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REMOVAL OF ESTER-BOUND FATTY ACYL RESIDUES FROM *Salmonella* LIPOPOLYSACCHARIDE BY ENZYME PREPARATIONS FROM *Acanthamoeba castellanii**

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ABSTRACT

Three enzyme preparations (EIa, EIIa, and EIII), which exhibited fatty acyl esterase activity towards *p*-nitrophenyl laurate and towards the lipopolysaccharide from *Salmonella minnesota* R4, were obtained from a cell-free lysate of *Acanthamoeba castellanii*. In the presence of Triton X-100, EIa and EIIa cleaved all ester-bound fatty acids from the lipopolysaccharide, and EIII cleaved non- and 2-hydroxylated fatty acids, but not ester- (and amide-)bound 3-hydroxymyristic acid. The content of heptose, 3-deoxy-2-octulosonic acid, glucosamine, and phosphate in the degraded preparations was unchanged, although phosphatase and *N*-acetyl-β-D-glucosaminidase activity was detectable in the enzyme preparations when tested with the respective *p*-nitrophenyl substrates.

INTRODUCTION

Endotoxin, *i.e.*, bacterial lipopolysaccharide, or its lipid A component, is one of the biologically most active bacterial components. In the late 1930's, W. T. J. Morgan and his associates[1,2] were pioneers in working out the isolation and purification of the endotoxic or O-antigen complex, composed of protein, carbohydrate, and lipid material. These studies formed the basis of further detailed work on the characteristic components of the complex, such as the lipopolysaccharides (LPS) and their lipid A component. The digestion of endotoxin-producing bacteria in phagocytyzing cells may lead to degradation of the lipopolysaccharide with concommittant gradual or complete loss of the characteristic endotoxin activities. Two different phagocytizing cell types have recently been investigated, *i.e.*, certain species of Amoebae[3,4], as well as neutrophils and macrophages of higher animals and man[5].

*Dedicated to Professor Walter T. J. Morgan.
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Over 10 years ago, we began studying the *in vivo* degradation of bacterial LPS in *Dictyostelium discoideum*. It was found that the amoebal enzymes cleaved ester- and amide-bound long chain fatty acids, and phosphate groups from lipid A, leaving intact the lipid A backbone and the polysaccharide linked to it[6]. The degraded product was nontoxic. Verret *et al.*[7] were able to isolate two amoebal amidases of high structural specificity for the respective amide-bound β-hydroxymyristoyl residues of lipid A, but they acted only on the *O*-deacylated product. The substrate was produced by extensive treatment of the lipopolysaccharide with alkali. In the present work, *Acanthamoeba castellanii* served as the source of enzymes. The preparation of fractions with acyl esterase activity from the cell-free lysate of the organism is described. These cleaved the ester-bound fatty acyl residues from lipid A, thus producing the substrate for the action of the amidases.

EXPERIMENTAL

Enzyme assays. — The assays were performed as described[4]. Fatty acyl esterase activity was tested with *p*-nitrophenyl laurate as substrate, fatty acyl amidase qualitatively with 2-deoxy-2-myristoylamino-D-glucose, phosphatase with *p*-nitrophenyl phosphate, *N*-acetyl-β-D-glucosaminidase with *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside, and proteolytic activity with casein (Hammersten, Merck). Lipopolysaccharide-degrading activity was tested in buffer *A* (0.05M sodium citrate–citric acid), the pH ranging from 2.5 to 7.4, and in buffer *B* (0.05M sodium acetate–acetic acid, pH 5), the substrate being the lipopolysaccharide from *Salmonella minnesota* R4. This lipopolysaccharide contains heptose, 3-deoxy-2-octulosonic acid (KDO), lipid A, and the folowing fatty acids: lauric (12:0), myristic (14:0), 2-hydroxymyristic (2-OH-14:0), palmitic (16:0), and 3-hydroxymyristic acid (3-OH-14:0).

Analytical procedures. — Protein was determined according to Lowry *et al.*[8], with bovine serum albumin as the standard, and total glucosamine after hydrolysis with 4M HCl (8 h, 100°) according to Strominger *et al.*[9]. Total ester- and amide-linked fatty acids present in the lipopolysaccharide, or in the degraded lipopolysaccharide after enzyme treatment, were transesterified with 2M methanolic HCl (18 h, 85°), extracted with chloroform, and analyzed by g.l.c.[10]. Free fatty acids liberated from LPS by esterases were extracted with chloroform, and esterified with distilled diazomethane in ether containing methanol, and also analyzed by g.l.c.[10]; 3-hydroxydecanoic and heptadecanoic acid served as internal standards.

Fractionation of the A. castellanii *crude enzyme preparation.* — In a preceding paper[4], we reported that a crude cell-free homogenate of *A. castellanii*, harvested at the early stationary phase of growth, exhibited activities on a variety of substrates, including *p*-nitrophenyl laurate, phosphate, and some D-glycosides. Proteolytic activity towards casein was also demonstrated. In the present study, attempts were made to separate and identify different fatty acyl esterases.

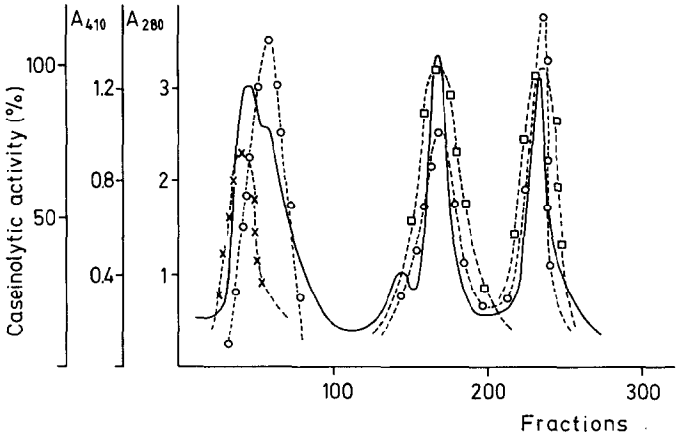

Fig. 1. Chromatography of crude enzyme preparation from *A. castellanii* on DEAE-Sephadex A50. (——) Protein (A_{280}), (O---O---O) activity towards *p*-nitrophenyl laurate (A_{410}), (x---x---x) activity towards *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside, and (□---□---□) activity towards casein. The three fractions obtained were designated as esterase preparation EI, EII, and EIII.

The organisms (*Acanthamoeba castellanii*) and the procedures for their axenic cultivation in a 22-L fermenter have been described previously[4] (yield 0.5 g wet weight/mL). The amoeba were lyzed by three cycles of freezing (−20°) and thawing in the presence of 0.1% Triton X-100. The homogenate was centrifuged (60 min, 30 000*g*, 4°), and the supernatant dialyzed against buffer *C* (0.02M Tris·HCl, pH 7.4; 20 vols.). After centrifugation (60 min, 30 000*g*) to remove insoluble protein, the supernatant (crude enzyme preparation) was kept frozen. For fractionation, the solution (500 mL, 18 mg protein/mL, 2.1×10^5 units of esterase) was applied to a column of DEAE-Sephadex A-50 (8 × 70 cm), equilibrated with buffer *C*. Initial elution was with the same buffer until the effluent was free of protein. The nonabsorbed protein contained some protease activity, but was devoid of the other enzyme activities tested.

The fatty acyl esterases bound to the column were sequentially eluted with 800-mL portions of buffer *C* containing 0.1, 0.2, 0.5, and 1M NaCl. Factions (8 mL, flow rate 60 mL/h) were collected and assayed for fatty acyl esterase, *N*-acetyl-β-D-glucosaminidase, phosphatase, and protease activity, and for protein (Fig. 1). Fatty acyl esterases were obtained in the eluates with 0.1, 0.2, and 0.5M NaCl. *N*-Acetyl-β-D-glucosaminidase and protease activities could also be detected in some fractions. No material was eluted with M NaCl.

The fractions containing high fatty acyl esterase activities were pooled and designated as esterases EI, EII, and EIII, respectively. They were concentrated by precipitation with cold (−20°) acetone (1.5 vols.). After 2 h, the precipitate was isolated by centrifugation, resuspended in buffer *B*, and dialyzed against the same buffer. Subsequent chromatography on a column (2.8 × 85 cm) of Sephadex G-100 with buffer *B* (40 mL/h) permitted the separation of esterase EI and EII each into

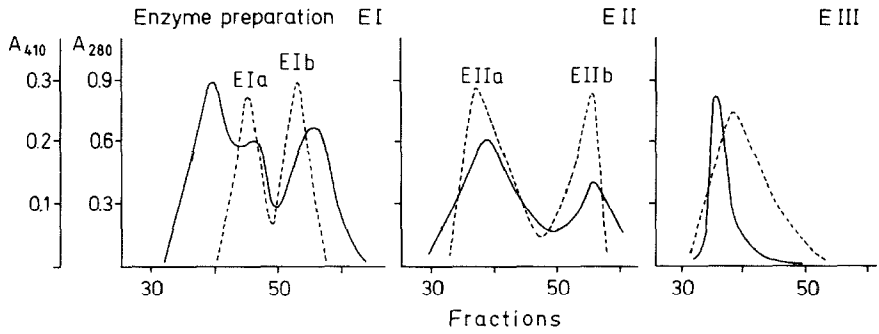

Fig. 2. Gel filtration of esterase fractions EI, EII, and EIII on Sephadex G-100. After precipitation with acetone, the fractions were applied to the column and elution was performed with buffer B: (——) Protein (A_{280}), and (------) activity towards *p*-nitrophenyl laurate (A_{410}).

two fractions (EIa and EIb, and EIIa and EIIb). Esterase EIII was eluted from the column as a single peak separated from a main protein peak (Fig. 2). The respective fractions were pooled and precipitated with acetone. In addition to acylester activity, fraction EIa exhibited also some acylamidase, *N*-acetyl-β-D-glucosaminidase, and phosphatase activity. Proteinase activity was detectable in fractions EIIa (at pH 2.7 and 7.4) and EIiI (at pH 7.4) (not shown). Table I shows the activities towards *p*-nitrophenyl laurate of the various preparations obtained during the separation procedures and the degree of purification achieved.

The pH dependancy of esterase activity of the fractions towards *p*-nitrophenyllaurate (buffer *A*) was determined. Fractions EIa, EIb, and EIII exhibited a pH optimum at about pH 6, fraction EIIb at pH 3–4, and fraction EIIa showed two optima, at pH 4 and at pH >7.

Activity the esterases towards lipopolysaccharide, pH dependency. — The presence of Triton X-100 in the reaction mixture containing lipopolysaccharide as the substrate was found to be essential for all fractions studied, as this stimulated the hydrolysis of acyl residues by 5 to 10 times (data not shown). This is in agreement with previous results[4] and, therefore, Triton X-100 was added routinely when lipopolysaccharide served a substrate. Fig. 3 shows the pH dependency of the liberation of different fatty acyl residues from lipopolysaccharide by enzyme preparations EIa, EIIa, and EIII. It is obvious that under comparable conditions, *i.e.*, buffer *A* (2 mL), LPS (4 mg), esterase (60 units), 2% Triton X-100, 2.5 h, and 35°, the pH profiles of the different preparations towards different acyl residues are distinct. As a consequence, the enzyme preparations differ significantly in their degree of hydrolysis of different *O*-acyl residues, *i.e.*, lauric, myristic, and palmitic acid were released to different degrees by the enzyme preparations, and 3-hydroxymyristic acid was liberated only by preparation Ia. Since preparations EIb and EIIb expressed only very low esterase activity towards lipopolysaccharide, they were not analyzed further.

Extensive treatment of lipopolysaccharide with the enzyme preparations. —

TABLE I

ACTIVITY TOWARDS *p*-NITROPHENYL LAURATE OF FRACTIONS ISOLATED FROM *A. castellanii* CELL-FREE HOMOGENATE

Fractionation step	*Esterase preparation*	*Total volume (mL)*	*Esterase activity*		*Total protein (mg)*	*Specific activity (units/mg)*	*Increase in activity*
			Units/mL	*Total units*			
Crude enzyme preparation		500	360	1.8	16 900	10.6	1.0
DEAE-Sephadex chromatography and acetone precipitation	EI	18	3000	5.4	520	103	9.6
	EII	12	3600	4.3	400	108	10.1
	EIII	14	4000	5.6	440	127	12.0
Sephadex G-100 filtration	EIa	20	500	1.1	48	229	21.5
	EIb	30	430	1.3	51	255	23.9
	EIIa	40	300	1.2	82	146	13.8
	EIIb	30	270	0.8	40	201	18.9
	EIII	55	650	3.6	190	190	17.8

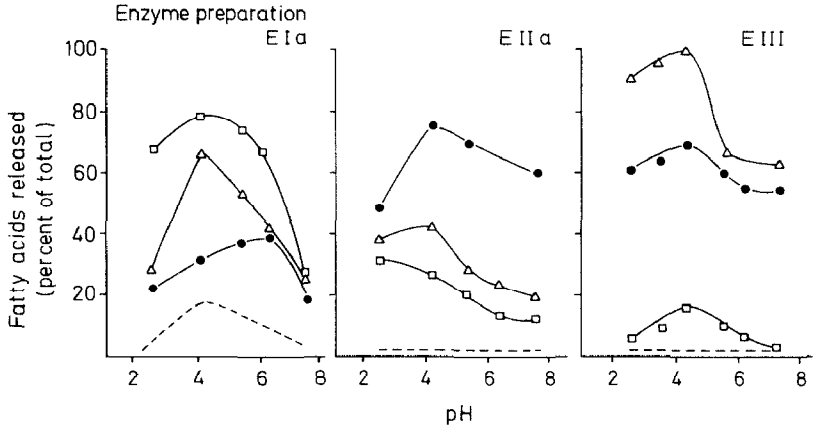

Fig. 3. The pH dependency of release of acyl residues from *S. minnesota* R4 LPS by esterase fractions EIa, EIIa, and EIII. The liberated fatty acids were determined by g.l.c.: (□—□—□) lauric acid, (○—○—○) myristic acid, (△—△—△) palmitic acid, and (------) 3-hydroxymyristic acid.

TABLE II

NATURE AND AMOUNTS OF FATTY ACIDS RELEASED FROM LIPOPOLYSACCHARIDE OF *S. minnesota* R4 AFTER DIGESTION WITH *A. castellanii* ESTERASE

Treatment of LPS with preparation	*Fatty acids*[a]									
	12:0		*14.0*		*2-OH-14:0*		*3-OH-14:0*		*16:0*	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*
EIa	286.6	88.8	182.1	88.5	69.9	82.3	764.9	60.9	143.2	86.2
EIb	170.6	55.9	128.4	63.3	46.9	55.3	136.4	10.8	56.6	34.1
EIIa	229.3	75.8	154.8	75.2	70.1	82.5	226.8	18.1	172.0	100.0
EIIb	88.0	28.8	136.6	66.2	51.2	60.3	60.8	4.8	113.0	68.0
EIII	120.9	39.6	166.4	80.9	64.2	75.6	86.9	6.8	165.9	99.9
No enzyme	302.3	100.0	205.7	100.0	84.9	100.0	1254.8	100.0	166.0	100.0

[a]A: nmol/mg; B: per cent of original LPS. Heptose, KDO, glucosamine, and phosphate values were unchanged.

Lipopolysaccharide (200 mg) in 0.02M acetate buffer, pH 5.0 (100 mL) was incubated with esterase (2×10^3 units) of the respective enzyme preparation in the presence of Triton X-100 (24 h, 35°). Free fatty acids were extracted with chloroform, esterified with diazomethane, and estimated by g.l.c. The results in Table II show that enzyme preparation EIa liberated essentially all of the ester-bound, nonhydroxylated fatty acids (including 2-hydroxymyristic acid), and about 50% of the 3-hydroxymyristic acid, presumably that part which was present in ester linkage. This was confirmed by analysis of the degraded lipopolysaccharide isolated from the incubation mixture, which was shown to be devoid of ester-bound fatty acids, and to contain only 2 mol of 3-hydroxymyristic acid in amide linkage (data

not shown). Preparation EIIa also cleaved a significant portion of the ester bonds in the lipopolysaccharide; the degree of liberation was, however, lower compared to that of preparation EIa (Table II). This is possibly due to the degradation of the enzymes during incubation by proteinases in the preparation. Preparation EIII expressed high activity towards the ester-bound, nonhydroxylated fatty acids, myristic and palmitic acid, whereas only 50% of lauric acid were released. On the other hand, no 3-hydroxymyristic acid was cleaved from the molecule. These data were confirmed by analysis of the degraded lipopolysaccharide, isolated from the incubation mixture; the proportions of heptose, KDO, and phosphate relative to 2-amino-2-deoxyglucose in all the degraded preparations were unchanged, as compared to the original lipopolysaccharide[4].

RESULTS AND DISCUSSION

It is well known that alkali treatment of endotoxic lipopolysaccharide removes the ester-linked fatty acyl residues from the lipid A moiety. The resulting products, which still contain 2 mol of amide-linked 3-OH-14:0 units, are devoid of endotoxic activities but still retain the corresponding antigenic specificity[11]. The present paper describes the enzymic preparation of a product that is analogous to an "alkali-treated lipopolysaccharide". This was achieved by treatment with acyl-esterases (enzyme preparation EIa), obtained from a cell-free lyzate of *Acanthamoeba castellanii*. The chemical analysis showed that the degraded lipopolysaccharide was essentially devoid of the ester-bound fatty acids, but still contained the original proportion of 2 mol of amide-bound 3-OH-14:0, heptose, KDO, glucosamine, and phosphate. Another enzyme fraction, EIII, was also isolated and found to remove only the non- and 2-hydroxyacyl residues without liberation of 3-OH-14:0 units, thus producing a degraded lipopolysaccharide corresponding to the (heptose- and KDO-containing) lipid A precursor molecule[12], which is characterized by the presence of 4 mol of 3-OH-14:0 residues. It is expected that, like the precursor, this degradation product will still retain endotoxin activities. Thus, the amoebal enzymes appear to be of general interest.

Amoebae, as well as animal phagocyting-cells contain all the enzymes necessary to transform the originally fully substituted lipid A compound into a non-substituted diglucosamine disaccharide still linked to the respective specific S or R form of the polysaccharide[5,6]. Obviously, these cells do not degrade the polysaccharide component of the lipopolysaccharide. So far, only certain phage enzymes have been described, which specifically cleave these polysaccharides[13]. Thus, of the whole bacterial lipopolysaccharide, the amoebae can only metabolize and make use of the lipid A substituents. This, they seem to perform, however, with high specificity and efficiency. Thus, these enzymes may be of interest for further studies on specific structural degradation procedures of the endotoxically active lipid A component. Their use will extend the synthetic approaches[14] by which the substrates or the end products of the enzyme action can be investigated in pure systems.

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