

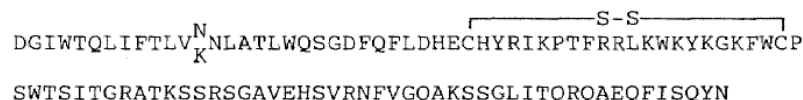
Primary Structure of Anti-Lipopolysaccharide Factor from American Horseshoe Crab, *Limulus polyphemus*¹

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The complete amino acid sequence of anti-lipopolysaccharide (LPS) factor purified from the hemocytes lysate of the American horseshoe crab, *Limulus polyphemus*, was determined by characterization of the NH₂-terminal sequence and the peptides generated after digestion of the protein with lysyl endopeptidase, clostripain, and *Staphylococcus aureus* V8 protease. Upon sequencing the peptides by the automated Edman method, the following primary structure was obtained:



During the sequence analysis, two species of the protein, which differed from each other at one locus, were found and characterized. *L. polyphemus* anti-LPS factor was a basic protein consisting of a single polypeptide chain of 101 residues and a calculated molecular weight of 11,786 or 11,800. The hydrophobic NH₂-terminal sequence and the clustering of positive charges found in the disulfide loop yielded a typical amphipathic character of this protein. Moreover, *L. polyphemus* anti-LPS factor showed 83% sequence identity with the *Tachypleus tridentatus* protein, and the sequence similar to that observed in the EF-hand structure was found to contain in the COOH terminal portions of these proteins, although its function is unknown.

The component of lipopolysaccharide (LPS) located in the cell surface of Gram-negative bacteria, has various biological activities, such as, pyrogenicity, adjuvant activity, activation of mac-

rophage and B lymphocyte, antitumor activity, and so on (1). It is also known that LPS induces the activation and degranulation of horseshoe crab hemocytes and results in the hemolymph coagula-

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Abbreviations: LPS, lipopolysaccharide; SV-8, *Staphylococcus aureus* V8 protease; HPLC, high performance liquid chromatography; PTH, phenylthiohydantoin.

tion (2, 3). This cellular event is thought to be one of the self-defense mechanisms of horseshoe crab. The recent studies on the limulus coagulation system indicate that it consists of the sequential activations of three serine protease zymogens and subsequent conversion of coagulogen, an invertebrate fibrinogen-like substance, to coagulin gel (4-8).

In 1982, a protein component that inhibits the coagulation cascade was found in the hemocyte lysate from Japanese (*Tachypleus tridentatus*) and American horseshoe crabs (*Limulus polyphemus*), and named anti-LPS factor (9). Afterward, this anti-LPS factor was purified as a single component from Japanese horseshoe crab, and it was shown that this protein specifically inhibits the LPS-mediated activation of limulus factor C (7) and has a strong anti-bacterial effect especially on the growth of Gram-negative R-type bacteria (10). Moreover, it was reported to have hemolytic activity on the red blood cells sensitized with LPS (11) and cytolytic activity on LPS-sensitized polymorphonuclear leukocytes, mononuclear cells, and human leukemia cells (12). The whole amino acid sequence of anti-LPS factor isolated from *T. tridentatus* was determined in 1986 and it was shown that this is a simple basic protein consisting of a single polypeptide chain with a molecular weight of 11,600 (13).

The present study was undertaken to determine the whole amino acid sequence of anti-LPS factor from other species of horseshoe crab, *L. polyphemus*, anticipating that the result may provide an information regarding the structure and function relationship of this protein.

EXPERIMENTAL PROCEDURES

Materials—Lysyl endopeptidase purified from *Achromobacter lyticus* M497-1 was purchased from Wako Pure Chemical Industries, Ltd., Osaka. Clostripain from *Clostridium histryticum* was from Sigma Chemical Co., Ltd., St. Louis, Mo. *Staphylococcus aureus* V8 protease was obtained from Miles Laboratories, Inc., Elkhart. A Cosmosil 5 C₁₈-P was purchased from Nakarai Chemicals, Ltd., Kyoto. A Chemcosorb 7-ODS-H column was from Chemco Scientific Co., Ltd., Osaka.

Purification from *L. polyphemus* Anti-LPS Factor and Its S-Pyridylethylation—The purifica-

tion procedures of anti-LPS factor were essentially the same as those described previously (10), and its reduction and S-pyridylethylation were previously described (13).

Enzymatic Digestions—The S-pyridylethylated sample (2.2 nmol) was digested with lysyl endopeptidase in 100 μ l of 50 mM Tris-HCl, pH 9.0, containing 4 M urea at 37°C for 24 h with the molar ratio of enzyme to substrate of 1 to 60 (14). For clostripain digestion, the S-pyridylethylated sample (2.2 nmol) was dissolved in 50 μ l of 50 mM Tris-HCl, pH 7.6, containing 4 M urea. Then, clostripain (0.2 mg/ml) dissolved in 20 mM CaCl₂ was added with the weight ratio of enzyme to substrate of 1 to 40 and 1 μ l of 2-mercaptoethanol was also added to the reaction mixture. After incubation at 37°C for 6 h, the same amount of the enzyme as used before was added and the incubation was continued further for 16 h. Final weight ratio of enzyme to substrate was 1 to 20.

The S-pyridylethylated sample (2.6 nmol) dissolved in 0.1 M NH₄HCO₃, pH 8.0, containing 2 M urea was also digested with SV-8. After addition of 3 μ g of the enzyme, incubation was performed at 37°C for 20 h. The reaction mixture was lyophilized and dissolved in 0.1 M NH₄HCO₃, pH 7.6, and then 3 μ g of the enzyme was added. After incubation at 37°C for 4 h, the same amount of the enzyme as used before was further added and incubated for 16 h. Final molar ratio of enzyme to substrate was 1 to 8.

Separation of Peptide Mixtures—Peptides obtained by proteolytic digestion were separated by reversed-phase HPLC, using the Beckman HPLC system Model 344 connected to a Gilson Model 201 fraction collector and a Linear Model 1200 recorder. Peptides were eluted by the gradient of acetonitrile containing 0.1% trifluoroacetic acid. A Chemcosorb 7-ODS-H column (2.1 $\times$ 150 mm) was used for lysyl endopeptidase peptides and clostripain peptides, and a Cosmosil 5C₁₈-P column (3.9 $\times$ 300 mm) for SV-8 peptides. One of the lysyl endopeptidase fraction, K-6, was rechromatographed with the Chemcosorb column, using 10 mM ammonium formate-acetonitrile system.

Amino Acid Analysis and Sequence Determination—Samples were hydrolyzed in 5.7 M HCl or 4 M methanesulfonic acid (15) in evacuated and sealed tubes at 110°C for 24 h. After evaporation, the hydrolyzates were analyzed on a Hitachi

835 automatic analyzer (16). Lysyl endopeptidase peptides and clostripain peptides were hydrolyzed in vapor of HCl at 110°C for 20 h. The hydrolyzates were analyzed by the phenylthiocarbamyl method (17) on the PICO-TAG system (Waters, Millipore Corp., Milford, Mass.) in conformity with the operator's manual.

Amino acid sequence was analyzed on a gas-phase sequencer (18), Applied Biosystems 470 A Protein Sequencer, connected to 120A PTH Analyzer and Spectra Physics SP 4270 Integrator.

Detection of Disulfide Bridge—The disulfide bond in the intact protein was detected by the method of Sueyoshi *et al.* (19).

Peptides Nomenclature—The following abbreviations were used for the various peptides: K, lysyl endopeptidase; Cl, clostripain; V8, *Staphylococcus aureus* V8 protease.

RESULTS

The purified anti-LPS factor isolated from the hemocyte lysate of *L. polyphemus* showed a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis either in the presence or absence of 2-mercaptoethanol (data not shown). Its apparent molecular weight estimated by the above method was approximately 15,000. The amino acid composition of anti-LPS factor is shown in Table I. No glucosamine and galactosamine were found.

The whole amino acid sequence of anti-LPS factor was determined by sequencing the S-alkylated protein and the peptides obtained by enzymatic cleavages of the protein with lysyl endopeptidase, clostripain, and SV-8. Automated Edman degradation of the intact protein resulted in the identification of 20 amino acid residues from the NH₂-terminal end, except for position 19 (Table IS). Both PTH-Asn and PTH-Lys were detected at position 13.

The S-alkylated protein (2.2 nmol) was digested with lysyl endopeptidase and the resulting peptide mixture was separated by reversed-phase HPLC (Fig. 1S). When the peptide K-6 was applied to the sequence analysis, two PTH-amino acids at every cycle were detected, indicating that the peptide contained two different peptides. Thus, the peptide K-6 was rechromatographed by the neutral pH buffer system, and the peptides K-6-1

TABLE I. Amino acid composition of S-pyridylethylated anti-LPS factor from *L. polyphemus*.

Amino acid	Analysis ^a (residues/molecule)	Sequence
Asp	7.2	3
Asn		4 (3) ^d
Thr	7.7	8
Ser	9.3	10
Glu	11.3	3
Gln		8
Pro	1.8	2
Gly	7.4	7
Ala	5.2	5
Val	4.2	4
Met	0	0
Ile	5.8	6
Leu	7.3	7
Tyr	3.0	3
Phe	7.0	7
Lys	7.3	7 (8) ^d
His	3.0	3
Trp	4.0 ^c	5
PeCys ^b	1.7	2
Arg	7.1	7
Total	100.3	101

^a The hydrolysis was performed in 5.7 M HCl for 24 h.

^b S-Pyridylethylcysteine. ^c The value from the hydrolysis in 4 M methanesulfonic acid for 24 h. ^d From the alternative sequence with a variant residue of Asn-13/Lys-13.

and K-6-2 were separated (data not shown). Finally, six major and two minor peptides were obtained in pure form and their amino acid compositions and sequence data are given in Tables IIS and IIIS, respectively. Through these procedures, all the peptides derived from the whole protein, except for a dipeptide corresponding Gly-48-Lys-49, were purified. The sum of the total residues of six major peptides and one additional glycine plus one additional lysine was in good agreement with that of the whole protein (Table I). The sequence analyses of eight peptides yielded 94% of the total sequence (95 out of 101 residues). The sequence of peptide K-11 was identical with

that of the NH₂-terminal portion of intact protein. As mentioned above, position 13 showed the microheterogeneity of Asn and Lys. The peptide K-11 contained Asn at position 13. On the other hand, the peptide K-9 corresponding Asn-14 to Lys-36 was also isolated, although the peptide corresponding to Asp-1-Lys-13 was not obtained. Apparently, the preparation contained two products derived from closely related genes or alleles.

To obtain overlaps and confirm the sequences of lysyl endopeptidase peptides, the *S*-alkylated protein (2.2 nmol) was digested with clostripain. The resulting peptide mixture was separated by reversed-phase HPLC (Fig. 2S) and five pure peptides were obtained. The amino acid composition and sequence analyses of these peptides are shown in Tables IVS and VS, respectively. The peptide Cl-5 overlapped the peptides K-5 and K-6-1. However, the other clostripain peptides did not overlap the lysyl endopeptidase peptides because of the cleavages of some of lysyl bonds.

To obtain further overlaps, the *S*-alkylated protein was digested with SV-8 and the digest was subjected to reversed-phase HPLC (Fig. 3S). Because the *S*-alkylated material was not dissolved in 2 M urea, the digestion did not occur completely. Thus, the digest was a mixture consisting of more than 10 peptides with various lengths. Among them, the peptides, V8-2, V8-3, V8-5, and

V8-7 were sequenced (Table VIS). The sequence of the peptide V8-7 was determined by Edman degradation up to 50 cycles, except for 19th, 44th, and 49th. This sequence corresponded to the NH₂-terminal portion of the whole protein and overlapped the peptides K-9, K-4, K-2, K-1, and K-6-2. The peptide V8-3 overlapped the peptides K-5 and K-6-1. V8-7 was further digested with lysyl endopeptidase and V8-7-K1 and V8-7-K2 were obtained (Table VIIS).

Even with the overlaps mentioned above, we still failed to obtain a complete overlap at the Lys-Ser (residues 64 and 65) bond. However, the whole amino acid composition determined by acid hydrolysis of this protein coincided well with that derived from the sequence data (Table I), and the amino acid sequence of this region was fairly well conserved in both proteins from *T. tridentatus* and *L. polyphemus* (Fig. 2), indicating that no other amino acid could be inserted between the Lys and Ser residues (residues 64 and 65).

The results described above permitted alignment of all the lysyl endopeptidase peptides and the whole amino acid sequence of *S*-alkylated anti-LPS factor, as shown in Fig. 1.

In this molecule two cysteine residues existed at the identical position with those of *T. tridentatus* anti-LPS factor. Free sulfhydryl groups were not detected in the absence of reducing reagent by the



Fig. 1. Amino acid sequence of anti-LPS factor from *L. polyphemus*. The residues marked with arrows are those identified by Edman degradation. Vertical lines represent the beginning and the end of the lysyl endopeptidase (K), clostripain (Cl), and SV-8 (V8) peptides, respectively. The peptides, for which the actual lengths are not known, give a dashed line and they do not have a vertical line at the COOH-terminal end.

method of Sueyoshi *et al.* (19), whereas they were detected in the presence of reducing reagent (data not shown). The result, therefore, indicated that these two cysteine residues must be linked with an intramolecular disulfide bridge as those of *T. tridentatus* anti-LPS factor.

DISCUSSION

L. polyphemus anti-LPS factor consists of a total of 101 amino acid residues with 2 half-cystines in a disulfide linkage and with two species as allo-types or isoproteins differing only at position 13. The molecular weight is therefore calculated to be 11,786 or 11,800.

The whole sequence analysis of this protein is provided by three overlapping sets of peptides. For lysyl endopeptidase digestion, we used a higher concentration of enzyme and longer incubation time than usual. As the result, the Lys-36-Pro-37 bond was cleaved significantly (Table IIS) and a slight cleavage of the Arg-76-Asn-77 bond was also found (data not shown). Although clostripain exhibited the expected primary specificity toward arginyl bonds, two lysyl bonds were cleaved apparently.

Three genera including four species of horseshoe crabs are living in the world. Out of four species, American horseshoe crab, *L. polyphemus*, is known to be evolutionarily distant from the other three Asian species, based on the immuno-chemical and structural studies of four coagulogens (20-24) and sialic acid binding lectins (25), in addition to their experimental hybridization (26). The sequence of *L. polyphemus* anti-LPS factor obtained here shows 83% sequence identity with that of Japanese horseshoe crab, *T. tridentatus* (13) (Fig. 2). This value is even high comparing with

that shown in their coagulogens (69%). As reported previously, coagulogen molecule has the lower homologous region consisting of the 28 amino acid residues long (named peptide C), which is cleaved off by a limulus clotting enzyme in its transformation to coagulin gel (27). If this region is ignored, the sequence homology of the remaining portion increases up to 73%, which become more comparable to the value obtained in this study. Nevertheless, the homology between two species of anti-LPS factors is high, which suggests that the entire molecule is closely related with the biological function.

Hydropathic characters of anti-LPS factors derived from *L. polyphemus* and *T. tridentatus* are given in Fig. 3. They show a typical amphipathic character, *i.e.* the NH₂-terminal region consisting of about 20 residues is highly hydrophobic and the remaining region contains positively charged residues and has relatively high content of serine. The region from Arg-41 to Lys-49 has basic amino acids at every second residues, and the region from Arg-61 to Arg-76 has them almost at every third residues. If these regions from Arg-41 to Lys-49 and from Arg-61 to Arg-76 make it possible to form β -sheet structure and α -helix, respectively, the positive charges would form a cluster at the same sides of the protein molecule. These two positive charge clusters may have an interaction with phosphate groups in the lipid A portion of LPS, and this character may be important to express the activity of this molecule for LPS-binding. Thus, it seems likely that anti-LPS factor interacts with LPS at the positively charged regions and perturbs a cell membrane structure at the NH₂-terminal hydrophobic region including up to approximately 27th residue. The region may have a sufficient length to go across a lipid bilayer like

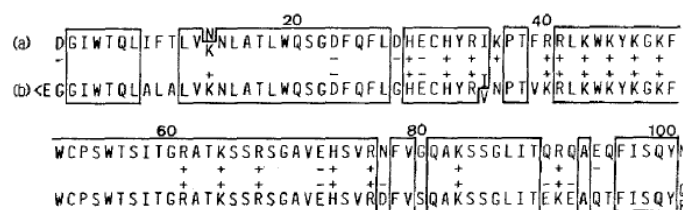


Fig. 2. Alignment of the sequences of anti-LPS factors from *L. polyphemus* (a) and *T. tridentatus* (b). Identical residues are boxed and charged residues are indicated by + or - between the two sequences.

transmembrane α -helices of bacteriorhodopsin molecule (35).

It has been reported that the amino acid sequence of *T. tridentatus* anti-LPS factor shows the homology with α -lactalbumin/lysozyme family (13). *L. polyphemus* anti-LPS factor also shows almost equivalent homology. Since α -lactalbumin has a high affinity Ca^{2+} binding site (28), we searched the potential Ca^{2+} binding site along the polypeptide chain of anti-LPS factor and found the existence of the sequence similar to that observed in the EF-hand structure (29), as shown in Fig. 4. Since the regions from Glu-72 to Tyr-100 and from Gln-90 to Asn-101 gave the EF-hand alignment score, 9 and 6, respectively, the regions from Gln-81 to Gln-92 and from Gln-90 to Asn-101 are thought to be a Ca^{2+} -binding loop (30, 31).

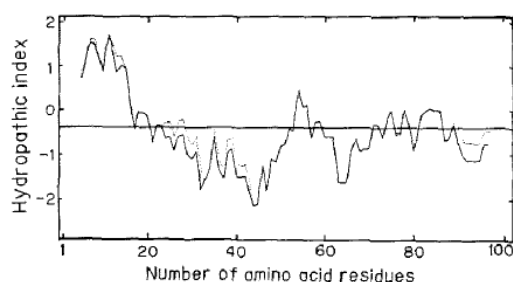


Fig. 3. Hydropathy profiles of anti-LPS factors from *L. polyphemus* (—) and *T. tridentatus* (13) (.....). Each hydropathic index is a mean hydropathy index value of 9 successive residues (34).

The latter loop, however, gave lower alignment score, because the polypeptide chain is terminated at Asn-101 so that there is no tail to form α -helix. In general, the scores of the region of other proteins which are known to actually bind Ca^{2+} are between 12 and 16. Our preliminary test on the Ca^{2+} binding ability of anti-LPS factor according to the method of Maruyama *et al.* (32) indicated that the dot blotted protein on a nitrocellulose membrane is not able to bind $^{45}\text{Ca}^{2+}$, although bovine α -lactalbumin used for the control can bind Ca^{2+} . Since anti-LPS factor contains lysine at the position Y (29), where is known to have an oxygen atoms as a Ca^{2+} ligand in other Ca^{2+} binding proteins, it might lose the high affinity Ca^{2+} binding site. There is a possibility, however, that the anti-LPS factor is an ancestral protein before duplications of EF-hand structure with Ca^{2+} binding, such as calmodulin and troponin C, which have four EF-hand structures. Recently, Stuart *et al.* have reported that the high affinity Ca^{2+} binding site in α -lactalbumin exists in the region from Lys-79 to Asp-88 (33), which indicates a novel form of the Ca^{2+} binding site. The amino acid sequence of the Ca^{2+} binding site found in α -lactalbumin is not homologous to the corresponding sequence of anti-LPS factor. Thus, it might be reasonable that anti-LPS factor does not bind Ca^{2+} .

	----- α -helix-----	--- Ca^{2+} binding loop---	----- α -helix-----	Score
TEST	E L - - L L - - L O - O - O G - L O - - O L - - L L - - L			
(a)	Ⓔ H S V R N F V G Ⓔ A K S Ⓔ Ⓒ L Ⓔ T Q R Ⓔ A E Q Ⓔ F Ⓔ S Q Y			9
(b)	Ⓔ H S V R D F V S Ⓔ A K S Ⓔ Ⓒ L Ⓔ T E K Ⓔ A Q T Ⓔ F Ⓔ S Q Y			9
(c)	Q A K S S G L I T Ⓔ R Ⓔ A Ⓔ Q F Ⓔ I Ⓔ S Q Y Ⓔ N			6
(d)	Q A K S S G L I T Ⓔ K Ⓔ A Ⓔ T F Ⓔ I Ⓔ S Q Y Ⓔ E			6

Fig. 4. EF-hand like structure found in anti-LPS factors. The sequences of anti-LPS factors from *L. polyphemus*, (a) residues 72–100 and (c) residues 81–101, and from *T. tridentatus* (13), (b) residues 73–101 and (d) residues 82–102 are aligned by their correspondence to the test sequence (TEST) (30, 31) to search EF-hand structures. Residues corresponding to the test sequence are circled and scored one point. The sum of scores are shown in the right. The abbreviations used for the test sequence are as follows: O, oxygen containing amino acids (D, N, E, Q, S, T); L, hydrophobic amino acids (L, I, V, F, M); G, glycine; E, glutamic acid.

J. Biochem.

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REFERENCES

1. Galanos, C., Freudenberg, M.A., Lüderitz, O., Rietschel, E.T., & Westphal, O. (1979) *Prog. Clin. Biol. Res.* **29**, 321-332
2. Levin, J. & Bang, F.B. (1964) *Bull. Johns Hopkins Hosp.* **115**, 265-274
3. Ornberg, R.L. & Reese, T.S. (1979) *Prog. Clin. Biol. Res.* **29**, 125-130
4. Nakamura, S., Morita, T., Harada-Suzuki, T., Iwanaga, S., Takahashi, K., & Niwa, M. (1982) *J. Biochem.* **92**, 781-792
5. Nakamura, T., Morita, T., & Iwanaga, S. (1985) *J. Biochem.* **97**, 1561-1574
6. Nakamura, T., Horiuchi, T., Morita, T., & Iwanaga, S. (1986) *J. Biochem.* **99**, 847-857
7. Nakamura, T., Morita, T., & Iwanaga, S. (1986) *Eur. J. Biochem.* **154**, 511-521
8. Nakamura, S., Iwanaga, S., Harada, T., & Niwa, M. (1976) *J. Biochem.* **80**, 1011-1021
9. Tanaka, S., Nakamura, T., Morita, T., & Iwanaga, S. (1982) *Biochem. Biophys. Res. Commun.* **105**, 717-723
10. Morita, T., Ohtsubo, S., Nakamura, T., Tanaka, S., Iwanaga, S., Ohashi, K., & Niwa, M. (1985) *J. Biochem.* **97**, 1611-1620
11. Ohashi, K., Niwa, M., Nakamura, T., Morita, T., & Iwanaga, S. (1984) *FEBS Lett.* **176**, 207-210
12. Aketagawa, J., Miyata, T., Nakamura, T., Morita, T., Iwanaga, S., Ohashi, K., Niwa, M., Takao, T., & Shimonishi, Y. (1985) *Proceedings of the 28th Symposium on Toxins* (in Japanese) pp. 123-129, Tokyo
13. Aketagawa, J., Miyata, T., Ohtsubo, S., Nakamura, T., Morita, T., Hayashida, H., Miyata, T., Iwanaga, S., Takao, T., & Shimonishi, Y. (1986) *J. Biol. Chem.* **261**, 7357-7365
14. Masaki, T., Fujihashi, T., Nakamura, K., & Soejima, M. (1981) *Biochim. Biophys. Acta* **660**, 51-55
15. Simpson, R.J., Neuberger, M.R., & Liu, T.-Y. (1976) *J. Biol. Chem.* **251**, 1936-1940
16. Spackman, D.H., Stein, W.H., & Moore, S. (1958) *Anal. Chem.* **30**, 1190-1206
17. Bidlingmeyer, B.A., Cohen, S.A., & Tarvin, T.L. (1984) *J. Chromatogr.* **336**, 93-104
18. Hewick, R.M., Hunkapiller, M.W., Hood, L.E., & Dreyer, W.J. (1981) *J. Biol. Chem.* **256**, 7990-7997
19. Sueyoshi, T., Miyata, T., Iwanaga, S., Toyo'oka, T., & Imai, K. (1985) *J. Biochem.* **97**, 1811-1813
20. Nakamura, S., Shishikura, F., Iwanaga, S., Takagi, T., Takahashi, K., Niwa, M., & Sekiguchi, K. (1980) in *Frontiers in Protein Chemistry* (Liu, T.-Y., Mamiya, G., & Yasunobu, K.T., eds.) Developments in Biochemistry, Vol. 10, pp. 495-514, Elsevier North-Holland, Amsterdam
21. Takagi, T., Hokama, Y., Miyata, T., Morita, T., & Iwanaga, S. (1984) *J. Biochem.* **95**, 1445-1457
22. Miyata, T., Hiranaga, M., Umezumi, M., & Iwanaga, S. (1984) *J. Biol. Chem.* **259**, 8924-8933
23. Miyata, T., Usui, K., & Iwanaga, S. (1984) *J. Biochem.* **95**, 1793-1801
24. Srimal, S., Miyata, T., Kawabata, S., Miyata, T., & Iwanaga, S. (1985) *J. Biochem.* **98**, 305-318
25. Dorai, D.T., Bachhawat, B.K., Bishayee, S., Kannan, K., & Rao, D.R. (1981) *Arch. Biochem. Biophys.* **209**, 325-333
26. Sekiguchi, K. & Sugita, H. (1980) *Evolution* **34**, 712-718
27. Nakamura, S., Takagi, T., Iwanaga, S., Niwa, M., & Takahashi, K. (1976) *J. Biochem.* **80**, 649-652
28. Hiraoka, Y., Segawa, T., Kuwajima, K., Sugai, S., & Murai, N. (1980) *Biochem. Biophys. Res. Commun.* **95**, 1098-1104
29. Kretsinger, R.H. & Nockolds, C.E. (1973) *J. Biol. Chem.* **248**, 3313-3326
30. Tufty, R.M. & Kretsinger, R.H. (1975) *Science* **187**, 167-169
31. Kretsinger, R.H. (1979) *Adv. Cyclic Nucleotide Res.* **11**, 1-26
32. Maruyama, K., Mikawa, T., & Ebashi, S. (1984) *J. Biochem.* **95**, 511-519
33. Stuart, D.I., Acharya, K.R., Walker, N.P.C., Smith, S.G., Lewis, M., & Phillips, D.C. (1986) *Nature* **324**, 84-87
34. Kyte, J. & Doolittle, R.F. (1982) *J. Mol. Biol.* **157**, 105-132
35. Engelman, D.M., Henderson, R., McLachlan, A.D., & Wallace, B.A. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2023-2027

Supplemental Materials

Table IS. NH₂-terminal sequence of anti-LPS factor

Cycle No.	Amino acid	Yield (pmol)
1	Asp	199
2	Gly	206
3	Ile	334
4	Trp	136
5	Thr	154
6	Gln	198
7	Leu	231
8	Phe	233
9	Thr	273
10	Thr	114
11	Leu	276
12	Val	198
13 ^a	Asn, Lys	61, 88
14	Asn	95
15	Leu	246
16	Ala	139
17	Thr	65
18	Leu	136
19	X ^b	
20	Gln	66
Repetitive Yield (%)		92.2 (Leu-7-Leu-18)
Position		1-20

^aTwo amino acids were detected.^bNot identified.Table IIS. Amino acid compositions of lysyl endopeptidase peptides^a

Amino acid	K-11	K-2	K-1	K-6-2	K-5	K-6-1	(minor peptides) K-9	K-4
Residues/molecule								
Asp	4.4 (5)				1.0 (1)	1.0 (1)	2.8 (3)	0.3 (0)
Glu	4.1 (4)				2.1 (2)	5.0 (5)	2.7 (3)	0.3 (0)
Ser	1.4 (1)	0.4 (0)		2.0 (2)	3.9 (4)	2.8 (3)	1.1 (1)	0.4 (0)
Gly	2.7 (2)			1.1 (1)	2.3 (2)	1.0 (1)	1.2 (1)	0.4 (0)
His	2.0 (2)				0.9 (1)		1.5 (2)	
Arg	3.1 (3)			1.1 (1)	2.0 (2)	1.0 (1)	1.0 (1)	1.5 (2)
Thr	3.6 (4)			3.0 (3)			1.0 (1)	0.7 (1)
Ala	1.3 (1)			1.1 (1)	2.1 (2)	1.0 (1)	1.1 (1)	0.3 (0)
Pro	1.2 (1)			1.0 (1)			0.3 (0)	0.7 (1)
Tyr	1.2 (1)		1.2 (1)			0.8 (1)	0.9 (1)	
Val	1.2 (1)				3.0 (3)			0.4 (0)
Met							0.6 (1)	
PeCys ^b	0.5 (1)			0.3 (1)			0.9 (1)	
Ile	2.8 (3)			1.0 (1)		1.8 (2)	2.9 (3)	0.7 (1)
Leu	6.2 (6)					1.0 (1)	2.9 (3)	1.1 (1)
Phe	4.1 (4)			0.9 (1)	1.0 (1)	0.9 (1)	2.2 (2)	1.1 (1)
Trp	N.D. ^c (2)	N.D. ^c (1)		N.D. ^c (2)			N.D. ^c (1)	
Lys	1.9 (2)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)		1.0 (1)	0.8 (1)
Total	43	2		15		18	23	7
Position	1-43	44-45	46-47	50-64	65-83	84-101	14-36	37-43
Yield (%)	32	58	56	79	64	95	16	32

^aValues in parenthesis are from sequence data.^bS-pyridylethylcysteine.^cNot determined.

Table IIIS. Amino acid sequence of lysyl endopeptidase peptides

Cycle No.	K-11	K-9	K-4	K-2	K-1	K-6-2	K-5	K-6-1
Amino acid (pmol)	Amino acid (pmol)	Amino acid (pmol)	Amino acid (pmol)	Amino acid (pmol)	Amino acid (pmol)	Amino acid (pmol)	Amino acid (pmol)	Amino acid (pmol)
1	Asp 267	Asn 167	Pro 157	Trp 541	Tyr 209	Phe 312	Ser 142	X
2	Gly 272	Leu 237	Thr 49	Lys 403	Lys 99	Trp 193	Ser 106	Ser 22
3	Ile 348	Ala 190	Phe 111			PeC N.Q.	Arg 155	Gly 142
4	Trp 145	Thr 71	Arg 159			Pro 110	Ser 70	Leu 281
5	Thr 145	Leu 263	Arg 192			Ser 48	Gly 170	Ile 208
6	Gln 268	Trp 67	Leu 63			Trp 124	Ala N.Q.	Thr 59
7	Leu 227	Gln 158	Lys 42			Thr 53	Val 274	Gln 100
8	Ile 218	X ^a				Ser 28	Glu 110	Arg 41
9	Phe 180	Gly 132				Ile 92	His N.Q.	Gln 88
10	Thr 61	Asp 30				Ser 37	Ala 144	
11	Leu 192	Phe 127				Gly 61	Val 164	Glu 68
12	Val 159	Gln 80				Arg 78	Arg 129	Gln 90
13	Asn 109	Phe 112				Ala 53	Asn 75	Phe 143
14	Asn 150	Leu 106				Thr 18	Phe 170	Ile 124
15	Leu 140	Asp 42				Lys 24	Val 137	Ser 17
16	Ala 142	X					Gly 78	Gln 66
17	Thr 37	Glu 59 ^c					Gln 62	Tyr 73
18	Leu 95	PeC ^b N.Q. ^c					Ala N.Q.	Asn 34
19	X						Lys 51	
20		Tyr 73						
21		Arg N.Q.						
22		Ile 55						
23		Lys 32						
Repetitive Yield (%)		92.4 (Leu-7-Leu-18)	93.5 (Leu-2-Leu-14)				91.7 (Val-7-Val-15)	94.4 (Ile-5-Ile-14)
Position		1-18	14-36	37-43	44-45	46-47	50-64	65-83

^aNot identified.^bS-pyridylethylcysteine.^cNot quantitated.Table IVS. Amino acid compositions of clostripain peptides^a

Amino acid	Cl-3	Cl-1	Cl-6	Cl-2	Cl-5	Cl-4
Residues/molecule						
Asp					1.0 (1)	1.2 (1)
Glu		0.3 (0)	0.3 (0)	0.9 (1)	2.2 (2)	4.1 (4)
Ser	0.3 (0)	0.4 (0)	1.8 (2)	1.8 (2)	1.9 (2)	1.1 (1)
Gly	0.3 (0)	1.2 (1)	1.2 (1)	1.0 (1)	1.9 (2)	
His				0.7 (1)		
Arg	1.0 (1)		1.0 (1)	1.0 (1)		
Thr	0.9 (1)		1.6 (2)		0.9 (1)	
Ala				1.0 (1)	1.1 (1)	1.1 (1)
Pro	0.9 (1)		0.8 (1)			
Val				1.9 (2)	0.3 (0)	0.9 (1)
Met ^b		0.3 (0)			0.9 (1)	
PeCys ^b			0.3 (1)			
Ile	0.9 (1)		0.9 (1)		1.1 (1)	1.0 (1)
Leu					1.1 (1)	
Phe	0.8 (1)			0.8 (1)	1.3 (1)	1.0 (1)
Trp			N.D. ^c (2)			
Lys	0.9 (1)	0.8 (1)			0.9 (1)	
Total	6	2	12	9	15	10
Position	35-40	48-49	50-61	68-76	77-91	92-101
Yield (%)	31	12	45	70	24	50

^aValues in parenthesis are from sequence data.^bS-pyridylethylcysteine.^cNot determined.

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Table VS. Amino acid sequence of clostripain peptides

Cycle No.	Cl-1		Cl-6		Cl-2		Cl-5		Cl-4	
	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)
1	Gly	87	Phe	299	Ser	287	Asn	101	Gln	176
2	Lys	23	Trp ^a	117	Gly	463	Phe	252	Ala	103
3			Val	164	Ala	688	Val	235	Glu	123
4			Pro	164	Val	490	Gly	142	Gln	89
5			Ser	59	Glu	319	Gln	108	Phe	139
6			Trp	68	His	111	Ala	288	Ile	296
7			Thr	40	Ser	92	Lys	150	Ser	63
8			Ser	44	Val	205	Ser	42	Gln	132
9			Ile	91			Ser	42	Tyr	118
10			Thr	23			Gly	81	Asn	105
11			Gly	52			Leu	118		
12			Arg	15			Ile	100		
13							Thr	32		
14							Gln	73		
Repetitive Yield (%)			90.7 (Ser-5-Ser-8)		80.4 (Val-4-Val-8)		95.7 (Gln-5-Gln-14)		96.0 (Gln-1-Gln-8)	
Position		48-49	50-61		68-75		77-90		92-101	

^aNot identified.Table VIS. Amino acid sequence of *S. aureus* V8 protease peptides

Cycle No.	V8-7		V8-5		V8-3		V8-2	
	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)
1	Asp	1657	PeC	N.Q.	His	268	Gln	406
2	Gly	1529	His	34	Ser	205	Phe	235
3	Ile	960	Tyr	71	Val	N.Q.	Ile	182
4	Trp	832	X	X	X	Ser	N.Q.	
5	Thr	965	Ile	89	X	Gln	145	
6	Gln	763	Lys	77	Phe	N.Q.	Tyr	201
7	Leu	880	Pro	63	Val	493	Asn	113
8	Ile	664	Thr	57	Gly	358		
9	Phe	744	Phe	69	Gln	189		
10	Thr	802	Arg	40	Ala	412		
11	Leu	774	Arg	60	Lys	418		
12	Val	585	Leu	45	X			
13 ^a	Asn, Lys	449, 282	Lys	30	X			
14	Asn	633	Trp	N.Q.	Gly	101		
15	Leu	644	Lys	21	Leu	164		
16	Ala	718	Tyr	15	Ile	132		
17	Thr	416	Lys	17	Thr	155		
18	Leu ^b	534	Gly	34	Gln	32		
19	X	X	Lys	17	Arg	55		
20	Gln	339	Phe	16	Gln	99		
21	Ser	226	Trp	N.Q.	Ala	81		
22	Gly	349	PeC	N.Q.	Glu	32		
23	Asp	158	Pro	12				
24	Phe	283						
25	Gln	240						
26	Phe	271						
27	Leu	235						
28	Asp	152						
29	His	69						
30	Glu	103						
31	PeC ^c	N.Q.						
32	His	59						
33	Tyr	108						
34	Arg	106						
35	Ile	103						
36	Lys	258						
37	Pro	56						
38	Thr	105						
39	Phe	95						
40	Arg	76						
41	Arg	94						
42	Leu	89						
43	Lys	164						
44	X	X						
45	Lys	170						
46	Tyr	33						
47	Lys	162						
48	Gly	104						
49	X	X						
50	Phe	57						
Repetitive Yield (%)		93.7 (Leu-7-Leu-42)	90.2 (Pro-7-Pro-23)		94.3 (Gln-9-Gln-20)			
Position		1-50	31-53		73-94		95-101	

^aTwo amino acids were detected.^bNot identified.^cS-pyridylethylcysteine.^dNot quantitated.

Table VIIS. Amino Acid Sequence of V8-3-K1 and V8-3-K2

Cycle No.	V8-3-K2		V8-3-K1	
	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)
1	His	50	Ser	232
2	Ser	56	Ser	163
3	Val	224	Gly	363
4	Arg	105	Leu	441
5	Asn	75	Ile	336
6	Phe	162	Thr	174
7	Val	117	Gln	208
8	Gly	70	Arg	297
9	Gln	62	Gln	248
10	Ala	82	Ala	N.Q. ^a
11	Lys	45	Glu	142
Repetitive Yield (%)		85.0 (Val-3-Val-7)		
Position		73-83	84-94	

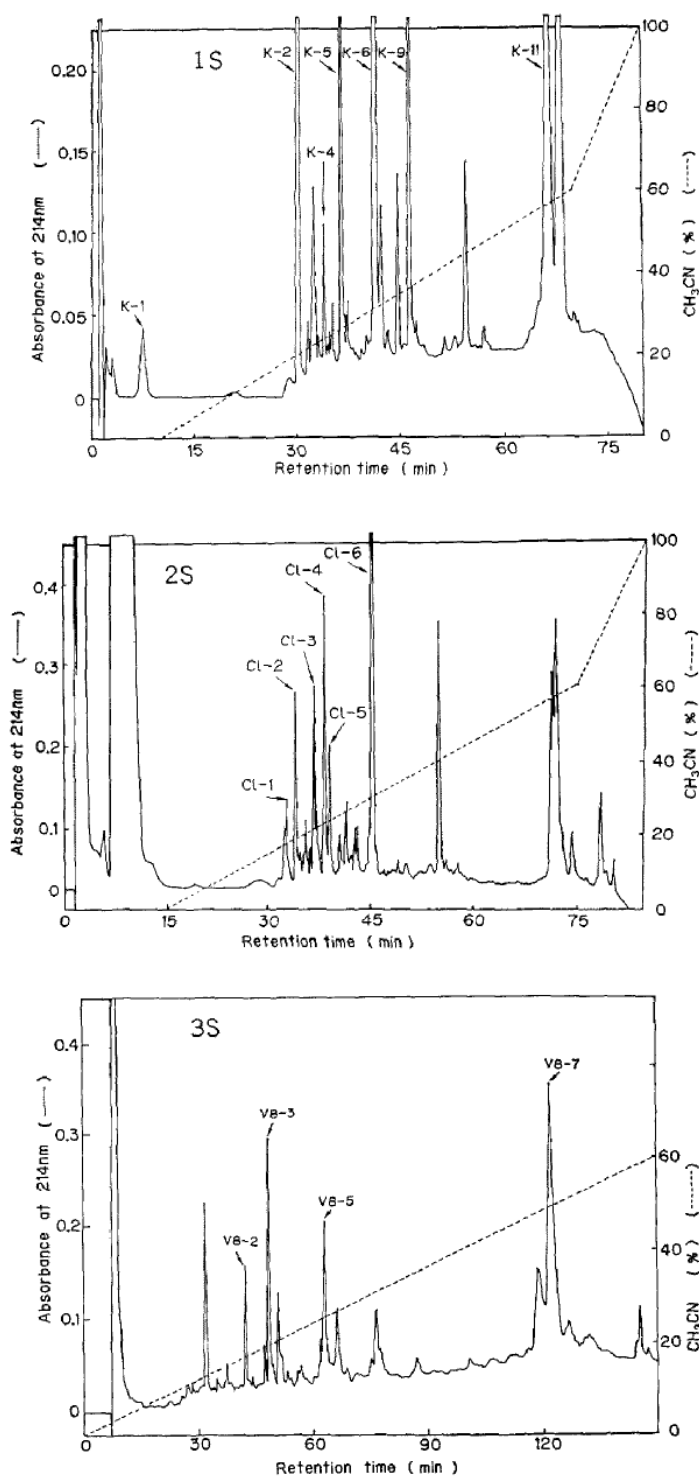
^aNot quantitated.

LEGENDS TO SUPPLEMENTAL FIGURES

Fig. 1S. Separation of lysyl endopeptidase digest of *S*-pyridylethylated anti-LPS factor by reversed-phase HPLC. A 100 μ l of the digest was applied to a Chemcosorb 7-ODS-H column (2.1 $\times$ 150 mm). Flow rate was 0.5 ml/min. For other conditions, see EXPERIMENTAL PROCEDURES.

Fig. 2S. Separation of clostripain digest of *S*-pyridylethylated anti-LPS factor by reversed-phase HPLC. A 50 μ l of the digest was applied to a Chemcosorb 7-ODS-H column (2.1 $\times$ 150 mm). Flow rate was 0.5 ml/min. For other conditions, see EXPERIMENTAL PROCEDURES.

Fig. 3S. Separation of SV-8 digest of *S*-pyridylethylated anti-LPS factor by reversed-phase HPLC. A 100 μ l of the digest was applied to a Cosmosil SC₁₈-P column (3 $\times$ 300 mm) after addition of 90 μ l of 6 M guanidine-HCl to dissolve insoluble material. Flow rate was 0.5 ml/min. For other conditions, see EXPERIMENTAL PROCEDURES.

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