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STUDIES ON THE PROTEIN MOIETY OF ENDOTOXIN FROM
GRAM-NEGATIVE BACTERIA:
CHARACTERIZATION OF THE ENZYMATICALLY DEGRADED PROTEIN MOIETY
ISOLATED BY PHENOL TREATMENT OF ENDOTOXIN FROM
S. marcescens 08

W. Wober and P. Alaupovic

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Technical Report No. 29
University of Oklahoma Medical Center THEMIS Contract

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August 17, 1970

Research sponsored by the Office of Naval Research
Contract N00014-68-A-0496
Project NR 105-516

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Abstract

The "simple" protein of endotoxin from Serratia marcescens O8 was treated successively with trypsin and pronase. The resulting trypsin and pronase cores contained increasing amounts of lipid A constituents firmly bound to the residual protein moiety. Since lipid A was separated as an entity from the protein moiety only after acid hydrolysis of the pronase core, it is proposed that in intact endotoxin lipid A is covalently linked to the protein moiety. The absence of any detectable glucosamine and fatty acids in the mixture of peptides and amino acids released by trypsin and pronase, indicated a single point attachment between the lipid A and protein moieties. Both trypsin and pronase cores were immunogenic and revealed the presence of a common antigenic determinant with the parent "simple" protein. The second set of antigenic determinant(s) different from the specific O-antigens is located in the protein moiety close to lipid A.

Although it is recognized that the protein moiety constitutes an integral part of the structure of undegraded endotoxin preparations, its site and mode of attachment to the lipopolysaccharide moiety is unknown (1, 2). It has been established in the preceding paper of this series (3) that the so-called "simple" proteins isolated by phenol treatment of endotoxins from S. marcescens 08 and E. coli O 141:K85(B) contain also lipid A constituents such as glucosamine, phosphorus and β -hydroxy myristic acid as the major fatty acid. Since these compounds were not separable from the protein moiety by solvent extraction or agarose electrophoresis, it was concluded that at least a portion of lipid A is firmly bound to the protein moiety. To explore further the mode of linkage between the protein moiety and lipid A constituents, "simple" protein from S. marcescens 08 was treated successively by proteolytic enzymes. The resulting tryptic and pronase cores contained increasing amounts of lipid A constituents firmly bound to the residual protein. Since lipid A was removed only

after acid hydrolysis of cores, we propose that the protein moiety is covalently linked to lipid A.

MATERIALS AND METHODS

Enzymes

Trypsin was a 2 X crystallized preparation treated with diisopropyl phosphofluoridate (DFP) and was purchased from Worthington (Freehold, N. J.). Pronase, B grade, from Streptomyces griseus was obtained from Calbiochem, Los Angeles, Calif. DFP-treated Carboxypeptidase A was purchased from Worthington, Freehold, N. J.

Isolation of "Simple" Proteins

"Simple" proteins of endotoxins from S. marcescens 08 (PX-S) and E. coli O 141:K85(B) (PX-E) were isolated according to a procedure previously described (3).

Preparation of the Tryptic and

Pronase Cores of PX-S

Fifty milligrams of PX-S were suspended in 15 ml 0.02 M ammonium carbonate buffer, pH 8.6, heated for 30 seconds in a

boiling water bath and cooled in an ice bath to 37°. Trypsin, dissolved in a small volume of the same buffer, was added to the suspended protein (trypsin/protein, 1:100, w/w) and the reaction mixture was stirred gently for 12 hours at 37° (digestion was completed after 8-10 hours, as determined photometrically by the ninhydrin reaction). After hydrolysis the clear reaction mixture was heated again for 30 seconds in a boiling water bath, cooled to room temperature and acidified with acetic acid (pH 3.5-3.8). The precipitate which formed upon standing overnight at 4° was removed by filtration through a membrane filter AM7 (Gelman Instrument Co., Chelsea, Michigan) or by centrifugation at 12,000 rpm (17,300 x g) for 20 minutes at 10°. The sediment was redissolved in ammonium carbonate buffer and precipitated again at pH 3.5-3.8 with acetic acid. After washing 3X with acetic acid and 2X with distilled water, the precipitate was lyophilized and designated tryptic core of PX-S. The combined, lyophilized supernates represented the tryptic peptides.

The tryptic core of PX-S was digested with pronase according to a similar procedure; since maximum release of amino acids was reached after 20-21 hours, the hydrolysis time was prolonged to 24 hours. The corresponding fractions were called pronase core of PX-S and pronase peptides. The tryptic and pronase cores were tested by high voltage electrophoresis for the possible presence of ninhydrin-positive impurities. If necessary, the cores were washed with distilled water until free of impurities.

Preparation of Carboxypeptidase Core of PX-S

Two milligrams of pronase core of PX-S were dissolved in a small volume of 0.05 M Tris buffer containing 0.5% sodium dodecyl sulfate (SDS), pH 7.6. To this solution 0.2 M N-ethyl-morpholine buffer, pH 8.5, was added to a final volume of 1 ml. The final concentration of SDS was 0.2%. Carboxypeptidase A was added in a substrate/enzyme ratio 80:1 and the mixture was incubated at 37°. Aliquots were taken at different time intervals within 72 hours and the undigested material was precipitated with 0.1 N HCl. The

combined supernates, evaporated to dryness and dissolved in citrate buffer pH 2.2, were used for the amino acid analysis. The final precipitate, washed with distilled water and lyophilized, represented the carboxypeptidase core of PX-S.

Isolation of Lipid A from "Simple" Protein

of S. marcescens 08

PX-S was added to preheated 0.1 N HCl (mg PX-S/0.2 ml acid) and hydrolyzed for 30 minutes at 85-90°. After cooling in an ice bath, the hydrolysate was centrifuged at 12,000 rpm (17,300 x g) for 10 minutes at 4°. The supernate and the remaining pellet were separately extracted 3X by chloroform. The combined chloroform extracts from both supernate and pellet were washed exhaustively with deionized water to remove traces of acid and evaporated to dryness under reduced pressure. The lipid residue was treated several times with boiling acetone. The acetone insoluble material (lipid A) was removed by filtration. The unhydrolyzed pellet (protein moiety) was washed with ice-cold water and lyophilized.

Electrophoresis and Infrared Spectroscopy

Agarose electrophoresis was carried out in 1% agarose according to the procedure by Grabar and Williams (4) employing Veronal buffer, pH 8.6, ionic strength 0.05, for 40 minutes. Protein samples (20 mg/ml) were dissolved in 0.05 M Tris buffer, pH 7.6, containing 0.5% SDS and diluted to a final concentration of 10 mg/ml with 1% agarose in Veronal buffer.

The infrared spectra of PX-S, tryptic and pronase cores and lipid A were obtained with a Beckman Infrared Spectrophotometer JR10 using potassium bromide pellets (1.0-1.8 mg of substance and 250 mg KBr).

Analytical Methods

Procedures for the quantitative analysis of amino acids, fatty acids, glucosamine, phosphorus, nitrogen, carbon, hydrogen and ash were described in a previous paper (3). Sephadex G-50 was used for the gel filtration column chromatography of the pronase core of PX-S.

Immunological Methods

Preparation of antibodies to PX-S and tryptic and pronase cores and double diffusion experiments in 1% agar gel were performed according to the procedures previously described (3). Antibodies against an essentially protein-free lipopolysaccharide (LPS-A) from S. marcescens 08 were prepared by immunizing white rabbits with four successive intraperitoneal injections of 0.5 mg of antigen dissolved in 2 ml 0.05 M Tris-HCl buffer containing 0.5% SDS and emulsified with 2 ml of Freund's complete adjuvant. The injections were administered at weekly intervals, and antibodies were detected after the fourth injection. Even a slight increase in dosage of LPS-A caused death of the animals.

Antigens and the homologous antibodies are symbolized by the same letters; capital letters designate antigens and small letters antibodies:

<u>Antigens</u>		<u>Antibodies</u>	
PX-S	A	Anti PX-S	a
Tryptic core	E	Anti tryptic core	e
Pronase core	F	Anti pronase core	f
LPS-A	M	Anti LPS-A	m

RESULTS

Preparation of Tryptic and Pronase Cores of PX-S

Tryptic core of PX-S, isolated by isoelectric precipitation at pH 3.5-3.8, accounted for 64.5% of PX-S. The purple color of the tryptic core indicated that prodigiosin or one of its derivatives was still firmly attached to this portion of the protein moiety. The corresponding peptide fraction was colorless. The purple-colored pronase core, precipitated at the same pH range (3.5-3.8), accounted for 28.7% of the tryptic core and for 18.5% of PX-S. To remove an additional amount of protein moiety, pronase core was treated successively with carboxypeptidase A; carboxypeptidase A released amino acids, which were in very small amount.

Tryptic and pronase digestion of "simple" protein from E. coli (PX-E) resulted in the isolation of a tryptic core (60.3% of PX-E) and a pronase core (20.4% of PX-E); both cores were isolated by isoelectric precipitation at pH 3.5-3.8.

Physical-chemical Characterization of the Tryptic
and Pronase Cores of PX-S

Both cores were readily soluble in 0.02 M ammonium carbonate buffer, pH 8.6, but were insoluble at a pH lower than 3.8, in water or in saline. However, after repeated lyophilizations, tryptic and pronase cores were soluble only if the ammonium carbonate buffer contained 0.1-0.2% SDS.

. On agarose electrophoresis, tryptic and pronase cores migrated as single bands with mobilities similar to that of PX-S (Fig. 1). Gel filtration of pronase core on Sephadex G-50 yielded a single fraction characterized by a symmetrical elution curve with absorption maximum at 280 m μ . The amino acid and glucosamine analyses showed the same values before and after column chromatography.

The infrared spectra of the tryptic and pronase cores were similar to that of PX-S (Fig. 2). Proteolytic shortening of the protein moiety (PX-S+pronase core) resulted in a decrease in the ν OH ass. and the ν NH ass. absorption at 3400-3280 cm^{-1} , and an increase in the ν $\text{CH}_3 + \text{CH}_2$ absorption at 2960-2850 cm^{-1} and the carbohydrate absorption at approximately 1060 cm^{-1} (ν OH and ν C-O). The most striking feature in the spectrum of the pronase core was the appearance of a strong ester absorption ν C = O at 1740 cm^{-1} which could be recognized in the spectra of PX-S and tryptic core only as a shoulder on the amide I absorption band at 1650 cm^{-1} . The pronounced ester absorption in the spectrum of the pronase core was obviously caused by a decreased protein and increased fatty acid ester content in accordance with the simultaneous increase of ν $\text{CH}_3 + \text{CH}_2$ absorption at 2960-2850 cm^{-1} as well as with the results of chemical analysis.

Immunological Properties of Tryptic and Pronase Cores of PX-S

Tryptic core (E) and pronase core (F) showed single, identical

precipitin lines with antibodies to PX-S (a) and exhibited identity with PX-S (A) (Fig. 3, pattern 1). In immunoelectrophoresis both cores gave single precipitin arcs with antibodies to PX-S. Tryptic and pronase cores were immunogenic. PX-S (A), tryptic core (E) and pronase core (F) showed complete coalescence of precipitin lines with antibodies to tryptic core (Fig. 3, pattern 2) and with antibodies to pronase core (Fig. 3, pattern 3). Since both cores were immunogenic, the antigenic determinant(s) may be located either on the residual protein fragment close to lipid A or on the lipid A moiety itself. To resolve this alternative, a lipopolysaccharide preparation (LPS-A) containing less than 2% amino acids and PX-S were tested with antibodies to LPS-A (m) and/or antibodies to PX-S (a). Since LPS-A consists of a polysaccharide moiety and lipid A, and PX-S of a protein moiety and lipid A, a reaction of partial identity would indicate the presence of a second set of antigenic determinants within the lipid A. In contrast, a non-identity reaction would indicate the occurrence of antigenic determinant(s) in the protein moiety. Results of these experiments showed that neither LPS-A (M)

reacted with antibodies to PX-S (a) nor PX-S (A) with antibodies to LPS-A (m) (Fig. 4, left pattern). Moreover, PX-S (A) and LPS-A (M) showed a non-identity reaction with a mixture of antibodies to PX-S (a) and LPS-A (m) (Fig. 4, right pattern). Thus, the second set of antigenic determinants in the endotoxin complex is located in the protein rather than the lipid moiety.

Chemical Characterization of the Tryptic and

Pronase Cores of PX-S

Results of the elementary analysis and chemical composition of the tryptic and pronase cores are compared with those of PX-S (Table 1). Shortening of the protein moiety (PX-S→pronase core) caused an increase in the relative content of carbon, hydrogen, phosphate, glucosamine and fatty acids and a decreased content of nitrogen and amino acids. Proteolysis of the tryptic core by pronase caused a more drastic compositional change than that caused by trypsin treatment of PX-S. This change was also clearly reflected in and paralleled by similar changes in the infrared

spectra and yields of tryptic (64% of PX-S) and pronase (18% of PX-S) cores. PX-S as well as its cores contained the characteristic components of lipid A with an approximate molar ratio of fatty acids/phosphate/glucosamine of 2:1:1. The relatively low recovery of the constituents of the pronase core (60.6%) may be explained by a loss of amino acids known to occur when hydrolysis is carried out in the presence of carbohydrates (5), and by the fact that the absolute amount of prodigiosin, present in the pronase core in the highest concentration, is unknown.

Results of the amino acid analysis of tryptic and pronase cores and their corresponding peptide fractions are shown in Table 2. Peptide fractions contained no detectable glucosamine or fatty acids. Aspartic acid was the major amino acid of all three cores. The relative content of glucosamine and the molar ratios of aspartic acid and glucosamine of PX-S and corresponding cores are shown in Table 3. From the molar ratios of glucosamine and major amino acids of pronase and carboxypeptidase cores (Table 4), it was

concluded that aspartic acid, glutamic acid and serine represented the amino acids most likely to be involved with glucosamine in a covalent linkage between the lipid A and protein moiety.

The fatty acid composition of PX-S and its cores was characterized by the presence of β -hydroxy myristic acid, the typical marker for lipid A, in a concentration higher than any other long-chain saturated or unsaturated fatty acid (Table 5).

Isolation of Lipid A from "Simple" Protein

Although chemical analysis of PX-S and its cores indicated clearly the presence of lipid A constituents, an intact lipid A moiety could not be separated from the protein moiety by electrophoresis or by exhaustive extraction with chloroform. However, when PX-S was hydrolyzed with 0.1 N HCl according to a standardized procedure used for the isolation of lipid A from lipopolysaccharide, a chloroform soluble but acetone insoluble fraction was obtained. The infrared spectrum (Fig. 2) of this fraction was identical with the spectrum of lipid A isolated from the lipopolysaccharide

fraction (LPS-A) and was very similar to that of the pronase core. The slightly higher ester/amide I ratio (0.86) of this lipid fraction in comparison with that (0.79) of pronase core could be explained by its decreased protein content and, therefore, a decreased amide absorption. The glucosamine content of this preparation was 27.7%. These results indicated that this fraction identified as lipid A is very likely covalently linked to the protein moiety.

DISCUSSION

The "simple" proteins PX-S and PX-E were only partially degraded by successive digestion with trypsin and pronase. A similar, partial degradation of the protein moiety by tryptic digestion has also been reported for the endotoxin complex of Salmonella typhosa (6, 7), Shigella paradysenteriae (8, 9), E. coli (10) and Neisseria gonorrhoeae (11). On the other hand, Morgan and Partridge (12) claimed that tryptic hydrolysis of endotoxin from Shigella dysenteriae resulted in an almost complete removal of the protein moiety. It is not known whether the partial tryptic hydrolysis

of the protein is due to a particular amino acid sequence or to an inhibitory effect of the protein-bound lipid A on the enzymatic activity. However, even pronase digestion of the tryptic core of PX-S resulted in an incomplete removal of the protein moiety. Recently, Rosselet et al., (13) isolated from E. coli a proteinaceous component by phenol-water treatment of bacterial extracts. This phenol-soluble fraction, called "protodyne," contained 87.7% protein, 1.2% carbohydrate, 0.3% amino sugars and 0.9% lipid and exhibited the ability to enhance nonspecific host resistance to infection. Upon pronase digestion, the remaining residue contained still an appreciable amount of protein (62.9%) but also an increased amount of lipid (8.6%). Since neither of these two substances contained 2-keto-3-deoxy-octonate (KDO), the authors concluded that "protodyne" contained no lipopolysaccharide and therefore differed from this cell wall constituent. However, this does not exclude the possibility that "protodyne" represents the protein moiety of endotoxin, because KDO, linking lipid A with the

polysaccharide moiety, is present in the water soluble lipopolysaccharide and not in the phenol-soluble protein moiety. It seems, therefore, that "protodyne" and its pronase core may be similar to "simple" proteins PX-S and PX-E and the corresponding pronase cores. Pronase digestion of both "protodyne" and PX-S preparations resulted in an incomplete removal of proteins.

Although the successive enzymatic degradation of "simple" proteins resulted in only partial removal of protein moieties, the tryptic and pronase cores contained increasing amounts of lipid A constituents which could not be separated from the remaining protein residue by isoelectric precipitation, gel filtration or by extraction with chloroform. Since lipid A constituents could be isolated as an entity only after hydrolysis of the pronase core of PX-S with 0.1 N HCl, we suggest that, at least in the intact endotoxin of S. marcescens 08, lipid A is covalently linked to the protein moiety. In contrast to the more usual random attachment of carbohydrate chains along the polypeptide backbones of several

structurally known glycoproteins (14), we propose a single point of linkage between lipid A and protein. This proposal is based on the following evidence: 1) peptides obtained by enzymatic digestion of PX-S with trypsin, pronase and carboxypeptidase A were free of glucosamine and fatty acids, and 2) the glucosamine/aspartic acid ratio of the carboxypeptidase core (10:1) was twice as high as that of the pronase core (5:1). The amino acid analysis of the carboxypeptidase core indicated aspartic acid, glutamic acid and serine as amino acids most likely to be linked with the terminal glucosamine unit of lipid A.

Antibodies prepared against "simple" protein and its tryptic and pronase cores revealed the presence of a common antigenic determinant(s) different from the determinants of the O-specific side chain of the polysaccharide moiety. Homma and his coworkers (15, 16) have demonstrated that endotoxins isolated from the autolysates of Pseudomonas aeruginosa contain antigenic determinants in the polysaccharide as well as in the protein moiety. Recently,

Maeland (17, 18) has established that endotoxin preparations obtained by either aqueous-ether or trichloroacetic acid extraction of Neisseria gonorrhoeae contain also two antigenic determinants, one of which (determinant a) is located in the polysaccharide and the other (determinant b) in the protein moiety. Like fraction LPS-A in this study, the lipopolysaccharide fragment isolated by phenol-water extraction contained only determinant a (O-specific antigen). The determinant located in the protein moiety of Neisseria gonorrhoeae was destroyed by pronase, but not by trypsin digestion. Since in the present study the pronase core of "simple" protein PX-S was still immunogenic, the antigenic site is most probably positioned close to the lipid A moiety.

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Table 1
Elementary Analysis and Chemical Composition of PX-S, Tryptic Core and Pronase Core

	C	H	N	PO ₄	Glucosamine	Fatty Acids	Amino Acids	Recovery ^a	Ash
	%	%	%	%	%	%	%	%	%
PX-S	51.2	6.9	13.4	2.0	2.9	9.2	72.9	87.0	4.9
PX-S Tryptic core	51.3	7.1	11.1	3.1	4.8	11.4	69.0	88.3	4.3
PX-S Pronase core	52.3	8.0	4.0	5.8	12.1	31.3	11.5	60.7	3.6

^a Recovery was calculated from phosphate, glucosamine, fatty acid and amino acids.

Table 2
 Amino Acid Composition of PX-S, Tryptic Core, Pronase Core, and Peptides

	PX-S		Tryptic peptides		Tryptic core		Pronase peptides		Pronase core	
	$\mu\text{Moles/g}$	$\text{mg}/100$								
Lysine	245	3.58	246	3.60	142	2.08	165	2.41	52	0.76
Histidine	74	1.15	56	0.87	80	1.24	75	1.16	13	0.20
Arginine	181	3.15	156	2.72	95	1.65	117	2.04	27	0.47
Aspartic acid	824	10.97	811	10.79	876	11.66	1640	21.83	142	1.89
Threonine	422	5.03	394	4.69	402	4.79	570	6.78	50	0.60
Serine	442	4.64	360	3.78	490	5.15	610	6.41	122	1.28
Glutamic acid	537	7.90	471	6.93	477	7.02	632	9.30	69	1.02
Proline	137	1.58	130	1.50	219	2.52	223	2.57	31	0.36
Glycine	657	4.93	598	4.49	600	4.50	867	6.51	89	0.67
Alanine	657	5.85	657	5.85	680	6.05	862	7.68	109	0.97
1/2 Cystine	-	-	-	-	-	-	-	-	-	-
Valine	378	4.43	419	4.91	307	3.60	482	5.65	60	0.70
Methionine	42	0.63	57	0.85	60	0.90	89	1.33	15	0.22
Isoleucine	238	3.12	227	2.98	182	2.39	268	3.52	45	0.59
Leucine	478	6.27	385	5.05	535	7.02	695	9.12	66	0.87
Tyrosine	240	4.35	222	4.02	235	4.26	350	6.34	17	0.31
Phenylalanine	228	3.77	188	3.11	235	3.88	322	5.32	32	0.53

Table 3
Percent Content of Glucosamine and Molar Ratios of Aspartic
Acid/Glucosamine in PX-S and Its Cores

	Glucosamine in %	Molar Ratio Aspartic Acid:Glucosamine
PX-S	2.87	6:1
Tryptic core	4.81	3:1
Pronase core	12.09	1:5
Carboxypeptidase core	16.12	1:10

Table 4
Molar Ratio of Glucosamine and Major Amino Acids in the
Pronase and Carboxypeptidase Cores of PX-S

	Pronase Core	Carboxypeptidase Core
Glucosamine	10	10
Aspartic acid	2	1
Serine	2	1
Glycine	1	1
Alanine	2	1
Glutamic acid	1	1

Table 5
 Percent Fatty Acid Composition of PX-S, Tryptic Core
 and Pronase Core

	PX-S %	Tryptic core %	Pronase core %
C ₁₂	2.6	3.7 [*]	3.4
C ₁₄	9.5	9.8	10.7
C ₁₄₋₁	Trace	0.0	0.0
Unknown	12.9	14.6	14.4
C ₁₆	16.5	18.2	13.5
C ₁₆₋₁	5.5	3.4	2.8
Unknown	Trace	3.1	5.7
Unknown	3.9	4.7	2.0
Unknown	4.5	3.7	5.8
C _{14-OH}	44.5	38.7	41.8

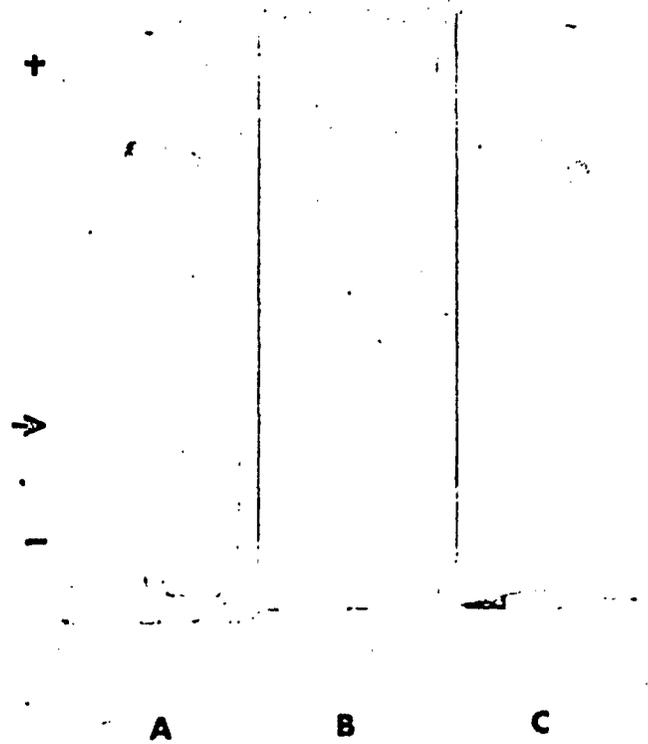


Figure 1. Agarose electrophoresis of "simple" protein PX-S (A), tryptic core (B) and pronase core (C). Protein preparations (20 mg/ml) were dissolved in 0.05 M Tris-Cl buffer, pH 7.6, containing 0.5% SDS, and diluted to a final concentration of 10 mg/ml with 1% agarose in Veronal buffer. Electrophoresis was carried out for 40 minutes in 1% agarose gel employing Veronal buffer, pH 8.6, ionic strength 0.05.

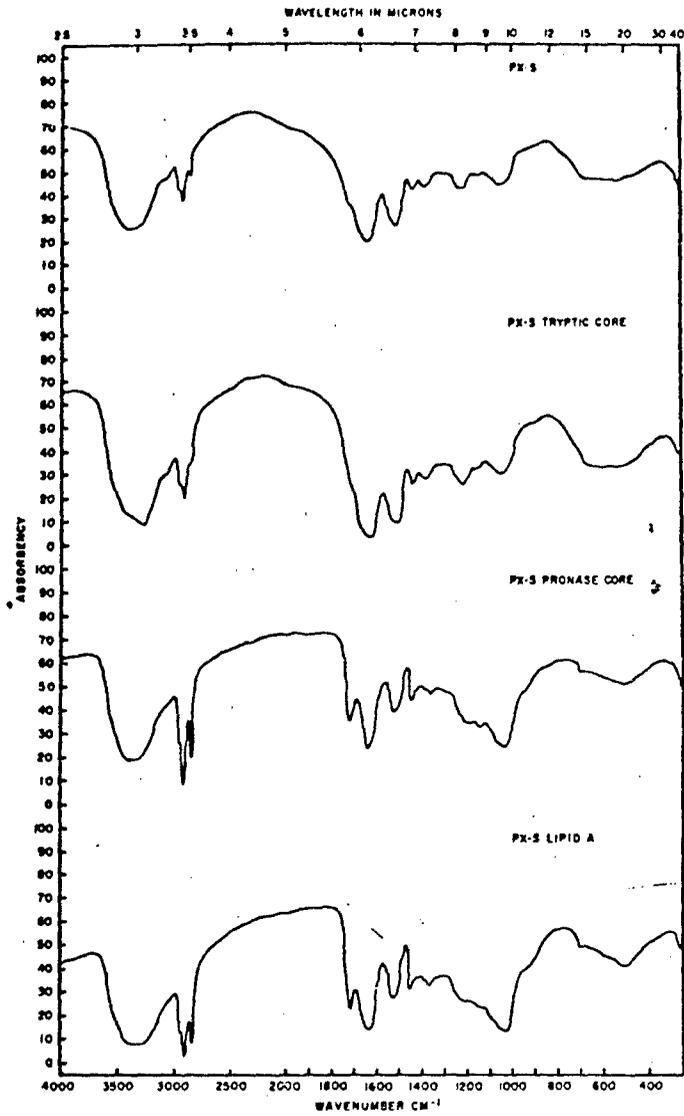


Figure 2. Solid infrared spectra of "simple" protein PX-S and its corresponding tryptic core, pronase core and lipid A.

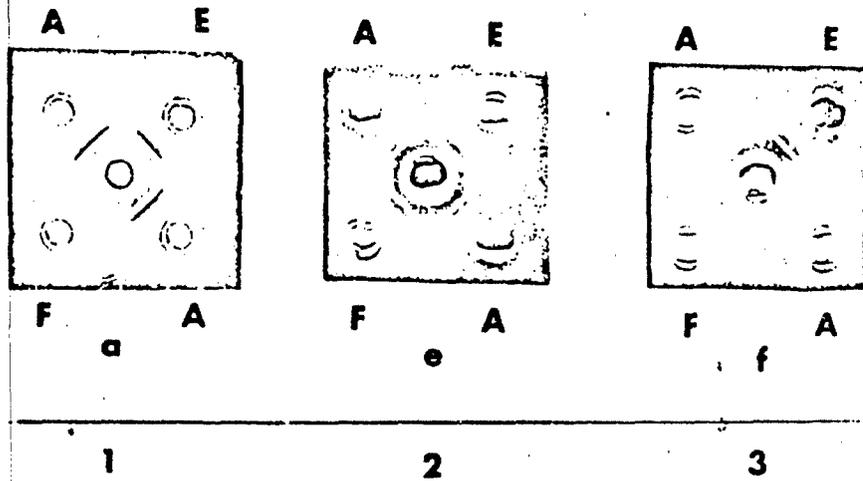


Figure 3. Immunodiffusion patterns of "simple" protein PX-S (A), tryptic core (E) and pronase core (F). Central wells contain antibodies to PX-S (a), tryptic core (e) and pronase core (f).

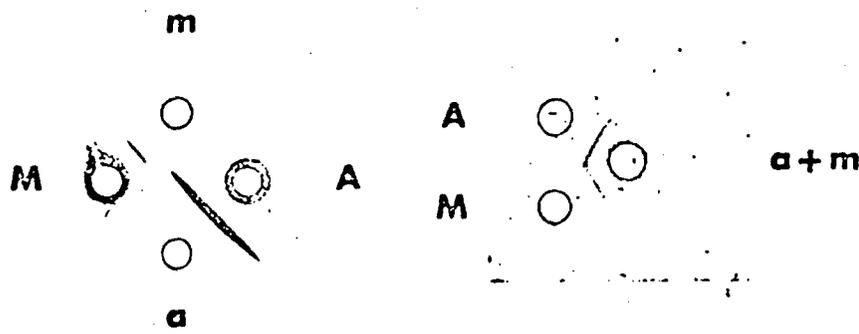


Figure 4. Immunodiffusion patterns of PX-S (A) and LPS-A (M). Antibodies to PX-S are present in well a and antibodies to LPS-A in well m. Well a+m contains a mixture of equal amounts of antibodies to PX-S and LPS-A.

UNCLASSIFIED

Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author) Medical Center Research and Development Office of the University of Oklahoma Foundation, Inc.		2a. REPORT SECURITY CLASSIFICATION Unclassified	
		2b. GROUP Unclassified	
3. REPORT TITLE STUDIES ON THE PROTEIN MOIETY OF ENDOTOXIN FROM GRAM-NEGATIVE BACTERIA: CHARACTERIZATION OF THE ENZYMATICALLY DEGRADED PROTEIN MOIETY ISOLATED BY PHENOL TREATMENT OF ENDOTOXIN FROM <i>S. marcescens</i> 08			
4. DESCRIPTIVE NOTES (Type of report and, inclusive dates) Technical Report			
5. AUTHOR(S) (First name, middle initial, last name) W. Wober and P. Alaupovic			
6. REPORT DATE August 17, 1970		7a. TOTAL NO. OF PAGES 33	7b. NO. OF REFS 18
8a. CONTRACT OR GRANT NO. N00014-68-A-0496		9a. ORIGINATOR'S REPORT NUMBER(S) 29	
b. PROJECT NO. NR 105-516		9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)	
10. DISTRIBUTION STATEMENT This document has been approved for public release and sale; its distribution is unlimited.			
11. SUPPLEMENTARY NOTES		12. SPONSORING MILITARY ACTIVITY Office of Naval Research	
13. ABSTRACT The "simple" protein of endotoxin from <i>Serratia marcescens</i> 08 was treated successively with trypsin and pronase. The resulting trypsin and pronase cores contained increasing amounts of lipid A constituents firmly bound to the residual protein moiety. Since lipid A was separated as an entity from the protein moiety only after acid hydrolysis of the pronase core, it is proposed that in intact endotoxin lipid A is covalently linked to the protein moiety. The absence of any detectable glucosamine and fatty acids in the mixture of peptides and amino acids released by trypsin and pronase, indicated a single point attachment between the lipid A and protein moieties. Both trypsin and pronase cores were immunogenic and revealed the presence of a common antigenic determinant with the parent "simple" protein. The second set of antigenic determinant(s) different from the specific O-antigens is located in the protein moiety close to lipid A.			

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S/N 0101-807-6811

UNCLASSIFIED

Security Classification

A-31408