *T.*-factor C (*6*). The structural properties of the *L.*-factor C molecule, including the entire amino acid composition and the partial NH_2-terminal sequences of their chain subunits, show similarities with those of *T.*-factor C (*6, 7*). They also exhibit common antigenicity, as revealed with the immunoblotting method and the Ouchterlony test (Supplementary Figs. 4 and 5). Like the zymogen *T.*-factor C, the purified *L.*-factor C is rapidly activated in the presence of *E. coli* LPS and synthetic lipid A analogue. These observations strongly indicate that both factor C's are the same protease zymogen and function as an initiator of the coagulation cascade in the horseshoe crab hemocytes. These findings also lead to the idea that the hemocytes of *L. polyphemus* probably contain a coagulation system similar to that established for the *T. tridentatus* hemocytes (*5-7*).

The present results are the first evidence that both the purified *L.*- and *T.*-factor C's are activated by α-chymotrypsin and not by trypsin. All the serine protease zymogens associated with mammalian coagulation, fibrinolysis, and complement systems are activated by trypsin-like enzymes. Thus, the horseshoe crab factor C seems to be a novel type of serine protease zymogen susceptible to α-chymotrypsin. The necessity of cleavege of the Phe-Ile bond (*7*) implies that the zymogen factor C cannot be activated by the horseshoe crab trypsin-like proteases (*4, 5*). Thus, positive feedback activation of the clotting system, which occurs in common in the mammalian coagulation cascade reaction, probably does not occur in horseshoe crabs, only the LPS-associated factor C appearing to be the initiator of this cascade reaction.

For the LPS-mediated activation of the zymogen factor C, it is now clear that no other protease is required for its conversion to its active form, factor $\bar{C}$, and only LPS-bound factor C is activated, probably through an intermolecular interaction between the two molecules (*8*), as is the case with activation of the complement C1r (*20*). The substrate specificity of factor $\bar{C}$ shows a close similarity to that of α-thrombin. As shown in Table I, the most reactive substrate for factor $\bar{C}$ is Boc-Val-Pro-Arg-NH-Np, with an extremely high $k_{cat}/K_m = 7{,}240{,}000$. This value is comparable to that of the sensitive fluorogenic substrate, Boc-Asp(OBzl)-Pro-Arg-NH-Mec, for bovine α-thrombin (*14*). Thus, apolar and non-aromatic residues such as Pro and Ala at the P2 site are the most favorable, as for α-thrombin (*16*). For the P3 site, bulky groups such as Val, Asp(OBzl), and Gln probably serve, as judged from their K_m values

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(Table I). Such a structural basis for a peptide substrate for factor C is also supported by the observation that D-Phe-Pro-Arg-chloromethyl ketone, which is sensitive to α-thrombin strongly inhibits factor $\bar{C}$ activity, the K_i value being 7.4×10^{-6} M. The entire cDNA sequence and deduced amino acid sequence of *T.*-factor C have been determined (manuscript submitted). Then, the factor C-derived B chain corresponding to a serine protease domain of this zymogen is the most homologous with human α-thrombin (38.5% identity) among various known mammalian serine proteases. Presumably, the serine protease domain of *T.*-factor C is closely related with α-thrombin, in terms of molecular structure and substrate specificity.

Although the active factor $\bar{C}$ shows the Arg-Xaa specificity toward synthetic peptide substrates, cleavage of the Phe-Ile bond by LPS-bound factor $\bar{C}$ is not unreasonable, because the residue preceding Phe is Pro (*7*). The reactive site of α_1-antitrypsin has been identified as Met-Ser with Met proceded by Pro (*21*). Moreover, the reactive site of heparin cofactor II is a Leu-Ser bond that is cleaved by α-thrombin. The rapid hydrolysis of MUTMAC by the LPS-bound factor C indicates that it should have intrinsic chymotrypsin-like activity. It seems likely that structural properties of horseshoe crab factor C beyond the specific cleavage site residues play an important role in the LPS-mediated activation of this zymogen. Further detailed studies on the molecular interaction of LPS and factor C are underway.

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REFERENCES

1. Bang, F.B. (1956) *Bull. Johns Hopkins Hosp.* **98**, 325-351
2. Levin, J. & Bang, F.B. (1964) *Bull. Johns Hopkins Hosp.* **115**, 265-274
3. Mürer, E.H., Levin, J., & Holme, R. (1975) *J. Cell. Physiol.* **86**, 533-542
4. Nakamura, T., Morita, T., & Iwanaga, S. (1985) *J. Biochem.* **97**, 1561-1574
5. Nakamura, T., Horiuchi, T., Morita, T., & Iwanaga, S. (1986) *J. Biochem.* **99**, 847-857
6. Nakamura, T., Morita, T., & Iwanaga, S. (1986) *Eur. J. Biochem.* **154**, 511-521
7. Tokunaga, F., Miyata, T., Nakamura, T., Morita, T., Kuma, K., Miyata, T., & Iwanaga, S. (1987) *Eur. J. Biochem.* **167**, 405-416
8. Nakamura, T., Tokunaga, F., Morita, T., & Iwanaga, S. (1988) *J. Biochem.* **103**, 370-374
9. Nakamura, T., Tokunaga, F., Morita, T., Iwanaga, S., Kusumoto, S., Shiba, T., Kobayashi, T., & Inoue, K. (1987) *Eur. J. Biochem.* **176**, 89-94
10. Tai, J.Y., Seid, R.C., Jr., Huhn, R.D., & Liu, T.-Y. (1977) *J. Biol. Chem.* **252**, 4773-4776
11. Miyata, T., Hiranaga, M., Umezu, M., & Iwanaga, S. (1984) *J. Biol. Chem.* **259**, 8924-8933
12. Tai, J.Y. & Liu, T.-Y. (1977) *J. Biol. Chem.* **252**, 2178-2181
13. Liang, S.-M., Liang, C.-M., & Liu, T.-Y. (1981) *J. Biol. Chem.* **256**, 4968-4972
14. Nakamura, S. & Levin, J. (1982) *Biochem. Biophys. Res. Commun.* **108**, 1619-1623
15. Nakamura, S. & Levin, J. (1982) *Biochim. Biophys. Acta* **707**, 217-225
16. Kawabata, S., Miura, T., Morita, T., Kato, H., Fujikawa, K., Iwanaga, S., Takada, K., Kimura, T., & Sakakibara, S. (1988) *Eur. J. Biochem.* **172**, 17-25
17. Woodbury, R.G., Everitt, M.T., & Neurath, H. (1981) *Methods Enzymol.* **80**, 588-597
18. Jameson, G.W., Roberts, D.V., Adams, R.W., Kyle, W.S.A., & Elmore, D.T. (1973) *Biochem. J.* **131**, 107-117
19. Walker, B., Wikstrom, P., & Shaw, E. (1985) *Biochem. J.* **230**, 645-650
20. Kashihara, Y., Odai, H., Takahashi, K., Nagasawa, S., & Koyama, J. (1985) *J. Biochem.* **97**, 365-372
21. Johnson, D. & Travis, J. (1978) *J. Biol. Chem.* **253**, 7142-7144
22. Laemmli, U.K. (1970) *Nature* **227**, 680-685
23. Eisenthal, R. & Cornish-Bowden, A. (1974) *Biochem. J.* **139**, 715-720

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Supplemental Materials

MATERIALS AND METHODS

Materials ——— American horseshoe crabs (*Limulus polyphemus*) were obtained from Taiyo Fishery Co., Ltd., Tokyo. *T. tridentatus* factor C (two chain form) from hemocytes of Japanese horseshoe crabs was highly purified, as described (6). The active enzyme, factor C, was prepared from the isolated factor C by treatment with LPS (6). The LPS (*Escherichia coli* 0111:B4), obtained from List Biochemical Laboratories, Inc., CA, was heated at 70 °C for 1 h before use. Synthetic *E. coli* type lipid A analog, LA-15-PP, and aprotinin were products of Daiichi Pharm., Co., Ltd., Tokyo, and Mochida Pharma., Co., Ltd., Tokyo, respectively. Bovine pancreatic α-chymotrypsin treated with *N*-tosyl-*L*-lysyl chloromethyl ketone and trypsin treated with *N*-tosyl-*L*-phenylalanyl chloromethyl ketone were obtained from Worthington Biochemical Corp., Freehold, NJ. Boc-Val-Pro-Arg-NH-Np and an endotoxin detection kit, Toxicolor® LS-1 set, which contains the *T. tridentatus* hemocyte lysate, were generous gifts from S. Tanaka, Seikagaku Kogyo Co., Ltd., Tokyo. Rat mast cell chymotrypsin-like enzyme, chymase (RMCP I), was kindly provided by Dr. N. Katunuma, Institute of Enzyme Research, The University of Tokushima. Fluorogenic tripeptide substrates (14) were obtained from Peptide Institute Inc., Osaka. 4-Methylumbelliferyl *p*-guanidinobenzoate (MUGB) 4-methylumbelliferyl *p*-(*N N N*-trimethylammonium) cinnamate (MUTMAC), and soybean trypsin inhibitor were purchased from Sigma Chemical, Co., St. Louis, MO. H-Ala-Ala-Phe-chloromethyl ketone and Z-Gly-Gly-Phe-chloromethyl ketone were from Bachem, Bubendorf, Switzerland, and D-Phe-Pro-Arg-chloromethyl ketone was from Calbiochem, La Jolla, CA. Sepharose CL-6B and CM-Sepharose CL-6B were purchased from Pharmacia LKB Biotechnology, Uppsala. All other chemicals were of the highest quality commercially available.

Factor C assay ——— The amidase activity of factor C generated after activation with LPS was measured using Boc-Val-Pro-Arg-NH-Np as a substrate, as described (6). The reaction mixture, comprising 10-20 µl of sample in Tris/HCl/SA buffer in a total volume of 200 µl, was preincubated at 37 °C for 10 min in the presence or absence of 4 µl of LPS (10 µg/ml). Then, 50 µl of 2 mM Boc-Val-Pro-Arg-NH-Np was added and the mixture was incubated further for 10 min. Finally, 0.8 ml of 0.6 M acetic acid was added to terminate the reaction and the absorbance at 405 nm was read. One unit of enzyme activity was defined as 1 µmol of *p*-nitroaniline liberated per min. The amidase activity of factor C (active form) was measured as described for factor C in the absence of LPS.

SDS-polyacrylamide gel electrophoresis and Western blotting ——— SDS-PAGE was performed according to the method of Laemmli (22). Western blotting was performed using rabbit antiserum raised against *T.*-factor C, as described (7). For Ouchterlony analysis, a 1 % agarose gel dissolved in 20 mM Tris-HCl, pH 8.0, and containing 0.15 M NaCl was used.

Reduction and S-alkylation ——— The sample was reduced and *S*-pyridylethylated and each chain was separated by reversed phase liquid chromatography on a TSK phenyl 5PWRP column (4.6 x 75 mm), as described (7).

Amino acid and sequence analyses ——— Samples were hydrolyzed in 5.7 M HCl at 110 °C for 24, 48 and 72 h or 3 M mercaptoethanesulfonic acid for 24 h in evacuated and sealed tubes. After evaporation, the hydrolyzates were analyzed using a Hitachi 835 automatic analyzer. Amino acid sequence determination was carried out with a gas-phase sequencer (Applied Biosystems 477A) and a 120A-phenylthiohydantoin analyzer on-line system (7).

Activation of factor C by trypsin, α-chymotrypsin and chymase ——— Factor C (206 µg/ml) was incubated at 37 °C with 1/200 (w/w) of trypsin, α-chymotrypsin or chymase, in a total volume of 350 µl of 0.1 M Tris/HCl buffer, pH 8.0. At the indicated times, a 10 µl sample was taken and mixed with 50 µg/ml of aprotinin for α-chymotrypsin and trypsin, or 50 µg/ml of soybean trypsin inhibitor for chymase. These protease inhibitors were dissolved in a total 200 µl of Tris/HCl/SA buffer. Each mixture was incubated for a further 5 min to inhibit the protease activity and finally the factor C activity generated was measured.

Activation of the horseshoe crab coagulation system by α-chymotrypsin ——— α-Chymotrypsin-mediated activation of horseshoe crab coagulation system was measured using Toxicolor® reagent, LS-1 set, obtained from Seikagaku Kogyo, Ltd., Tokyo, according to the manufacturer's manual. The LS-1 set contained *Tachypleus* hemocyte lysate and Boc-Leu-Gly-Arg-NH-Np, the latter of which is an appropriate substrate for the clotting enzyme generated during activation of the coagulation system. The LS-1 set (100 µl) and various concentrations of α-chymotrypsin dissolved in 100 µl of 20 mM Tris/HCl buffer, pH 8.0, containing 0.15 M NaCl, were mixed and incubated for 30 min at 37 °C, and then 0.8 ml of 0.6 M acetic acid was added and the absorbance at 405 nm was read.

Kinetic analysis ——— The factor C activity was measured at 37 °C in 50 mM Tris/HCl buffer, pH 8.0, containing 0.15 M NaCl and 0.1 mg/ml of bovine serum albumin (BSA), with various fluorogenic peptide substrates. The initial rate of hydrolysis was measured with a Hitachi 650 fluorometer with excitation at 380 nm and emission at 440 nm at 37 °C, as described in detail elsewhere (14). Substrate concentrations of 1 µM to 2 mM and 2.7 nM factor C were used. Kinetic parameters were determined by direct linear plotting (23). The concentration of active factor C was determined with MUTMAC from the initial burst of 4-methylumbelliferone in 0.1 M sodium barbiturate buffer, pH 8.3, containing 0.1 mg/ml of BSA at 25 °C (18). Kinetics analysis for irreversible inhibitors, such as *D*-Phe-Pro-Arg-chloromethyl ketone, was performed by the method of Walker *et al.* (19).

Inhibitory effects of natural and synthetic inhibitors on factor C ——— The Screening of various inhibitors was performed with an inhibitor concentration of 0.2 mM and a factor C concentration of 10 nM in Tris/HCl/SA buffer. Each mixture was incubated at 37 °C for 10 min, in a total volume of 200 µl, and then the remaining factor C activity was measured.

RESULTS

Purification of *L. polyphemus* factor C ——— Purification of *L.*-factor C was carried out under sterilized conditions according to the method of Nakamura *et al.* (6). The lysate (595 ml) was first applied to a dextran-sulfate Sepharose CL-6B column (7.6 x 24.0 cm). Stepwise elution was performed with NaCl concentrations of from 50 mM, 0.25 M, 0.5 M and 2 M, as shown in Supplementary Fig. 1. Factor C in the 0.5 M NaCl fractions was collected, pooled and concentrated by Diaflo ultrafiltration. The concentrated fraction was then applied to a Sepharose CL-6B column (2.6 x 141 cm), equilibrated with 20 mM acetate buffer, pH 5.0, containing 0.1 M NaCl (Supplementary Fig. 2). The pooled factor C fraction was finally applied to a CM-Sepharose CL-6B column (2.0 x 24.0 cm), equilibrated with the same buffer as used in Supplementary Fig. 2 and eluted with a linear gradient of NaCl (0.1-0.35 M) in 20 mM acetate buffer, pH 5.0 (Supplementary Fig. 3). A summary of the purification of factor C is shown in Supplementary Table I. The yield of purified factor C was 5.2 mg from 80 g hemocytes (wet weight), 14.7-fold purification from the dextran-sulfate Sepharose CL-6B fraction (Supplementary Fig. 1) being achieved (6).

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Supplementary Table I

Summary of the purification procedure for L. polyphemus factor C.

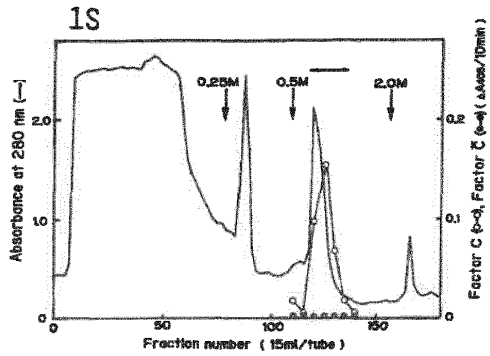
Step	Volume	Total protein	Total activity	Specific activity	Purification	Yield
	ml	mg	units	units/mg	-fold	%
Hemocyte lysate	298	—	—	—	—	—
Dextran-sulfate-Sepharose CL-6B	138	140	397	2.8	1	100
Sepharose CL-6B	90	17.3	421	24.3	8.7	106
CM-Sepharose CL-6B	130	5.2	214	41.2	14.7	54

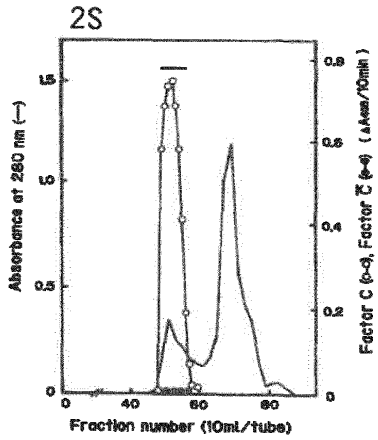
Supplementary Table II

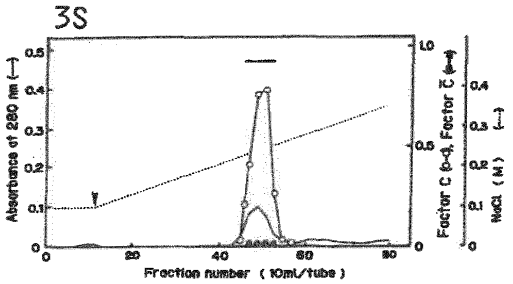
Amino acid compositions of L. polyphemus factor C and its S-alkylated derivatives[a].

Amino acid	Factor C			Factor $\bar{C}$	
	Whole	H chain	L chain	A chain	B chain
	Residues/molecule				
Asp	101.0 (101)	69.4 (67)	39.4 (34)	4.8 (5)	34.6 (29)
Thr	53.6 (59)	36.7 (37)	20.5 (22)	2.2 (2)	18.7 (20)
Ser	88.0 (82)	59.7 (55)	31.5 (27)	11.2 (13)	25.1 (14)
Glu	92.1 (89)	60.7 (58)	34.1 (31)	5.8 (5)	30.8 (26)
Pro	60.8 (69)	47.4 (52)	23.7 (17)	6.9 (6)	20.1 (11)
Gly	89.9 (88)	55.4 (57)	32.6 (29)	7.7 (7)	27.6 (22)
Ala	57.4 (56)	34.7 (35)	22.1 (21)	3.0 (3)	18.3 (18)
Val	61.3 (64)	44.7 (42)	23.8 (22)	3.9 (4)	20.4 (18)
Met	9.3 (10)	6.3 (7)	3.5 (3)	— (0)	3.2 (3)
Ile	39.5 (41)	22.9 (23)	16.0 (18)	2.2 (2)	13.3 (16)
Leu	69.0 (71)	45.4 (47)	25.4 (24)	3.9 (4)	22.2 (20)
Tyr	36.3 (37)	22.1 (22)	14.6 (15)	4.2 (4)	11.4 (11)
Phe	29.4 (28)	20.4 (20)	12.3 (8)	1.9 (1)	14.6 (7)
Lys	54.7 (50)	37.8 (38)	15.9 (12)	3.4 (2)	15.0 (10)
His	17.7 (17)	13.1 (12)	5.3 (5)	1.1 (1)	5.1 (4)
Trp	16.0 (23)	11.9 (12)	8.9 (11)	1.6 (1)	8.0 (10)
Pe-Cys	n.d.[b] (56)	37.5 (45)	13.5 (11)	3.4 (4)	12.3 (7)
Arg	44.4 (53)	25.6 (34)	15.8 (19)	6.8 (8)	11.8 (11)
Total	920.4 (994)	651.7 (665)	358.9 (329)	74.0 (72)	312.5 (257)
$GlcNH_2$	+	+	+	-	+
$GalNH_2$	+	+	+	-	+

a: Numbers in parentheses are taken from the sequence data for T.-factor C (manuscript summitted).
b: not determined.







Supplementary Fig. 1. Dextran-sulfate Sepharose CL-6B chromatography of the L. polyphemus hemocyte lysate. Details of the procedure are given under Methods. The fractions indicated by a solid bar were pooled and concentrated. The activities of factor C (○) and its active form, factor $\bar{C}$ (●), were assayed, using Boc-Val-Pro-Arg-NH-Np as a substrate. the absorbance at 280 nm (——) was measured.

Supplementary Fig. 2. Sepharose CL-6B gel filtration of the L.-factor C fraction obtained from a dextran-sulfate Sepharose CL-6B column. The zymogen factor C (○) and the activity of factor $\bar{C}$ (●) were assayed, using Boc-Val-Pro-Arg-NH-Np as a substrate. The absorbance at 280 nm (——) was measured.

Supplementary Fig. 3. CM-Sepharose CL-6B chromatography of the L.-factor C fraction obtained from a Sepharose CL-6B column. The zymogen factor C (○) and the activity of factor $\bar{C}$ (●) were assayed, using Boc-Val-Pro-Arg-NH-Np as a substrate. The absorbance at 280 nm (——) was measured and gradient elution (·····) with NaCl was started at the position indicated by the arrowhead.

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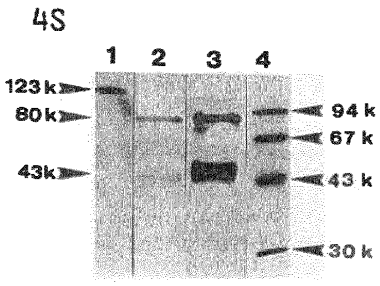
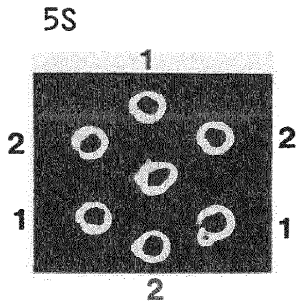
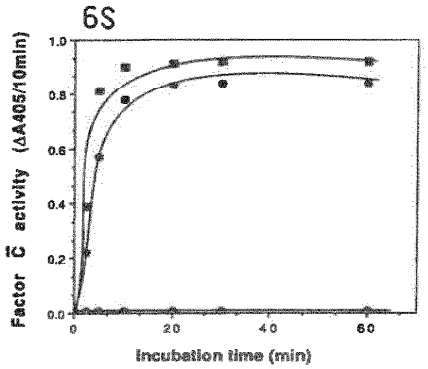
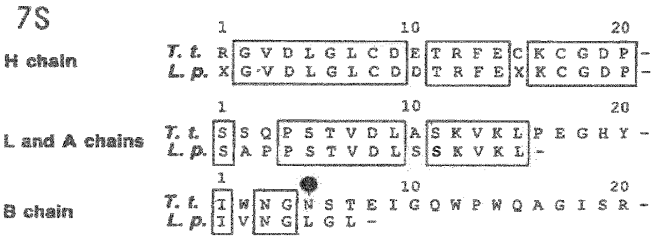
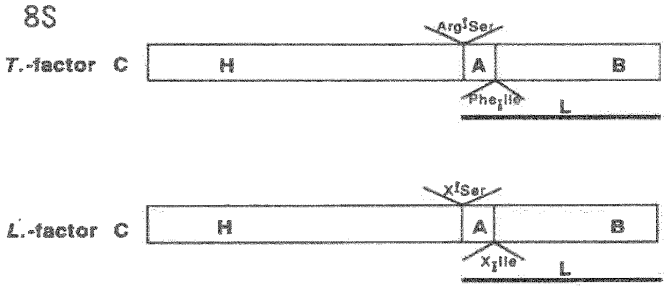
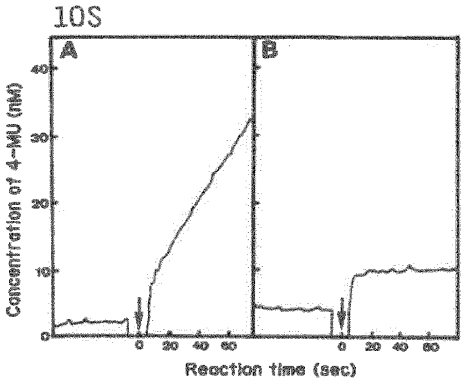












Supplementary Fig. 4. SDS-PAGE and Western blotting of the purified L.-factor C. Ten μg of L.-factor C was subjected to SDS-PAGE in the absence (lane 1) and presence (lane 2) of 2-mercaptoethanol. Western blotting (lane 3) of the reduced L.-factor C was also performed using rabbit anti-T-factor C serum as the 1st antibody, and the positions of co-electrophoresed standards of known molecular weight (lane 4) are indicated.

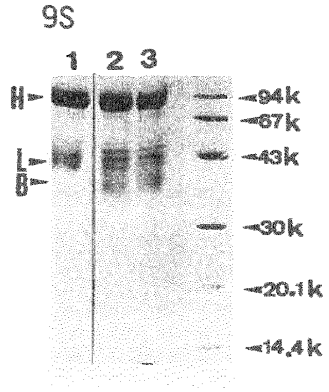
Supplementary Fig. 5. Immunodiffusion of the purified L.-factor C (1) and T.-factor C (2) against rabbit anti-T.-factor C antiserum (center well).

Supplementary Fig. 6. Time course of activation of the L.-factor C in the presence of LPS and a lipid A analogue. The purified L.-factor C (95.6 μg/ml) was incubated at 37 °C in the presence (■) and absence (○) of LPS (10 μg/ml). E. coli type-synthetic lipid A analogue, LA-15-PP (● , 10 μg/ml), was also used. At the indicated times, 20 μl aliquots were withdrawn and assayed for factor C activity.

Supplementary Fig. 7. Comparison of the NH_2-terminal sequences of the heavy, light and B chains derived from each of L.-factor C and T.-factor C. Residues identical in the two factor C's are boxed. The solid circle indicates the carbohydrate attachment site (7).

Supplementary Fig. 8. Gross structure of T.- and L.- factor C's. H, A, B and L denote the heavy, A, B and light chains of factor C. The arrowheads and solid bars indicate the cleavage sites and the locations of the light chains of the factor C's, respectively.

Supplementary Fig. 9. SDS-PAGE of T.-factor C activated by α-chymotrypsin. Samples of the zymogen factor C (lane 1) and the factor C treated with α-chymotrypsin (weight ratio of enzyme/substrate = 1/200) for 30 min (lane 2) and 60 min (lane 3) were subjected to SDS-PAGE. H, L and B denote the heavy, light and B chains of the factor C, respectively.



Supplementary Fig. 10. Active site titration of T.-factor C. A, 4-methylumbelliferyl p-guanidinobenzoate (MUGB) and B, 4-methylumbelliferyl p-(N N N-trimethylammonium) cinnamate (MUTMAC) were used as titrants, being added, respectively, to the reaction mixture containing T.-factor C at the times indicated by arrows after preincubation for 60 sec.

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