

Lipopolysaccharide-sensitive serine-protease zymogen (factor C) found in *Limulus* hemocytes

Isolation and characterization

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(Received September 9/November 4, 1985) — EJB 85 1003

Bacterial endotoxin (lipopolysaccharide, LPS) induces coagulation of horseshoe crab hemolymph. Our previous studies had demonstrated that a hemolymph factor, designated factor B, was associated with the LPS-mediated activation of the *Limulus* clotting system [Ohki et al. (1980) *FEBS Lett.* 120, 318–321]. On further purification of factor B we found that an additional component, designated factor C, was required to generate factor B activity in the presence of LPS in order to activate the proclotting enzyme. To elucidate the role of factor C in the LPS-mediated reaction, factor C was isolated and characterized from the hemocyte lysate under sterile conditions. The preparation exhibited a single band on sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) in the absence of 2-mercaptoethanol, while two protein bands on SDS-PAGE were observed after reduction. Thus, factor C had a M_r of 123 000 consisting of a heavy chain of $M_r = 80 000$ and a light chain of $M_r = 43 000$. Factor C was converted to an activated form in the presence of LPS with a $M_r = 123 000$, designated factor $\bar{C}$. Upon activation, cleavage of the light chain occurred resulting in the accumulation of two new fragments of $M_r = 34 000$ and 8500 on reduced SDS-PAGE. A diisopropylfluorophosphate-sensitive active site was localized in the light chain ($M_r = 34 000$) of factor $\bar{C}$. The reconstitution experiments, using factor C, factor B, proclotting enzyme and LPS, demonstrated that all of these proteins are essential for the endotoxin-mediated coagulation system. On the basis of these results we propose that a cascade pathway of LPS-induced activation of the *Limulus* clotting system consists of three sequential activations of hemolymph serine protease zymogens.

The bacterial endotoxin-induced clotting phenomenon of the hemocyte lysate from horseshoe crab is thought to be a defense mechanism that serves to immobilize invading Gram-negative bacteria [1]. Because of this specific property and its extreme sensitivity to endotoxins, *Limulus* hemolymph has been applied in the last decade to estimate or quantify nanogram quantities of endotoxins in human body fluids and pharmaceutical products [2–4]. We have studied the *Limulus* clotting system to define the overall molecular events in the hemolymph clotting and to compare these reactions with those of mammalian blood coagulation [5, 6]. Our recent studies [5, 6, 10, 11] have shown that there are sequential activations of intracellular serine protease zymogens similar to the mammalian coagulation system [7] and that a new factor, factor B, is involved in this system [5] in addition to coagulogen and proclotting enzyme [8–10]. Here we describe the existence of another protein, designated factor C, which is sensitive to lipopolysaccharide (LPS), and delineate its involvement in the LPS-mediated initiation reaction of the

Limulus clotting system. Furthermore, the isolation, characterization and the mechanism of its activation by LPS are reported herein. We propose a *Limulus* clotting cascade system on the basis of the reconstitution experiments and our biochemical studies on proclotting enzyme and factor B¹. A preliminary account has been presented in abstract form [11]. The Experimental Procedure and some of the Results are presented in the miniprint supplement.

RESULTS

In the previous paper [5] we reported that the *Limulus* intracellular clotting system, mediated with LPS, consists of at least three protein components, factor B, proclotting enzyme and coagulogen. Among them, the latter two components have been purified and characterized [9, 10]. On further purification of factor B we found that an additional component, factor C, is required to generate the factor $\bar{B}$ activity in the presence of LPS. In order to clarify further the LPS-mediated clotting system, we isolated and characterized factor C from the hemocyte lysate.

Purification of factor C

The hemocyte lysate was first fractionated on a dextran-sulfate–Sephacrose CL-6B column, as shown in Fig. 1.

¹ Factor $\bar{C}$ activates factor $\bar{B}$ of $M_r = 64 000$ to its activated form, designated factor $\bar{B}$ of $M_r = 56 000$ (T. Nakamura, T. Horiuchi, T. Morita and S. Iwanaga (1986) *J. Biochem. (Tokyo)*, March issue, in the press).

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Abbreviations. SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Dip-F, diisopropylfluorophosphate; Nan, *p*-nitroanilide; Boc, *N*-*tert*-butoxycarbonyl; Z, benzyloxycarbonyl; Bz, benzoyl; Bzl, benzyl; Cm, carboxymethyl; LPS, lipopolysaccharide; Mca, 4-methylcoumaryl-7-amide; AMC, 7-amino-4-methylcoumarin; ClHgBzOH, *p*-chloromercuribenzoate; HPLC, high-performance liquid chromatography.

Enzymes. Thrombin (EC 3.4.21.5); coagulation factor Xa (EC 3.4.21.6); plasmin (EC 3.4.21.7); plasma kallikrein (EC 3.4.21.34).

Factors B and C were separately eluted with the buffer containing 0.5 M NaCl. Fractions containing factor C and devoid of factor B were pooled as indicated in Fig. 1 and applied to a Sepharose CL-6B column. Fig. 2 shows the elution profile of factor C. While the factor C preparation in the first peak produced a single band in the unreduced sample on sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE), several bands were observed in the reduced

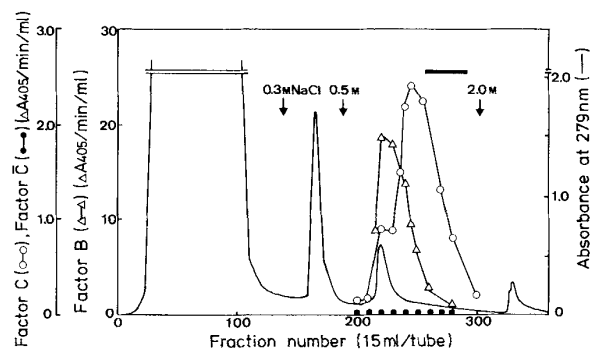


Fig. 1. Dextran-sulfate-Sepharose CL-6B chromatography of *T. tridentatus* hemocyte lysate. Details of the procedure are described in the supplement under Experimental Procedure. The fractions indicated by a solid bar were pooled and concentrated. Factor C (○), factor C-bar (●), and factor B (△) activities were assayed by using Boc-Leu-Gly-Arg-Nan as a substrate by the method described. Absorbance at 279 nm (—) was measured.

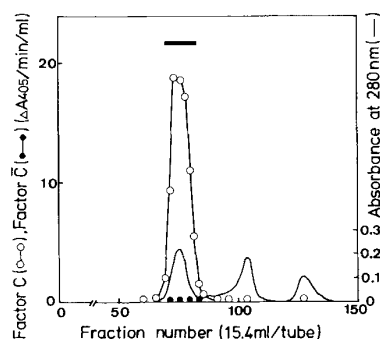


Fig. 2. Sepharose CL-6B gel filtration of the factor C fraction obtained from a dextran-sulfate-Sepharose CL-6B column. Details of the procedure are described in the supplement under Experimental Procedure. The fractions indicated by a solid bar were pooled. The activities of factor C (○) and factor C-bar (●) were assayed, using Boc-Val-Pro-Arg-Nan as a substrate by the method described. Absorbance at 280 nm (—) was measured.

sample (data not shown). Thus, further purification was performed by using a CM-Sepharose CL-6B column. Fractions containing factor C (Fig. 2) were applied to a CM-Sepharose CL-6B column, and eluted with linear salt gradient of the buffer containing NaCl. Fig. 3 shows that factor C was eluted as a single major peak with the activity of factor C. A summary of the procedures for purification of factor C is shown in Table 1. The yield of purified factor C was 15.5 mg. The purification procedures for factor C were repeated three times using 1.2 l lysate and reproducible results were obtained. The mean yield was 20.7 mg from 173 g hemocytes.

The purity of the final preparation was examined by PAGE in the presence and absence of SDS. On SDS-PAGE the purified zymogen gave a single band before reduction and two protein bands after reduction by 2-mercaptoethanol (Fig. 4A). This suggests that factor C consists of two polypeptide chains connected by disulfide linkage(s). As shown in Fig. 4B the purified factor C gave a single protein band on disc-PAGE at pH 4.3, and the factor C activity was observed at the same position as the protein band stained with Coomassie brilliant blue. Factor C preparation did not exhibit any amidase activity towards either Boc-Leu-Gly-Arg-Nan or Boc-Val-Pro-Arg-Nan. Furthermore, no apparent molecular change in factor C was observed on SDS-PAGE following incubation at 37°C for 3 h under LPS-free conditions. These results indicate that the factor C preparation did not contain the activated form of factor C and was not contaminated with LPS.

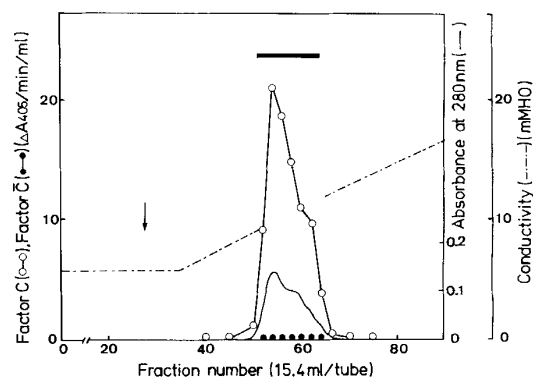


Fig. 3. CM-Sepharose CL-6B chromatography of the factor C fraction obtained from a Sepharose CL-6B column. The fractions indicated by a solid bar were collected. The activities of factor C (○) and factor C-bar (●) were assayed, using Boc-Val-Pro-Arg-Nan as a substrate by the method described in the supplement under Experimental Procedure. Absorbance at 280 nm (—) and the conductivity (---) were measured. The gradient elution of NaCl was started at the position indicated by an arrow.

Table 1. Summary of the purification procedure for the Tachyplesus factor C

Step	Volume	Total protein	Total activity	Specific activity	Purification	Yield
	ml	mg	units	units/mg	-fold	%
Hemocyte lysate	1150	6095	—	—	—	—
Dextran-sulfate-Sepharose CL-6B	525	98.4	767	7.8	1	100
Sepharose CL-6B	215	25.4	572	22.5	2.9	74.6
CM-Sepharose CL-6B	215	15.5	443	28.6	3.7	57.8

Molecular mass

The molecular mass of the purified factor C was estimated using Ferguson plots [12] from the relative mobilities of the samples on SDS-PAGE. The factor C preparation and marker proteins gave a straight line (data not shown). Using this calibration line, the relative molecular mass of factor C was calculated to be 123 000 in the absence of 2-mercaptoethanol. Upon reduction, the relative molecular masses of the two polypeptide chains were calculated to be 80 000 (designated heavy chain) and 43 000 (designated light chain).

Separation of heavy and light chains of factor C

To ascertain the polypeptide chain structure of factor C, the S-alkylated sample was applied to a TMS-250 column. The result is shown in Fig. 5. Two major peaks were obtained. The first peak gave a single band on SDS-PAGE while the

second peak gave one major band and one minor band on SDS-PAGE (Fig. 5, inset). According to the mobilities of these bands on SDS-PAGE the first and second peaks contained the heavy chain of $M_r = 80\,000$ and the light chain of $M_r = 43\,000$. The minor protein band in the second peak is most probably an aggregated form of the light chain, since the apparent M_r of the minor protein band was 86 000, which is consistent with the molecular mass of a light-chain dimer.

Amino acid and carbohydrate compositions

Table 2 shows the amino acid and carbohydrate compositions of the intact molecule of factor C and its heavy and light chains. A total of 1027 amino acid residues and 24 and 31 residues of hexose and hexosamine per molecule of factor C were calculated respectively. No neuraminic acid was detected (less than 0.1 residue/mol). The sum of the total amino acid

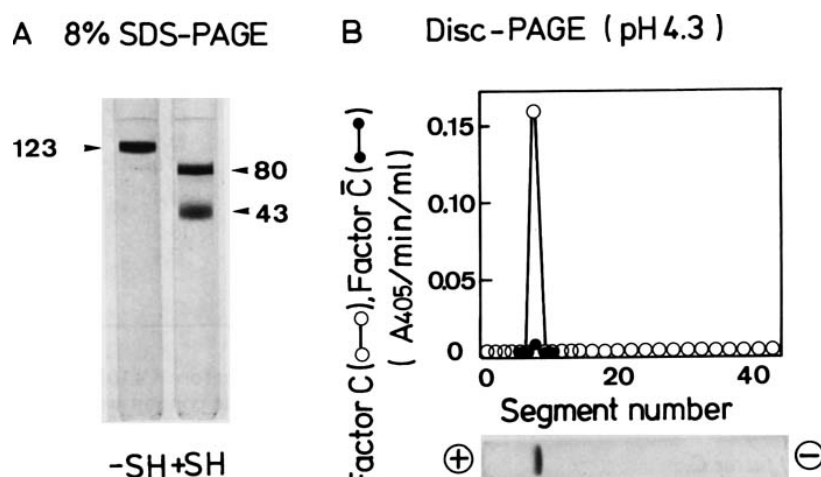


Fig. 4. PAGE of the purified factor C. (A) SDS-PAGE (13- μ g sample) was performed in the presence (right) or absence (left) of 2-mercaptoethanol. (B) PAGE (7.5% gel) was performed at pH 4.3. One of the two gels was sliced into 1.5-mm segments and the segments were soaked in 0.4 ml 0.5 M Tris/HCl buffer, pH 8.0, containing 0.05% bovine serum albumin, and kept at 4°C for 24 h. Aliquots of the extracts (50 μ l) were assayed for factor C (○) and factor C (●) by using Boc-Val-Pro-Arg-Nan as a substrate. The other gel was stained with Coomassie brilliant blue R-250. Arrows show $M_r \times 10^{-3}$.

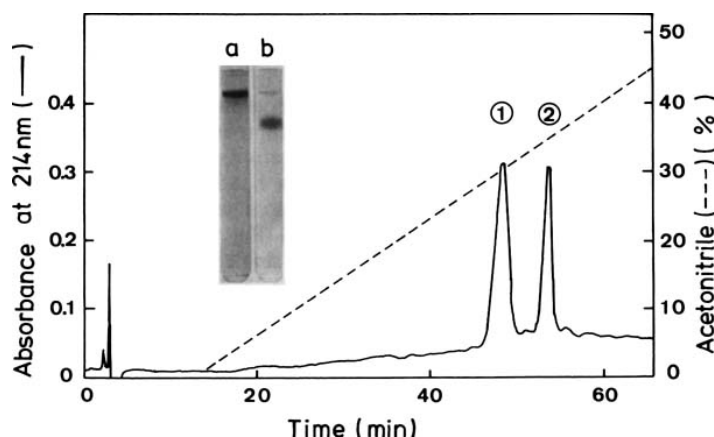


Fig. 5. Reverse-phase HPLC pattern of S-alkylated factor C on a TMS-250 column. (A) The S-alkylated factor C (64 μ g) was injected onto the column and eluted with a linear gradient of acetonitrile/0.1% trifluoroacetic acid. SDS-PAGE of two peaks is shown in the insert. Lanes a and b were proteins from peaks 1 and 2 respectively.

Table 2. Amino acid compositions of S-alkylated factor C and heavy and light chains of factor C

Amino acid and hexosamine compositions were calculated from extrapolated or average values estimated on samples of 24-h, 48-h and 72-h hydrolysates. Hexosamine, hexose and neuraminic acid were calculated as N-acetylhexosamine, mannose and N-acetylneuraminic acid respectively. Residue values are based on $M_r = 112000$ for the factor C polypeptide, 73000 for the heavy chain polypeptide and 39000 for the light chain polypeptide

Amino acid or carbohydrate	Factor C	Sum of L and H chains	Heavy chain (H)	Light chain (L)
Residues per molecule				
Cm-cysteine	65.3	65.2	48.9	16.3
Aspartic acid	109.0	108.8	69.4	39.4
Threonine	64.4	59.8	37.9	21.9
Serine	90.7	88.7	57.8	30.9
Glutamic acid	93.3	93.0	59.8	33.2
Proline	65.1	65.6	43.2	22.4
Glycine	92.4	93.7	59.9	33.8
Alanine	53.7	57.4	34.2	23.2
Valine	59.4	58.7	38.6	20.1
Methionine	10.5	9.8	6.7	3.1
Isoleucine	38.1	37.1	21.8	15.3
Leucine	67.3	69.2	43.6	25.6
Tyrosine	37.0	35.9	21.2	14.7
Phenylalanine	29.4	29.0	19.6	9.4
Lysine	57.1	57.8	40.5	17.3
Histidine	21.0	21.1	14.7	6.4
Tryptophan	23.0	24.8	13.3	11.5
Arginine	50.4	51.1	30.0	20.8
Total	1027.1	1026.8	661.4	365.4
Glucosamine	20.2	20.6	13.3	7.3
Galactosamine	11.3	11.0	7.4	3.6
Hexose	24.4	—	—	—
Neuraminic acid	< 0.1	—	—	—

and hexosamine residues of the two chains essentially corresponded to the total residues of intact factor C.

LPS-mediated activation of factor C

The activation of factor C in the presence of LPS (2 µg/ml) was followed by measuring the generation of amidase activity due to newly generated activated factor C (factor $\bar{C}$). In parallel with this experiment the structural change of the zymogen during the activation was examined by SDS-PAGE. As shown in Fig. 6A the activation reaction proceeded rapidly and was complete in 90 min. No generation of amidase activity was observed in the absence of LPS. During the activation the molecular mass of factor C remained unchanged in unreduced SDS-PAGE (Fig. 6B). In reduced SDS gels, however, the factor C light chain of $M_r = 43000$ was converted to 34000, while the heavy chain of $M_r = 80000$ did not change (Fig. 6C). In this experiment we could not detect small fragment(s) derived from the light chain by SDS-PAGE. However, the appearance of the new fragment ($M_r = 34000$) correlated with the generation of factor $\bar{C}$ activity. These results indicate that the activation of factor C to factor $\bar{C}$ in the presence of LPS involves limited proteolysis of the light chain of factor C.

Isolation and characterization of activated form of factor C, factor $\bar{C}$

Factor C was partially purified essentially as described in Purification of factor C, using dextran-sulfate–Sephacrose CL-6B and Sepharose CL-6B. The factor-C-containing sample from Sepharose CL-6B was applied to a benza-

midine–CH-Sepharose CL-6B column. Factor C was first passed through the column with the equilibration buffer, as shown in Fig. 7A. Then, the factor-C-containing sample was incubated with LPS (2 µg/ml) to activate factor C to factor $\bar{C}$ at 37°C for 4 h. The reaction mixture containing factor $\bar{C}$ was applied again to the same benzamidine–CH-Sepharose CL-6B column (Fig. 7B). In this column, contaminating proteins were unadsorbed, whereas factor $\bar{C}$ was tightly bound to the column. The column was washed with buffer containing 1 M NaCl to elute trace quantities of contaminating proteins, and factor $\bar{C}$ was subsequently eluted from the benzamidine-Sepharose column with buffer containing 1.0 M guanidine hydrochloride. The yield of factor $\bar{C}$ was 8.8 mg.

The purity of the final preparation was examined by PAGE in the presence and absence of SDS. In SDS-PAGE the preparation gave a single protein band in the absence of 2-mercaptoethanol. In the presence of 2-mercaptoethanol the preparation produced two major protein bands and one faint protein band as shown in Fig. 8A. This faint protein band was also detected by measuring the absorbance at 590 nm on the gel (Fig. 9B). As shown in Fig. 8B, the purified factor $\bar{C}$ gave a single protein band on disc-PAGE at pH 4.3. Moreover, a single peak with factor $\bar{C}$ activity was observed at the same position as the protein band stained with Coomassie brilliant blue. These data suggest that factor $\bar{C}$ consists of three polypeptide chains connected by disulfide bridges.

Molecular mass

The molecular masses of factor $\bar{C}$, the heavy chain, the light chain and a small fragment of factor $\bar{C}$ were estimated using Ferguson plots from the relative mobilities of the

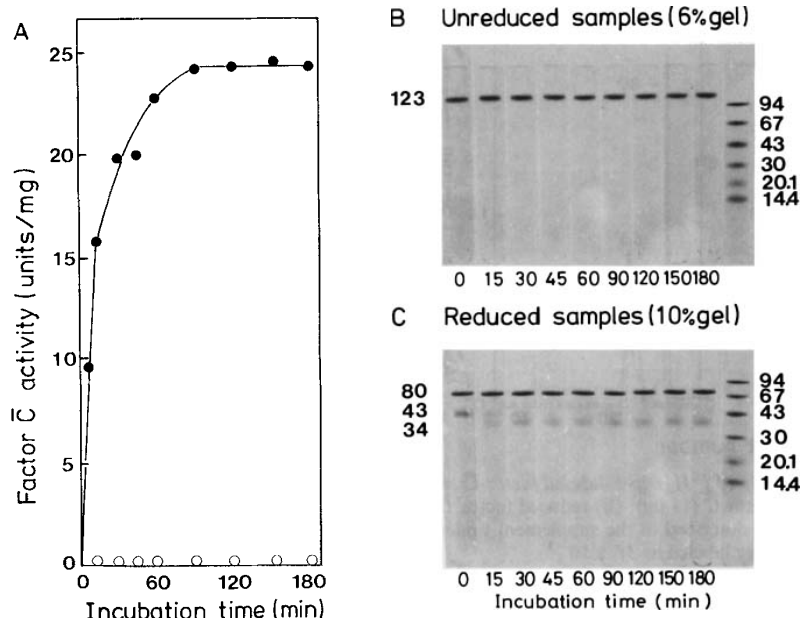


Fig. 6. Time course for activation of factor C to factor $\bar{C}$ in the presence of LPS. Factor C (248 $\mu\text{g}/\text{ml}$) was incubated at 37°C in the presence (●) or absence (○) of LPS (2 $\mu\text{g}/\text{ml}$) in a total volume of 0.05 M Tris/HCl/0.1 M NaCl (pH 8.0). At the indicated time 5- μl aliquots were withdrawn and assayed for factor $\bar{C}$ activity, and the structural change of factor C was analyzed on SDS-PAGE (6% gels for non-reduced samples and 10% gels for reduced samples). Bands are labeled as $M_r \times 10^{-3}$.

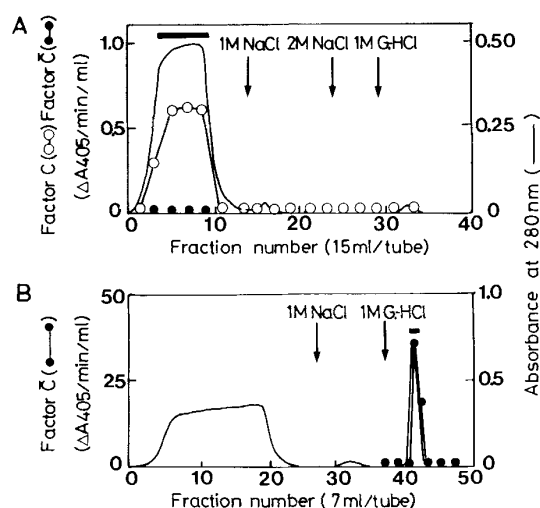


Fig. 7. First benzamidine-CH-Sepharose CL-6B chromatography of the factor-C-containing fraction obtained from a Sepharose CL-6B column (A) and second benzamidine-CH-Sepharose CL-6B chromatography of factor- $\bar{C}$ -containing reaction mixture (B). (A) Details of the procedure are described in the supplement under Experimental Procedure. The fractions indicated by a solid bar were pooled. The activities of factor C (○) and factor $\bar{C}$ (●) were assayed by using Boc-Leu-Gly-Arg-Nan as a substrate by the method described. Absorbance at 280 nm (—) was measured. (B) Details of the procedure as described. The factor- $\bar{C}$ -containing mixture was applied to the same benzamidine-CH-Sepharose column as described in Fig. 8A. The elution of factor $\bar{C}$ was performed with 0.02 M Tris/HCl buffer, pH 8.0, containing 1.0 M NaCl and 1.0 M guanidine/HCl. The activity of factor C (●) was assayed by the method described. Absorbance at 280 nm (—) was measured.

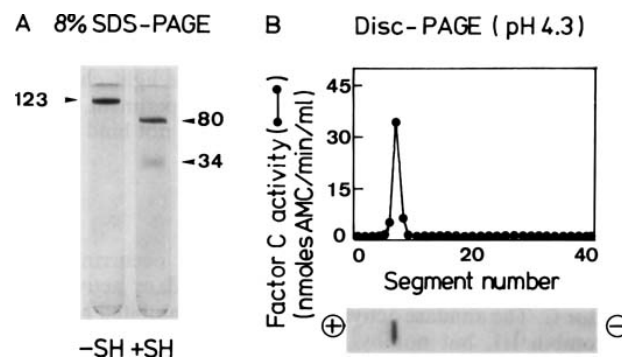


Fig. 8. PAGE of the purified factor $\bar{C}$. (A) SDS-PAGE (11- μg sample) was performed in the presence (right) or absence (left) of 2-mercaptoethanol. Bands are labeled as $M_r \times 10^{-3}$. (B) PAGE (6% gel) was performed at pH 4.3. One of the two gels was sliced into 1.5-mm segments and the segments were soaked in 0.4 ml 0.2 M Tris/HCl buffer, pH 8.0, containing 0.1% bovine serum albumin, and kept at 4°C for 45 h. Aliquots of the extracts (50 μl) were assayed for the factor $\bar{C}$ by using Boc-Val-Pro-Arg-Mea as a substrate. The other gel was stained with Coomassie brilliant blue R-250.

samples on SDS-PAGE. In the absence of 2-mercaptoethanol the M_r of factor $\bar{C}$ was the same as that of factor C ($M_r = 123000$). Upon reduction, the M_r of the heavy chain of factor $\bar{C}$ was identical to the heavy chain of factor C ($M_r = 80000$), whereas the M_r of the light chain of factor $\bar{C}$ ($M_r = 34000$) was smaller than the light chain of factor C ($M_r = 43000$). In addition, a new fragment of factor $\bar{C}$ ($M_r = 8500$) was detected. Furthermore, the amino acid composition of factor $\bar{C}$ was essentially identical to that of factor C (data not shown).

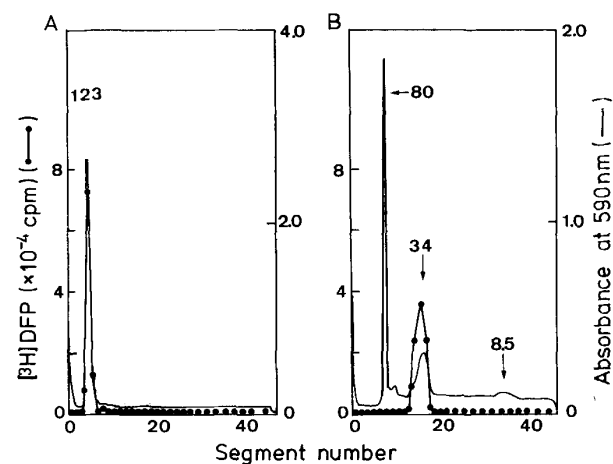


Fig. 9. Distribution of radioactivity of [^3H]Dip-F-labeled factor $\bar{C}$ on SDS-PAGE. (A) Nonreduced factor $\bar{C}$ (11 μg); (B) reduced factor $\bar{C}$ (11 μg). Experimental details are described in the supplement under Experimental Procedure. Peaks are labeled as $M_r \times 10^{-3}$.

Incorporation of [^3H]diisopropylfluorophosphate ([^3H]Dip-F) into the light chain

Factor $\bar{C}$ was treated with [^3H]Dip-F under the conditions described in Experimental Procedure (see supplement) and the resulting product was subjected to SDS-PAGE. As shown in Fig. 9A a single peak of radioactivity before reduction was found at the position corresponding to factor $\bar{C}$. Upon reduction the radioactive peak migrated to the position which corresponded to the mobility of the light chain, indicating that the Dip-F-reactive site is located in the light chain of $M_r = 34000$ (Fig. 9B). In parallel with this experiment, factor $\bar{C}$ was also treated with [^3H]Dip-F, but it did not bind Dip-F (data not shown).

The effect of inhibitors on factor $\bar{C}$

Table 3 shows the effect of naturally occurring and synthetic proteinase inhibitors on the amidase activity of factor $\bar{C}$. The amidase activity was strongly inhibited by antithrombin III, but not by α_2 -antiplasmin. Soybean trypsin inhibitor and hirudin had no inhibitory effect on factor $\bar{C}$ activity. Dip-F had a potent inhibitory effect among the three synthetic inhibitors tested. Benzamidine and leupeptin also had inhibitory activity at high concentrations (5 mM and 0.23 mM respectively), while ClHgHzOH had no apparent inhibitory effect. These results clearly indicate that factor $\bar{C}$ is a serine protease.

Substrate specificity of factor $\bar{C}$

The purified factor $\bar{C}$ activated factor B to factor $\bar{B}$ as described in the next section, but it did not activate Limulus proclotting enzyme [10] and various mammalian plasma coagulation factors, such as factor IX, factor X, prothrombin, plasminogen, protein C and prekallikrein. The mobility of these zymogens remained unchanged in SDS-PAGE after incubation with factor $\bar{C}$ (1/50, w/w, 37°C, 60 min). Moreover, no specific amidase activity appeared after the incubation of factor $\bar{C}$ with each zymogen, and factor $\bar{C}$ did not form any fibrin gel from fibrinogen after incubation (1/50, w/w, 37°C,

Table 3. Effect of various inhibitors on the activity of factor $\bar{C}$. The activity of factor $\bar{C}$ (0.05 μM) was measured using Boc-Val-Pro-Arg-Nan as a substrate as described in the supplement in Experimental Procedure. The inhibitory activity of antithrombin III was measured in the presence of heparin (12.5 units/ml)

Inhibitors	Concentration	Remaining activity
		%
None		100
Antithrombin III	0.14 μM	71
	0.27 μM	41
α_2 -antiplasmin	0.42 μM	100
Hirudin	15 ATU/ml	100
Benzamidine	5 mM	49
	10 mM	31
	20 mM	17
Dip-F	0.5 mM	1
	1.0 mM	0
ClHgBzOH	1.0 mM	100
	4.0 mM	95
Leupeptin	0.06 mM	88
	0.11 mM	80
	0.23 mM	66

60 min). In addition, factor $\bar{C}$ had no effect on coagulogen. These data indicate an extremely high specificity of factor $\bar{C}$ towards Limulus clotting factor B. Table 4 shows the amidase activity of factor $\bar{C}$ toward synthetic fluorogenic peptide substrates for factor Xa, α -thrombin, plasmin, activated protein C and kallikrein [13–16]. Of the peptide substrates examined at 0.2 mM substrate concentration, factor $\bar{C}$ hydrolyzed Bz-Thr-Ser-Arg-Mca most efficiently. Factor $\bar{C}$ possessed an apparent affinity for hydroxyamino acids at the P_2 (Ser) and P_3 site (Ser or Thr) (nomenclature of Schechter and Berger [17]). We also examined four peptidyl-Nan chromogenic substrates for comparison with Mca substrates. Factor $\bar{C}$ most strongly hydrolyzed Boc-Val-Pro-Arg-Nan (Table 4).

Evidence of factor $\bar{C}$ for involvement in the LPS-mediated initiation step

To elucidate the relationship among the Limulus coagulation factors, i.e. factor C, factor B [18] and proclotting enzyme [10], and to obtain evidence for factor C as the initiation factor, reconstitution experiments were performed using LPS as a trigger for the clotting reaction and Boc-Leu-Gly-Arg-Nan as a substrate for clotting enzyme generated in this reaction. The results are shown in Table 5. No amidase activity toward Boc-Leu-Gly-Arg-Nan was observed in the combination of proclotting enzyme and factor B (experiment 4) or factor C (experiment 5) and factors B and C (experiment 6). However, strong amidase activity was observed in the reaction mixture containing the above three components in the presence of LPS (experiment 7). These results indicate that three components are required for activation of proclotting enzyme. To further confirm this conclusion and to examine factor $\bar{C}$ as an initiation enzyme, the recombination experiments were performed using factor $\bar{C}$, factor B and proclotting enzyme in the absence of LPS (Table 6). A mixture of factor $\bar{C}$, factor B and proclotting enzyme produced strong amidase activity. However, a mixture of factor $\bar{C}$ and proclotting enzyme

Table 4. Amidase activity of factor $\bar{C}$ toward peptidyl-Mca and peptidyl-Nan substrates

The activity of factor $\bar{C}$ was measured under the conditions described in the supplement in Experimental Procedure against the peptidyl-Mca or peptidyl-Nan substrates at 0.2 mM or 0.4 mM, respectively. Amidase activity is given as the rate of AMC or *p*-nitroaniline release

Substrates	Amidase activity
	$\mu\text{mol min}^{-1} \text{mg}^{-1}$
Boc-Val-Pro-Arg-Mca	5.8
Boc-Asp-Pro-Arg-Mca	1.3
Boc-Leu-Gly-Arg-Mca	1.0
Z-Leu-Gly-Arg-Mca	1.0
Boc-Glu(OBzl)-Gly-Arg-Mca	3.4
Boc-Gln-Gly-Arg-Mca	1.4
Boc-Met-Gly-Arg-Mca	1.4
Boc-Gly-Ser-Arg-Mca	1.0
Bz-Ser-Ser-Arg-Mca	6.6
Bz-Thr-Ser-Arg-Mca	7.2
Bz-Ser-Thr-Arg-Mca	1.7
Bz-Thr-Thr-Arg-Mca	2.5
Boc-Val-Pro-Arg-Nan	19.7
Boc-Leu-Gly-Arg-Nan	11.8
Boc-Thr-Ser-Arg-Nan	7.7
Boc-Leu-Thr-Arg-Nan	1.1

Table 5. Recombination experiments with factor C, factor B and proclotting enzyme

The amidase activity of the clotting enzyme generated in these experiments was measured by the method described in the supplement in Experimental Procedure. The substrate for clotting enzyme was Boc-Leu-Gly-Arg-Nan (0.4 mM). Amidase activity is given as the rate of *p*-nitroaniline release

Experiments	Recombination system	Amidase activity	
		with LPS	without LPS
		nmol/min	
1	proclotting enzyme	0	0
2	factor B	0	0
3	factor C	0.1	0
4	proclotting enzyme + factor B	0	0
5	proclotting enzyme + factor C	0.5	0
6	factor B + factor C	0	0
7	proclotting enzyme + factor B + factor C	14.9	0

Table 6. Recombination experiments with factor $\bar{C}$, factor B and proclotting enzyme

The amidase activity of the clotting enzyme generated in these experiments was measured by the method described in the supplement in Experimental Procedure. The substrate for clotting enzyme was Boc-Leu-Gly-Arg-Nan (0.4 mM). Amidase activity is given as the rate of *p*-nitroaniline release

Experiments	Amidase activity
	nmol/min
1. Factor $\bar{C}$ + factor B + proclotting enzyme	5.53
2. Factor $\bar{C}$ + proclotting enzyme	0.58

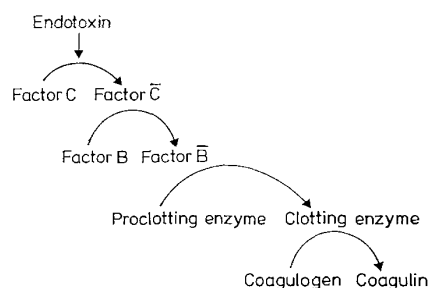


Fig. 10. A tentative mechanism for the coagulation cascade of the *Limulus* hemocyte lysate

showed weak amidase activity. These results indicate that all three components, factor C, factor B and proclotting enzyme, were required to activate proclotting enzyme, and that factor C is an initiation factor of the *Limulus* clotting system.

DISCUSSION

LPS has been reported to directly mediate the activation of proclotting enzyme resulting in the transformation of coagulogen to coagulin gel [19–21]. However, our recent studies have indicated that both hemocyte lysates from *Limulus polyphemus* and *Tachypleus tridentatus* contain another component, designated factor B, beside coagulogen and proclotting enzyme. All of these proteins are closely associated with the clotting system [5] and proclotting enzyme is insensitive to endotoxin [10]. In the present study we identified and separated an LPS-sensitive component, factor C, from a factor-B-containing fraction of dextran-sulfate–Sephacrose CL-6B. The reconstitution experiments demonstrate that factor C is an initiation factor for the activation of proclotting enzyme in the presence of factor B and LPS. The biochemical characterization of factor C shows that it is a glycoprotein with an apparent $M_r = 123000$, and that it consists of two polypeptide chains; a heavy chain of $M_r = 80000$ and a light chain of $M_r = 43000$. Factor C is converted to its activated form (factor $\bar{C}$) in the presence of LPS alone, while no activation occurs without addition of LPS. These data indicate that the activation of factor C is LPS-dependent and possible contaminants, such as a contaminating protease in the factor C preparation, is excluded. Furthermore, factor C binds to LPS to form a high-molecular-mass complex (T. Nakamura, unpublished observations). The activation process is accompanied by a change in the molecular mass of factor C, and the light chain of $M_r = 43000$ is converted to a species with $M_r = 34000$. Thus, limited proteolysis of the light chain of factor C occurs during the activation process. Factor $\bar{C}$ activates factor B to factor $\bar{B}$ with proclotting enzyme activator activity. On the basis of these results and our previous results [5, 10, 18] we propose a plausible mechanism for the coagulation cascade found in the hemocyte lysate of horseshoe crab (Fig. 10). In this mechanism, bacterial endotoxins induce the activation of factor C and its active form converts factor B to factor $\bar{B}$, which subsequently activates proclotting enzyme to clotting enzyme. This sequential activation of serine protease zymogens results in the transformation of coagulogen to coagulin gel. Therefore, this cascade system found in the hemocytes may impart an extremely high sensitivity of the lysate to nanogram quantities of endotoxins.

In *Limulus* the LPS-mediated clotting does take place in cell adhesion, aggregation, degranulation and gel formation, and locally occurs in response to damage. In addition the *Limulus* clotting system found in the hemocytes appears to be a complex amplification process comparable to the mammalian blood coagulation cascade. These properties of the *Limulus* hemocytes are very similar to those of mammalian monocytes and macrophages, which may play an important role in extravascular coagulation. It is known that monocytes produce tissue-factor-like activity when the cells are exposed with endotoxins [22]. Macrophages are also known to synthesize factor X and prothrombin [23, 24]. Therefore, a similar coagulation cascade, induced with bacterial endotoxins, may be contained within both *Limulus* hemocytes and mammalian monocytes. Because of the unique properties and the sensitivity of factor C to LPS with the concomitant activation to a serine protease, it is of great interest to investigate the zymogen activation mechanism of factor C by LPS. A study on the interaction of LPS and factor C may provide a new approach to increase our understanding of the activation mechanism of other serine protease zymogens.

This work was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan. We wish to express our thanks to Toshihiro Horiuchi for his valuable assistance in performing the study on several inhibitors of factor C, to Kazuko Usui-Kawaguchi for her skillful analyses of amino acids, and to Mizumo Akiyoshi for her expert secretarial assistance. We thank Dr W. Kisiel (Department of Pathology, University of New Mexico, Albuquerque, NM) for his help in preparing the English manuscript.

REFERENCES

- Levin, J. & Bang, F. B. (1964) *Bull. Johns Hopkins Hosp.* 115, 265–274.
- Reinhold, R. B. & Fine, J. (1971) *Proc. Soc. Exp. Biol. Med.* 137, 334–340.
- Harada, T., Morita, T., Iwanaga, S., Nakamura, S. & Niwa, M. (1979) *Prog. Clin. Biol. Res.* 29, 209–220.
- Takagi, K., Moriya, A., Tamura, H., Nakahara, C., Tanaka, S., Fujita, Y. & Kawai, T. (1981) *Thrombosis Res.* 23, 51–57.
- Ohki, M., Nakamura, T., Morita, T. & Iwanaga, S. (1980) *FEBS Lett.* 120, 217–220.
- Morita, T., Tanaka, S., Nakamura, T. & Iwanaga, S. (1981) *FEBS Lett.* 129, 318–321.
- Jackson, C. M. & Nemerson, Y. (1980) *Annu. Rev. Biochem.* 49, 765–811.
- Nakamura, S., Iwanaga, S., Harada, T. & Niwa, M. (1976) *J. Biochem. (Tokyo)* 80, 1011–1021.
- Takagi, T., Hokama, Y., Miyata, T., Morita, T. & Iwanaga, S. (1984) *J. Biochem. (Tokyo)* 95, 1445–1457.
- Nakamura, T., Morita, T. & Iwanaga, S. (1985) *J. Biochem. (Tokyo)* 97, 1561–1574.
- Nakamura, T., Morita, T. & Iwanaga, S. (1983) *Thromb. Haemostasis* 50, 405.
- Ferguson, K. A. (1964) *Metabolism* 13, 985–1002.
- Morita, T., Kato, H., Iwanaga, S., Takada, K., Kimura, T. & Sakakibara, S. (1977) *J. Biochem. (Tokyo)* 82, 1495–1498.
- Kato, H., Adachi, N., Ohno, S., Iwanaga, S., Takada, K. & Sakakibara, S. (1980) *J. Biochem. (Tokyo)* 88, 183–190.
- Iwanaga, S., Morita, T., Kato, H., Harada, T., Adachi, N., Sugo, T., Maruyama, I., Takada, K., Kimura, T. & Sakakibara, S. (1979) *Adv. Exp. Med. Biol.* 120A, 147–163.
- Ohno, Y., Kato, H., Morita, T., Iwanaga, S., Takada, K., Sakakibara, S. & Stenflo, J. (1981) *J. Biochem. (Tokyo)* 90, 1387–1395.
- Schechter, I. & Berger, A. (1967) *Biochem. Biophys. Res. Commun.* 27, 157–162.
- Horiuchi, T., Nakamura, T., Morita, T. & Iwanaga, S. (1984) *Seikagaku* 56, 891 (in Japanese).
- Tai, J. Y., Seid, R. C. Jr, Huhn, R. D. & Liu, T.-Y. (1977) *J. Biol. Chem.* 252, 4773–4776.
- Tai, J. Y. & Liu, T.-Y. (1977) *J. Biol. Chem.* 252, 2178–2181.
- Liang, S.-M. & Liu, T.-Y. (1982) *Biochem. Biophys. Res. Commun.* 105, 553–559.
- Schwartz, B. S., Levy, G. A., Curtiss, L. K., Fair, D. S. & Edgington, T. S. (1981) *J. Clin. Invest.* 67, 1650–1658.
- Østerud, B., Lindahl, U. & Seljelid, R. (1980) *FEBS Lett.* 120, 41–43.
- Lindahl, U., Kolset, S. O., Bøgwald, J., Østerud, B. & Seljelid, R. (1982) *Biochem. J.* 206, 231–237.
- Thaler, E. & Schmer, G. (1975) *Br. J. Haematol.* 31, 233–243.
- Holmberg, L., Bladh, B. & Astedt, B. (1976) *Biochim. Biophys. Acta* 445, 215–222.
- Bradford, M. M. (1976) *Anal. Biochem.* 72, 248–254.
- Reisfeld, R. A., Lewis, U. V. & Williams, D. E. (1962) *Nature (Lond.)* 195, 281–283.
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* 244, 4406–4412.
- Spackman, D. H., Stein, W. H. & Moore, S. (1958) *Anal. Chem.* 30, 1190–1206.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956) *Anal. Chem.* 28, 350–356.
- Warren, L. (1959) *J. Biol. Chem.* 234, 1971–1975.
- Skoza, L. & Mohos, S. (1976) *Biochem. J.* 159, 457–462.

Supplementary Material to
Lipopolysaccharide-sensitive serine-protease zymogen
(factor C) found in limulus hemocyte : Isolation and
characterization
by
Takanori NAKAMURA, Takashi MORITA and Sadaaki IWANAGA

EXPERIMENTAL PROCEDURE

Materials

Horseshoe crabs (*Tachypleus tridentatus*) were collected in Fukuoka prefecture and bled by cardiac puncture in the presence of caffeine (10 mM). The hemocytes were sedimented at 1,500 rpm for 10 min at 4°C, and the lysate was prepared according to the method previously described (10). Sepharose CL-6B, CM-Sepharose CL-6B, and marker proteins for the molecular weight determination were from Pharmacia Fine Chemicals, Uppsala. LPS prepared from *Escherichia coli* (*E. coli*) 0111:B4 was a product of List Biological Laboratories, Inc. Fluorogenic and chromogenic substrates were from the Protein Research Foundation, Minoh, Osaka. Soybean trypsin inhibitor and hirudin (1,000 antithrombin units/mg) were from Sigma Chemical Co., Ltd., St. Louis. Leupeptin sulfate was purchased from Nippon Kayaku Co., Ltd., Tokyo. Antithrombin III was prepared by a published method (25). Human α_2 -antiplasmin was a kind gift from Mr. T. Sasaki, Daiichi Pharmaceutical Co., Ltd., Tokyo. Benzamidine-CH-Sepharose CL-6B and benzamidine-CH-Sepharose 4B were prepared by the method of Holmberg *et al.* (26). Dextran sulfate-Sepharose CL-6B was prepared as described previously (10). [^{3} H] Dip-F, NCS Tissue solubilizer and ACS II were products of Amersham Co., USA. All other chemicals were of highest quality commercially available. The proclotting enzyme-containing fraction used for assay of factor B was partially purified by a dextran sulfate-Sepharose CL-6B column chromatography (10). This proclotting enzyme fraction did not possess any detectable levels of the activated form, factor B and factor C. The factor B-containing fraction used for recombination experiments (in Tables 5 and 6) was partially purified by a combination of dextran sulfate-Sepharose CL-6B, Sepharose CL-6B, and benzamidine-CH-Sepharose 4B column chromatography. This factor B-containing fraction had no detectable activated factor B and factor C.

Methods

Factor C assay

The amidase activity of factor C after activation in the presence of LPS was measured using Boc-Val-Pro-Arg-Nan as the substrate. A reaction mixture containing 10–20 μ l of sample, 20 μ l of 1 M Tris-HCl (pH 8.0) containing 50 mM $MgCl_2$, and 4 μ l of LPS (10 μ g/ml), in a total volume of 200 μ l, was preincubated at 37°C for 10 min. Then, 50 μ l of 2 mM Boc-Val-Pro-Arg-Nan was added, and this mixture was incubated further for 7 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 in a Hitachi 220 A spectrophotometer. One unit of the enzyme activity was defined as 1 μ mole of p-nitroaniline liberated per min.

The amidase activity of factor $\bar{C}$ was measured as that described for factor C with the exception that no LPS was added.

Factor B assay

Factor B activity was expressed as the amidase activity of the proclotting enzyme induced by activated factor B under the given conditions. A reaction mixture containing 10 μ l of sample, 20 μ l of 1 M Tris-HCl (pH 8.0) containing 50 mM $MgCl_2$, and 10 μ l of factor $\bar{C}$ (22 μ g/ml), in a total volume of 200 μ l, was preincubated at 37°C for 10 min to activate factor B. To the mixture, 20 μ l of 5 mM Boc-Leu-Gly-Arg-Nan and 30 μ l of partially purified proclotting enzyme (0.96 mg/ml) were added and incubated further for 3 min. Finally, 0.8 ml of 0.6 M acetic acid was added and the appearance of amidase activity was measured.

Determination of protein concentration

Protein concentration of factor C was determined from the absorbance at 280 nm. The extinction coefficient $E_{280}^{1\%} = 21.3$ for factor C was calculated from the amino acid and carbohydrate mass/ A_{280} . An internal standard, norleucine, was included in the protein hydrolysates to allow correction for losses. The same value was assumed for factor $\bar{C}$. The protein concentrations of other protein samples were determined according to Bradford (27), using bovine serum albumin as a standard protein.

Electrophoresis

PAGE was performed at pH 4.3 by the method of Reisfeld *et al.* (28). SDS-PAGE was performed according to Weber and Osborn (29). The gels were stained with Coomassie brilliant blue R-250.

Determination of molecular weight

The molecular weights of factor C, factor $\bar{C}$, and the heavy and light chains of factor C (or factor $\bar{C}$) were estimated using Ferguson plots from the relative mobilities of the samples on 4 %, 6 %, 8 % and 10 % gels, using heavy meromyosin ($M_r = 145,000$), phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100) and α -lactalbumin (14,400) as reference proteins.

Amino acid and carbohydrate analyses

Samples were hydrolyzed for 24, 48 and 72 h at 110°C in evacuated, sealed tubes with 100 μ l of 4 M methanesulfonic acid containing 0.2 % tryptamine. Excess 4 M methanesulfonic acid was neutralized by adding 200 μ l of 3.5 M NaOH followed by the addition of 200 μ l of 0.02 M HCl. Analyses were performed with a Hitachi model 835 amino acid analyzer according to Spackman *et al.* (30). Neutral sugar was determined by the phenol-sulfuric acid method of Dubois *et al.* (31), and neuraminic acid was determined by the thiobarbituric acid method (32) as modified by Skoza and Mohos (33).

The effect of factor $\bar{C}$ on several mammalian plasma coagulation factors

The possible activation of factor IX, factor X, prothrombin, fibrinogen, plasminogen, protein C, and plasma prekallikrein by purified factor $\bar{C}$ was examined. The activation reactions were performed with a weight ratio of factor $\bar{C}$ to plasma zymogen of 1 to 50. After incubation at 37°C for 60 min, aliquots of each

reaction mixture, except for plasma prekallikrein, were subjected to SDS-PAGE, in the presence and absence of 2-mercaptoethanol, in order to detect any possible structural change in the plasma zymogens. Other aliquots (except for factor IX and fibrinogen) were taken to determine the enzyme activities generated using specific fluorogenic substrates for factor Xa, α -thrombin, plasmin, activated protein C, and kallikrein. The substrates used here were Boc-Leu-Gly-Arg-Mca for factor Xa, Boc-Val-Pro-Arg-Mca for α -thrombin, Boc-Val-Leu-Lys-Mca for plasmin, Boc-Leu-Ser-Thr-Arg-Mca for activated protein C, and Z-Phe-Arg-Mca for kallikrein.

The effect of inhibitors on the activity of factor C

The effect of various inhibitors on the amidase activity of factor C was measured as follows. A reaction mixture containing 20 μ l of factor C (38 μ g/ml), 20 μ l of 1 M Tris-HCl (pH 8.0), 10 μ l of human serum albumin (10 mg/ml) and 150 μ l of various inhibitors, in a total volume of 200 μ l, was incubated at 37°C for 5 min. Then, 50 μ l of 2 mM Boc-Val-Pro-Arg-Nan was added and the amidase activity was measured.

Hydrolysis of peptidyl-Mca and -Nan substrates

A reaction mixture containing 50 μ l of 1 M Tris-HCl, pH 8.0, and 25 μ l of various Mca substrates (4 mM), in a total volume of 475 μ l, was incubated at 37°C in a cuvette. The reaction was started by adding 25 μ l (56 μ g/ml) of factor C, and the change of the fluorescence at 440 nm was measured following excitation at 380 nm in a Hitachi 650-10 M fluorescence spectrophotometer. For peptidyl-Nan substrates, a reaction mixture containing 20 μ l of 1 M Tris-HCl, pH 8.0, 10 μ l of human serum albumin (10 mg/ml), and 20 μ l of various Nan substrates (5 mM), in a total volume of 190 μ l, was preincubated at 37°C for 5 min. The reaction was started by the addition of 10 μ l of factor C (45 μ g/ml) to the reaction mixture, and then the amidase activity was measured.

Effects of pH on the stability of factor C and factor C, the amidase activity of factor C, and the activation of factor C

To examine the pH stability, factor C (30 μ g/ml) or factor C (77 μ g/ml) was incubated at 37°C for 6 h at pH 1.5-11.0. After incubation, the remaining amidase activity of factor C or factor C was measured at pH 8.0 as described above. The optimum pH of the amidase activity of factor C between pH 1.5-11.0 and the optimum pH for the activation of factor C by LPS between pH 4.0-11.0 were also examined. The activation of factor C by LPS (10 μ g/ml) was performed at 37°C for 60 min, and the amidase activity of factor C generated was measured at pH 8.0 using Boc-Val-Pro-Arg-Nan.

Purification of factor C from hemocyte lysate

All the following procedures were made under sterile conditions. The lysate (1,150 ml) was applied to a dextran sulfate-Sepharose CL-6B column (5.0 x 23.5 cm), and the clotting factors were eluted as shown in Fig. 1. The factor C-containing fractions indicated by a solid bar were collected and concentrated by Diaflo ultrafiltration. The concentrated fraction was applied to a Sepharose CL-6B column (4.0 x 127 cm), equilibrated with 0.02 M sodium acetate (pH 5.0)

containing 0.1 M NaCl. Elution was carried out at a flow rate of 60 ml/h. Factor C activity appeared in the first protein peak, as shown in Fig. 2. Fractions 69 to 82 were collected for further purification. The pooled factor C fraction was applied to a CM-Sepharose CL-6B column (2.0 x 25.5 cm), equilibrated with 0.02 M sodium acetate-0.1 M NaCl (pH 5.0). Factor C was eluted with a linear NaCl gradient (0.1 to 0.35 M) in 0.02 M sodium acetate (pH 5.0) (500 ml/chamber) at a flow rate of 72 ml/h. As shown in Fig. 3, one major protein peak appeared and factor C activity coincided with this peak.

Preparation and purification of factor C from hemocyte lysate

The lysate (624 ml) prepared from 58 g (wet weight) of hemocytes was applied to a dextran sulfate-Sepharose CL-6B column (5.0 x 23.5 cm). The elution of factor C was performed by the procedure described in Fig. 1. The pooled and concentrated factor C sample was applied to a Sepharose CL-6B column (3.5 x 122 cm), equilibrated with 0.05 M Tris-HCl (pH 8.0) containing 0.2 M NaCl. Elution was carried out with the same buffer at a flow rate of 55 ml/h. The fractions containing factor C activity were collected and NaCl was added to give a final concentration of 0.5 M. This sample was applied to a benzamidine-CH-Sepharose CL-6B column (1.5 x 14 cm), equilibrated with 0.02 M Tris-HCl-0.5 M NaCl (pH 8.0). The column was then washed with 100 ml of the equilibrating buffer. As shown in Fig. 7A, factor C was eluted in the unadsorbed fraction of the column under these conditions. The proteins adsorbed on the column were eluted in stepwise fashion sequentially with 0.02 M Tris-HCl-1.0 M NaCl (pH 8.0) (150 ml), 0.02 M Tris-HCl-2.0 M NaCl (pH 8.0) (100 ml), and finally 0.02 M Tris-HCl-2.0 M NaCl (pH 8.0) containing 1.0 M guanidine hydrochloride (100 ml) to elute any factor C present in the applied sample. To the factor C pool, MgCl₂ and LPS of *E. coli* O111:B4 were added to give a final concentration of 5 mM and 2 μ g/ml, and the mixture was incubated at 37°C for 4 h to activate factor C. This reaction mixture was applied to the same benzamidine-CH-Sepharose CL-6B column (1.5 x 14 cm), equilibrated with 0.02 M Tris-HCl-0.5 M NaCl (pH 8.0). The column was initially washed with the equilibrating buffer (100 ml) and subsequently with 0.02 M Tris-HCl-1.0 M NaCl (pH 8.0) (100 ml). Factor C was then eluted from the column with 0.02 M Tris-HCl-1.0 M NaCl (pH 8.0) containing 1.0 M guanidine hydrochloride, as shown in Fig. 7B. Factor C-containing fractions were collected and dialyzed against 0.02 M Tris-HCl-0.1 M NaCl (pH 8.0).

Separation of heavy and light chains of factor C

Factor C (4.4 mg) was reduced with dithiothreitol and alkylated with monoiodoacetamide in 5.5 ml of 0.5 M Tris-HCl (pH 8.0) containing 6 M guanidine hydrochloride and 2 mM EDTA (10). The resulting S-alkylated protein was desalted in a Sephadex G-25 column (2.5 x 18.5 cm), previously equilibrated with 0.1 M ammonium bicarbonate. Then, 200 μ l of S-alkylated factor C (64 μ g) was applied to a TMS-250 column equilibrated with 0.1 % trifluoroacetic acid. Elution was performed with a linear acetonitrile gradient (0-50 %) in 0.1 % trifluoroacetic acid at flow rate of 0.5 ml/min by Beckman HPLC system.

Activation of factor C in the presence of LPS

A reaction mixture containing 200 μ l of factor C (621 μ g/ml), 200 μ l of 0.05 M Tris-HCl (pH 8.0) / 0.25 % Tween-20, and 100 μ l of LPS (10 μ g/ml), were incubated at 37°C, and samples (5 μ l) of the mixture were removed at selected intervals and assayed for factor C activity. At the same time, a 50 μ l aliquot was subjected to SDS-PAGE in the presence or absence of 2-mercaptoethanol.

Treatment of factor C with [^{3} H]Dip-F

The purified factor C (22 μ g) in 100 μ l of 0.02 M Tris-HCl (pH 8.0) containing 0.1 M NaCl, was incubated with 5 μ l of 0.834 mM [^{3} H] Dip-F at 37°C for 30min. After incubation, SDS-PAGE analyses of the aliquots (50 μ l) were performed before and after reduction with 2-mercaptoethanol. The gels were stained with Coomassie brilliant blue R-250, destained, sliced into 1.5 mm segments, and the radioactivity of the gel slices was counted as described (10).

Recombination experiments of factor C, factor B and proclotting enzyme

A series of reaction mixtures containing either 5 μ l of purified factor C (14 μ g/ml), 20 μ l of partially purified factor B (200 μ g/ml), 30 μ l of partially purified proclotting enzyme (960 μ g/ml) or combinations of the above proteins, and 20 μ l of 1 M Tris-HCl (pH 8.0) with or without 4 μ l of LPS of *E. coli* 0111:B4 (10 μ g/ml), in a total volume of 200 μ l, was incubated at 37°C for 15 min. Then, 50 μ l of 2 mM Boc-Leu-Gly-Arg-Nan was added to the reaction mixture and incubated further at 37°C for 2 min. Finally, 0.8 ml of 0.6 M acetic acid was added to terminate the reaction, and the absorbance at 405 nm was measured.

Recombination experiments of factor C, factor B and proclotting enzyme

A series of reaction mixtures containing either 10 μ l of purified factor C (22 μ g/ml), 20 μ l of partially purified factor B (200 μ g/ml) and 20 μ l of partially purified proclotting enzyme (960 μ g/ml) or combined mixture, 20 μ l of 1 M Tris-HCl (pH 8.0) containing 50 mM MgCl₂, and 50 μ l of 2 mM Boc-Leu-Gly-Arg-Nan, in a total volume of 250 μ l, was incubated at 37°C for 4 min. Finally, 0.8 ml of 0.6 M acetic acid was added, and the absorbance at 405 nm was measured.

RESULTS

Effect of pH on the stability and the activation of factor C

The pH stability of factor C was estimated with the various buffer systems at 37°C. Factor C was stable at pH 4.0-10.0 (data not shown). The effect of pH on activation of factor C was also estimated using Boc-Val-Pro-Arg-Nan. The optimum pH for the activation of factor C in the presence of LPS was 7.2 (data not shown).

Effect of pH on the stability and the amidase activity of factor C

The pH stability of factor C was estimated with the various buffer systems at 37°C. Factor C was found to be stable between pH 1.5 and 10.0 (data not shown). The effect of pH on the amidase activity of factor C was also examined using Boc-Val-Pro-Arg-Nan. The optimum pH of the amidase activity of factor C was 8.5 (data not shown).