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Isolation and Purification of Endotoxin by Hydrolytic Enzymes

SAMUEL LEHRER[1] AND ALOIS NOWOTNY

Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

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Various commercial hydrolases were used in an attempt to degrade the endotoxic lipopolysaccharide macromolecule. Some inert components, such as peptides and nucleic acids, could be removed from endotoxin preparations. As a result, endotoxic activity, measured by pyrogenicity, Shwartzman reaction, and mouse lethality, was increased. The remarkable resistance of endotoxin to hydrolases led to the use of such enzymes for the liberation and purification of endotoxin from whole bacterial cells.

An increasing amount of evidence shows that the active site of bacterial endotoxic lipopolysaccharides lies in the lipid moiety of the molecule. No procedures are available presently for the isolation of the lipid moiety in intact form. Acid hydrolysis degrades the polysaccharides and will precipitate a lipid-rich fraction, which was called fraction A by Boivin and associates (3) or lipid A by Westphal and Lüderitz (24). This precipitate, which consists of over 40 different fractions, shows 1 to 10% of the original activity of the parent endotoxin, as determined by various laboratories. These breakdown products derived from the lipid moiety of the molecule may resemble it, but are obviously not identical to it.

Other attempts to isolate the lipid-rich moiety of endotoxin without degrading it employed hydrolytic enzymes. In our laboratories, over thirty commercially available hydrolytic enzymes were used, and, after digestion with these enzymes, their effect on endotoxin preparations was followed by (i) determination of changes in the most characteristic endotoxicity parameters and (ii) analyses of breakdown products liberated by the enzymes from the endotoxin preparation. The enzymes selected were those which are capable of cleaving certain linkages known or assumed to be present in the endotoxic lipopolysaccharide macromolecule. As will be shown, a remarkable resistance of endotoxin to these hydrolytic enzymes was observed. Although some contamination of the endotoxin preparations could be digested away and removed by enzymatic treatment, an enzyme-resistant core remained, which showed no loss of biological activity. In several instances, with the right enzyme combination, an increase in biological potency could be observed.

Based on these observations, a procedure was worked out for the direct extraction of endotoxin from bacterial cells by using enzymes, and the further purification of the crude preparation obtained by enzymatic procedures was developed.

MATERIALS AND METHODS

Hydrolytic enzymes. All enzyme preparations used in these studies were obtained from commercial sources. Determination of the activity of the enzyme preparations using recommended substrates under optimal conditions for the given enzyme-substrate system was carried out as described in the literature or as instructed by the vendor. Only sufficiently active enzyme preparations were used.

Extraction of endotoxin by the Boivin procedure. *Serratia marcescens* 08 cells were grown by Merck, Sharp & Dohme, Rahway, N.J., and kindly provided for this entire project. The lyophilized cells were extracted with chloroform-methanol (1:1) in a Soxhlet to remove noncovalently bound lipids. The trichloroacetic acid (TA) extraction procedure of the lipid-extracted and dried bacterial cells was basically that described by Boivin et al. (2), as later modified by Nowotny et al. (17). This product was further purified by precipitation with alcohol and by sedimentation in an ultracentrifuge (TA endotoxin). Purification of trichloroacetic acid-extracted endotoxin by phenol-water was carried out as described (16) at 70 C by using freshly distilled phenol (TA-PW). The trichloroacetic acid endotoxin preparation was purified also by Pronase, in 0.1 M ammonium acetate buffer (*p*H 8.5). Enzyme (1 mg) was incubated with 100 mg of endotoxin dissolved in 25 ml of buffer at 37 C for 10 hr (TA-

[1] Present address: Scripps Clinic and Research Foundation, La Jolla, Calif. 92037.

ENZ). The purified endotoxins, TA-PW and TA-ENZ, were precipitated with methanol and centrifuged in ultracentrifuge.

Extraction of endotoxin by hydrolytic enzymes. A 1-g amount of lyophilized, lipid-extracted, and water-washed bacteria was suspended in 100 ml of 0.1 M ammonium acetate buffer, *p*H 8.5, and homogenized. The ammonium acetate buffer was prepared by adjusting the *p*H of 1 N ammonium hydroxide to 8.5 with 1 N acetic acid, and the molarity was adjusted to 0.1 M with ion-depleted water. A 10-mg amount of Pronase (Calbiochem, Los Angeles, Calif.) and 10 mg of three-times crystallized lysozyme (General Biochemicals, Chagrin Falls, Ohio) were added to the suspension. The suspension, to which 1 ml of toluene had been added to prevent extensive bacterial growth, was shaken at 37 C for 10 hr. The digested material was centrifuged at 8,000 × *g* for 30 min. The pellet was discarded. Two further enzymes were added to the supernatant fluid, venom phosphodiesterase and alkaline phosphatase (from *Escherichia coli*). The ratios of these two enzymes to the original dried bacterial substrate in the case of phosphodiesterase was 1:500, and in the alkaline phosphatase was 1:200. The flasks were shaken for 2 hr at 37 C. The material was dialyzed against ion-depleted water for 3 days at 5 C temperature, the water being changed twice daily. The contents of the dialysis bags were filtered through Whatman no. 4 paper, adjusted to *p*H 7.5 with 0.1 M NaOH, and concentrated by vacuum distillation to approximately two-fifths of the original volume.

The purification of this concentrated extract was achieved by adding two volumes of cold methanol. The precipitate was dissolved in water and centrifuged at 10,000 × *g* at 4 C for 30 min. The supernatant fluid was recentrifuged for 3 hr at 4 C at 105,000 × *g*. This lyophilized pellet obtained from the supernatant fraction was our enzyme-extracted and enzyme-purified endotoxin (ENZ-ENZ).

Chemical analysis. Analysis of the preparations obtained included determination of phosphorus by the method of Chen, Toribara, and Warner (4). The quantitative determination of the amino acid content was by the method of Moore and Stein (11). The amino sugar content was measured by the method of Rondle and Morgan (19). Carbohydrate content was measured after hydrolysis with 1 N sulfuric acid at 100 C for 8 hr. The hydrolysates were analyzed by paper chromatography as well as by the quantitative reducing carbohydrate method of Park and Johnson (18). Heptose was measured by the procedure of Dische (6) and the 2-keto-3-deoxy-octonate was measured by the thiobarbituric acid procedure of Weissbach and Hurwitz (23). Long-chain carboxylic acids were identified by gas-liquid chromatography and were quantitatively measured after transesterification with boron trifluoride in methanol (7).

Ion-exchange column chromatographic analysis of the endotoxic preparations obtained. Amberlite XE220, kindly provided by Rohm and Haas Company, Philadelphia, Pa., was used to fractionate the endotoxin preparations, as described earlier (14–16).

Biological activity of endotoxin. Biological activity was determined by using three different parameters. Mouse lethality in ICR male mice, the local Shwartzman skin assay, and the pyrogenicity assay in rabbits were carried out by routine procedures (13), and the febrile response was expressed as fever index (FI_{20}; 1, 5) or minimal pyrogenic dose (MPD; 10).

RESULTS

An attempt has been made to combine all the findings related to enzymatic treatment of trichloroacetic acid endotoxin (TA-endotoxin) in Table 1. The only remarkable hydrolysis was obtained by proteolytic enzymes, especially with Pronase. Other enzymes, such as pepsin or ficin, were also active in liberating amino acids and peptides from the endotoxin. A cellulase preparation (of microbiological origin), obtained through the courtesy of Rohm and Haas Company, Philadelphia, Pa., as well as a fungal hydrolase liberated some amino acids and peptides, most probably due to contamination with proteolytic enzymes. The treatment with phosphatases set free hydrolytic products. Venom phosphodiesterase and spleen phosphodiesterase pretreatment rendered the endotoxin preparations sensitive to phosphomonoesterases, which liberated inorganic phosphorus.

No significant cleavage of the polysaccharide or the lipid moiety could be detected by the analytical procedures applied here. The change in mouse lethality of the endotoxin preparations after treatment with hydrolytic enzymes is not shown in the table. Elevation of the lethality could be observed after proteolytic enzyme and phosphatase treatments. None of the other enzymatic hydrolyses changed the toxicity of the preparations measurably.

Similarly, as was done by using phosphatases, some other enzymes were also used in combination or in sequence. Pretreatment with proteolytic enzymes was followed by lipases or lipase-pretreated preparations were further subjected to proteolytic treatments. None of these experiments resulted either in enhanced liberation of cleavage products or in changes in the biological activities tested.

In these experiments, the activity of the second enzyme applied was measured in the presence of the previously used enzyme to determine its activity under these conditions. Those combinations in which no interference could be observed were used.

Since such remarkable resistance of endotoxicity to the applied hydrolytic enzymes could be observed, and since mostly inert components (such as proteins or nucleic acids) of the trichloroacetic acid-extracted endotoxin prepara-

TABLE 1. *Enzymatic hydrolysis of trichloroacetic acid endotoxin*

Enzyme	Enzyme activity (units/mg)	Reduction of turbidity	Cleavage products detected	
			Qualitative analysis	Quantitative analysis
Aminopeptidase (hog kidney)	1.66	None	None	NT[a]
Carboxypeptidase (bovine pancreas)	9.16	None	Amino acids	NT
Pronase (*Streptomyces griseus*)	0.72	Yes	Amino acids, peptides	85.94 μg of leucine[b]/mg of endotoxin
Pepsin (hog stomach)	1,575	Yes	Peptides	40.08 μg of leucine/mg of endotoxin
Trypsin	5,200	Yes	Peptides	6.42 μg of leucine/mg of endotoxin
Alkaline protease (*Bacillus*)	0.33	Yes	Amino acids, peptides	NT
Papain (papaya latex)	6	Yes	Amino acids, peptides	8.65 μg of leucine/mg of endotoxin
Ficin (fig latex)	1.7	Yes	Amino acids peptides	22.14 μg of leucine/mg of endotoxin
Acid phosphatase (wheat germ)	0.05	None	None	0.18 μg of P/mg of endotoxin
Venom phosphodiesterase (venom)	0.03	None	None	1.37[c] μg P/mg endotoxin
Spleen phosphodiesterase (spleen)	3.4	None	None	1.25[c] μg P/mg endotoxin
Phospholipase C (*Clostridium perfringens*)	0.51	None	None	NT
Phospholipase D (cabbage)	0.0012	None	None	NT
Lipase (wheat germ)	0.11	None	Phosphate	NT
Lipase (steapsin)	0.165	Yes	Amino acids, peptides	NT
Lipase (calf gland)	0.083	None	None	NT
Lipase (pork pancreas)	1.36	Yes	Amino acids, peptides	NT
Acylase (hog kidney)	10.4	None	Amino acids, peptides	NT
α-Amylase (bacterial)	1,500	None	None	NT
β-Amylase (sweet potato)	2,000	None	None	NT
Cellulase 35 (microbiological)	NT	Yes	Amino acids, peptides, phosphate	0.70 μg of P/mg of endotoxin, 14.28 μg of leucine/mg of endotoxin, 1.67 μg of glucose[d]/mg of endotoxin
Chitinase (fungal)	17	None	None	NT
Lysozyme (egg white)	43.4	None	None	NT
β-Glucosidase (almond)	2.2	None	Phosphate	0.44 μg of P/mg of endotoxin

[a] NT, Not tested.

[b] Total amino acid remaining in the supernatant fluid after enzyme digestion and sedimentation expressed as micrograms of leucine per milligrams of endotoxin.

[c] Total phosphorus released by alkaline phosphatase from phosphodiesterase pretreated endotoxin.

[d] Increase in reducing activity after enzyme digestion expressed as micrograms of glucose per milligram of endotoxin.

TABLE 1. *Continued*

Enzyme	Enzyme activity (units/mg)	Reduction of turbidity	Cleavage products detected	
			Qualitative analysis	Quantitative analysis
Alkaline phosphatase (intestinal)	0.44	None	Phosphate	0.75 μg of P/mg of endotoxin
Lipase (pancreas)	8.7	None	None	NT
Pectin methyl esterase (tomato)	30.0	Yes	Amino acids, peptides	NT
Carboxylesterase (liver)	0.28	None	None	NT
Lysozomal enzymes (liver)	0.26	None	None	NT
Hydrolase (fungal)	3,550	Yes	Amino acids, peptides	2.57 μg of glucose/mg of endotoxin, 26.85 μg of leucine/mg of endotoxin

tion (TA-endotoxin) seemed to undergo hydrolysis, efforts were concentrated on purification of endotoxins by enzymatic removal of those components which do not seem to belong to the active core.

Chemical purification, such as phenol-water extraction of the trichloroacetic acid-extracted endotoxin (TA-PW), resulted in enhanced biological activity and reduced amino acid content. Similar effects could be obtained by exposing trichloroacetic acid-extracted endotoxin to those enzymes which liberated amino acids and peptides, such as Pronase, as well as phosphatases, such as venom phosphodiesterase and alkaline phosphatase. The endotoxic activity (Table 2), as well as the data of chemical analyses (Table 3) of these endotoxin preparations have been compared with the enzyme-extracted and purified ENZ-ENZ preparations.

As these tables show, considerable enhancement of mouse lethality could be obtained by exposing trichloroacetic acid-extracted endotoxin to enzymes (TA-ENZ) or by obtaining the endotoxin from whole bacterial cells by enzymatic extraction and purification. The yield in toxic material which expresses the number of median lethal doses isolated from 1 g of lyophilized bacterial cells was also highest with enzymatic liberation. The differences in activities are shown especially well in the case of pyrogenicity. Expressing the MPD in micrograms, by far the most active preparation is the one which was liberated and purified from bacterial cells by enzymes. The pyrogenicity determined by the FI_{20} method did not agree with the MPD results. The FI_{20} of the ENZ-ENZ preparation was lower than that of the TA-PW. The local Shwartzman skin reactivity, which was expressed as the maximal dilution that still showed a positive skin reaction, did not reveal a significant difference between the different preparations. The ENZ-ENZ endotoxin did not show a greater activity here than other preparations.

The chemical composition of the different endotoxin preparations is compared in Table 3. The trichloroacetic acid-extracted endotoxin, which was the lowest in biological activity, had the highest amount of phosphorus and a considerably high amount, almost 20%, of amino acids. Purification of this perparation by phenol-water extraction greatly reduced the amino acid content, but it was still close to 6%. Enzymatic treatment of the trichloroacetic acid-extracted endotoxin, which greatly enhanced the biological activity, did not reduce the amino acid content to a great degree. The highest amino acid content, over 27%, was detected in the enzyme-extracted and enzyme-purified endotoxin. Carbohydrate analysis revealed that the highest content is present in the TA-PW preparation. The lowest carbohydrate content was in the ENZ-ENZ preparation, which was also the most active endotoxin. The fatty acid content was highest in the TA-PW and in the ENZ-ENZ preparations.

Column chromatographic analysis of the four preparations was carried out by using Amberlite XE220 anionic ion-exchange resin. Figure 1 shows the carbohydrate content of the chromatographic effluent, monitored by a Technicon AutoAnalyzer, as reported earlier (41). The carbohydrate content of the effluent reveals the presence of several carbohydrate-containing peaks in the effluent, and, as was observed earlier, the component which leaves the column shortly after 4 hr retention time showed endotoxic activity. The pattern obtained correlates with the

TABLE 2. *Comparisons of endotoxic activities of various preparations*

Endotoxin	Mouse lethal toxicity (LD_{50} in mg)	Toxic material yield (a)	Local Shwartzman reactivity (maximal dilution in μg)	FI_{20} (μg)	Pyrogenicity MPD (μg)
TA-endotoxin	0.267	155.7	2.50	0.28	0.116
TA-PW	0.203	99.2	1.25	0.13	0.0028
TA-ENZ	0.154	159.7	1.25	0.14	0.0003
ENZ-ENZ	0.153	223.6	2.50	0.32	0.00002

[a] The number of median lethal doses (LD_{50}) isolated from 1 g of bacterial cells.

TABLE 3. *Chemical analyses of the various endotoxin preparations*[a]

Endotoxin	P	N	Amino acids	Total reducing carbohydrates	Hexosamines	Heptoses	2-keto-3-deoxy carbohydrates	Fatty acids
TA-endotoxin	1.63	6.68	19.2	43.7	24.7	2.9	3.8	9.6
TA-PW	0.72	3.47	5.7	57.8	25.8	3.8	4.8	14.7
TA-ENZ	0.90	4.80	12.8	49.6	20.9	3.3	4.6	11.5
ENZ-ENZ	0.78	6.38	27.1	41.6	20.8	3.5	6.4	14.1

[a] All values expressed as percentages.

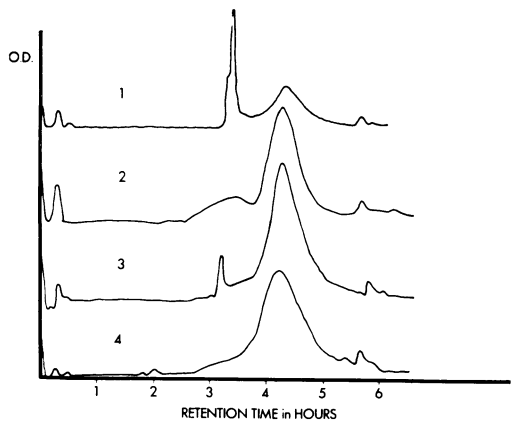

FIG. 1. *Column chromatographic fractionation of various endotoxin preparations. Endotoxins were fractionated by using an Amberlite XE 220 resin, with a continuous gradient as described. Carbohydrate in the effluent was detected by the orcinol reaction, and the resulting chromogen was monitored at 420 nm by using the Technicon autoanalysis system. 1, 3.75 mg of TA-endotoxin. 2, 3.75 mg of TA-PW endotoxin. 3, 3.75 mg of TA-ENZ endotoxin. 4, 3.75 mg of ENZ-ENZ endotoxin.*

biological activities observed. The greatest amount of this component is present in the fourth preparation, which is the ENZ-ENZ endotoxin. In view of the fact that only minor peaks are observed in this preparation in zones which are inert as endotoxins, this preparation shows considerably greater homogeneity in comparison to all other materials. From the shape of the curve as well as from additional analysis, it could be seen that the band leaving after 4 hr of retention time is still not a chromatographically homogeneous substance.

DISCUSSION

A few reports in the literature describe inactivation or degradation of endotoxin by serum fractions (9, 21) or by organs (8, 20). Some of these are attributed to enzymatic processes, but isolation of the enzymes or characterization of their specificities has not yet been achieved. A microbial enzyme preparation was reported by Vincent and Cameron (22) to be able to degrade endotoxin, but reproducibility of the findings was not satisfactory. *Dictyostelium discoideum*, a slime mold which utilizes bacterial cells as nutrients, appears to contain, among others, an endotoxin-inactivating enzyme, since no active endotoxin could be extracted from these bacteria plus slime mold cocultures by the phenol-water procedure. The substance obtained is nontoxic, and has a low percentage of fatty acid-containing, serologically fully active bacterial polysaccharide (A. M. Abdelnoor, Ph.D. thesis, University of Michigan, Ann Arbor, 1969; 12). Attempts to isolate the inactivating enzyme were not successful.

Experiments were performed in our laboratories to find a microorganism which would provide the desired endotoxin-degrading enzyme. Several strains were isolated from soil samples, which could utilize crude trichloroacetic acid-

endotoxin preparation as the sole organic nutrient. These bacteria and fungi were able to utilize endotoxin as a source of nutrient; however, these culture filtrates could not reduce endotoxin lethality. Details of these experiments were described elsewhere (C. B. Lehrer, Ph.D. thesis, Temple University, Philadelphia, Pa., 1971).

The fact that the macromolecule of endotoxin cannot be degraded or inactivated by known hydrolytic enzymes suggests that it requires more specific enzymes, possibly an endotoxinase. Biological inactivation and degradation of endotoxin, which probably takes place in the reticuloendothelial system, may require either a specific enzyme or a specific sequential effect of a number of hydrolases present in the cells of the reticuloendothelial system.

The benefit of the high resistance of endotoxin to common hydrolases is that endotoxin can be liberated and purified from bacterial cells by exclusively enzymatic processes, avoiding harsh chemical treatments. The endotoxin obtained by such a procedure is more active in mouse lethality and in MPD determination than any other preparations tested in our laboratory earlier.

The results reported here were obtained by using *Serratia marcescens* 08 cells. Whether the described enzymatic procedure can be applied to other endotoxin-containing gram-negative bacterial strains remains to be investigated.

The high amino acid content of the ENZ-ENZ is probably due to the fact that native proteins, which are known to be more resistant, were subjected to proteolysis. If one assumes that these amino acids left in the ENZ-ENZ do not participate in endotoxin activity, their removal should further enhance the activity. Experiments with this aim are being conducted in our laboratory at the present time.

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