Onderstepoort J. vet. Res., 54, 39–43 (1987)

PURIFICATION OF THE ALPHA TOXIN OF *CLOSTRIDIUM PERFRINGENS* TYPE A BY ULTRAFILTRATION AND GEL CHROMATOGRAPHY

M. W. ODENDAAL, Roodeplaat Research Laboratories, P.O. Box 13873, Sinoville 0129

ABSTRACT

ODENDAAL, M. W., 1987. Purification of the alpha toxin of *Clostridium perfringens* type A by ultrafiltration and gel chromatography. *Onderstepoort Journal of Veterinary Research*, 54, 39–43 (1987)

Clostridium perfringens type A toxin produced in Jayko & Lichstein medium was subjected to various concentration and purification procedures.

The results obtained with 3 different ultrafiltration membranes followed by gel filtration showed that by using Millipore PSED OHV10 and Amicon XM-100 filter membranes in combination, a three-hundred-and-fivefold purification could be achieved as against a twelvefold increase obtained with ammonium sulphate/acetone precipitation.

The lecitovitelin test was more sensitive than the haemolytic activity in determining the alpha toxin activity. The optical density, measured at 280 nm, did not reveal any alpha toxin activity in the relevant toxic fractions.

INTRODUCTION

The *in vitro* production of phospholipase C depends to a great extent on the composition of the medium in which *C. perfringens* type A is grown. The use of a complex medium, as opposed to the use of a synthetic medium for alpha toxin production (Murata, Yamamoto, Soda & Ito, 1965), has definite advantages, and is usually the medium of choice amongst researchers, with each one making his own minor alterations (Jayko & Lichstein, 1959; Nakamura, Cook & Cross, 1968; Nakamura, Schultze & Cross, 1969; Nord, Möllby, Smyth & Wadström, 1974). Physical factors also play an important role in toxin production, especially as regards the oxidation-reduction potential, the pH of the medium, and the temperature and length of incubation (Dolby & MacFarlane, 1956; Murata *et al.*, 1965; Schultze & Nakamura, 1968; Nakamura *et al.*, 1969; Möllby, Holme, Nord, Smyth & Wadström, 1976; Mahoney, 1977). The regulation of pH in a continuous batch culture has also been found to improve the extracellular toxin production of *C. perfringens* types A, B, C and D (Pivnick, Habeeb, Gorenstein, Stuart & Hauschild, 1964; Pivnick, Hauschild, Gorenstein & Habeeb, 1965; Nord *et al.*, 1974; Möllby *et al.*, 1976).

The first purification of the alpha toxin from a toxic culture supernatant was achieved by Prigg (1937), as quoted by MacFarlane & Knight, (1941), using sodium sulphate and ammonium sulphate, and subsequently by Van Heyningen (1941) with ammonium sulphate alone. They prepared a crude toxin with these precipitation reactions, which took place at various saturation levels. Dixon (1953) calculated a nomogram to facilitate the determination of the exact quantities of ammonium sulphate to achieve a certain degree of saturation. The ammonium sulphate precipitation reaction was often used to prepare a crude toxin for application to gel filtration chromatography (Ikezawa, Yamamoto & Murata, 1964; Ito, 1968; Diner, 1970; Mitsui, Mitsui & Hase, 1973; Katsaras & Hartwigk, 1979) or electrofocusing (Smyth & Arbuthnott, 1974). During the ammonium sulphate precipitation, the alpha toxin is subjected to considerable biochemical manipulation, with possible resultant deterioration of the molecular structure. This is reflected in the rather poor recovery rates obtained by Van Heyningen (1941) 54 %, Bangham & Dawson (1962) 56 %, Roth & Pillemer (1953) 14 %, and Smyth & Arbuthnott (1974) 56,6 %.

As far as could be ascertained, Stephen (1961) was one of the first to use high pressure ultrafiltration to obtain crude concentrates rich in alpha toxin for examination by zone electrophoresis and immunoelectrophoresis.

Received 6 November 1986—Editor

It is important to appreciate that ultrafiltration is not a high resolution separation technique. Its biggest advantage is the delicate way in which the biological molecules are treated, because it involves no phase change, maintains a constant pH and ionic strength and does not expose the molecules to temperature fluctuations or large surface areas. Also large volumes of culture supernatant can be filtrated in a short period. Ultrafiltration has been used quite successfully in the initial purification of the alpha toxin of *Clostridium oedimatiens* (Izumi, Kondo, Ohishi & Sakaguchi, 1983) as well as tetanus toxin and toxoid (Cox, Liefman, Premier, Chandler, Herrington, Middleton & Hurrell, 1984) as a means of concentrating the active substance in the culture supernatant for further purifying procedures.

The purpose of this paper was to compare the relative efficiency of 3 different ultrafiltration membranes for concentrating the alpha toxin sufficiently for subsequent gel filtration chromatography. A comparison was also made between these results and those obtained by the ammonium sulphate and acetone precipitation technique prior to gel filtration chromatography.

MATERIALS AND METHODS

Bacterial strains

Over a period of 3 years, 122 different isolates of *C. perfringens* type A were isolated from various animal sources, identified by means of their biochemical reactions (Smith, 1975) and typed according to the intradermal toxin neutralization test in guinea pigs (Sterne & Batty, 1975). Eventually, Strain 142 was chosen because of its ability to produce high levels of toxin under specified conditions (Odendaal, 1983).

Culture media

Blood tryptose agar[1] containing 10 % defibrinated sheep blood was used for the initial growth of the *C. perfringens* type A cultures under anaerobic conditions. Jayko & Lichstein's (1959) medium, modified by substituting sucrose with D(+) glucose[1], was used for toxin production.

Toxin production

Blood tryptose agar[1] plates were inoculated in batches of 12, 2 of which were incubated at 37 °C under aerobic conditions for detecting the presence of contaminants. The remaining 10 plates were incubated overnight in an anaerobic stainless steel flask[2] under anaerobic conditions at 37 °C (Sutter, Vargo & Finegold, 1975). After ensuring macroscopically that the 10 inoculated plates were free of contaminants based on colony morphology and gram-stained smears (Carter, 1973), all the bacterial

[1] Merck Chemicals, Johannesburg

[2] Baird & Tatlock, supplied by Merck Chemicals, Johannesburg

growth was transferred under laminar flow[3] to the 1 ℓ of medium in the fermenter culture vessel[4] by means of an angular, bent, glass rod.

The pH (7,0) and temperature (35 °C) were automatically regulated throughout the 3 h growth period. Anaerobic subcultures were made to monitor the purity of the culture. The culture containing the crude toxin was centrifuged at 10 400 × g[5] in 200 mℓ polypropylene flasks at 8 °C and the supernatant fluid was stored at 6–8 °C.

Phospholipase C standard curve

The establishment of a phospholipase C standard curve with a commercial product for the quantitative determination of phospholipase C was a standard procedure that was repeated in this study (Weiss & Strong, 1967; Nakamura & Cross, 1968; Mitsui *et al*., 1973). The correlation coefficient (r=0,99) was calculated (Clarke, 1977), as well as the regression line from which the concentrations of phospholipase C were determined in μg/mℓ.

Protein standard curve

A standard curve for the determination of protein concentration, using bovine serum albumen, was also standard procedure necessary for calculating the purity of the toxin and establishing a ratio in terms of mg phospholipase C per mg protein. The correlation coefficient was calculated between the 2 variables (r = 0,99). After construction of the line of regression, the protein concentration was calculated in mg/mℓ.

Assay of crude and purified alpha toxin

Lecitovitelin reaction (phospholipase C activity). The egg yolk or lecitovitelin suspension (LV) was made up according to the method of Sterne & Batty (1975), and standardized spectrophotometrically[6], at a wavelength of 470 nm (Mitsui *et al*., 1973). The stock solution was prepared fresh every week, whereas the standardized suspension was prepared daily. A standard curve was prepared, using a commercial *C. perfringens* type A phospholipase C[7] in concentrations ranging from 0,01 mg/mℓ to 0,1 mg/mℓ. The correlation coefficient and linear regression were calculated according to Clarke (1977). The LV reaction was performed as described by Mitsui *et al*., (1973), and the phospholipase C concentration was calculated from the standard curve in μg/mℓ.

Haemolytic activity of alpha toxin. Sheep red blood cells were collected in an equal volume of Alsevers solution (Garvey, Cremer & Sussdorf, 1977) and washed twice in normal physiological saline. A 1 % red blood cell suspension was prepared according to Mitsui *et al*., (1973), and subsequently used to assay the haemolytic activity of the alpha toxin in crude and purified form. This activity was expressed in haemolytic units which can be defined as the highest dilution of alpha toxin causing haemolysis exceeding an absorbance of 0,47 measured spectrophotometrically at a wavelength of 550 nm.

Protein determination. The protein contents of all toxic fractions, whether crude or pure, were determined by using the method of Lowry, Rosebrough, Farr & Randall, (1951). A standard curve, using bovine serum albumen (fraction V)[8], was established with concentrations ranging from 0,01 to 0,4 mg/mℓ. The correlation coefficient and linear regression line were calculated according to Clarke (1977).

Optical density. Measuring the optical density of a substance at 280 nm is a convenient method of indicating the presence of certain polypeptides and nucleic acids. Although no quantitative determination can be made by this method, it is often used (Diner, 1970; Zwaal, Roelofsen, Comfurius & Van Deenen, 1971). The optical density of most fractions eluted from the gel filtration column was measured at 280 nm.

Purification of the alpha toxin

Ammonium sulphate and acetone fractionation. One ℓ of culture supernatant prepared in a fermenter was subjected to fractionation by ammonium sulphate and acetone, according to the method by Mitsui *et al*., (1973). The final product was freeze-dried and kept at −20 °C for later gel filtration chromatography.

Ultrafiltration. Three different ultrafiltration membranes from 2 different systems were used. The XM-50 and XM-100 filter[9] membranes have a molecular cut-off level of 50 000 and 100 000 Dalton units, respectively. Ultrafiltration was accomplished by a positive nitrogen pressure of 2–3 kg/cm^2, in a filter unit with a maximum capacity of 550 mℓ. After filtration, these filters were preserved in 96 % ethyl alcohol.

The 3rd type of filter was a PSED OHV 10 filter[10] with a molecular cut-off level of 25 000 Dalton units. Here, ultrafiltration took place with a peristaltic pump with a pressure of 2 kg/cm^2 and was supplied by a filterunit with a maximum capacity of 10 ℓ. After ultrafiltration these filters were preserved in 1 % formalin.

The retentate, which was obtained after a single run through a XM-50 filter membrane and contained the concentrated alpha toxin, was freeze-dried and kept at −20 °C until its application on the gel filtration column.

The 2nd ultrafiltration method comprised in succession filtration by both the PSED OHV 10 and XM-100 membranes. The retentate, collected after passing the culture supernatant through the PSED OHV 10 filter, was passed through the XM-100 filter. The resultant filtrate was freeze-dried for further gel chromatography.

In the final ultrafiltration method, the XM-100 filter was used alone, and the filtrate was collected, freeze-dried and kept at −20 °C for gel filtration.

Gel filtration. A glass column (3,5 × 100 cm) with a bed volume of 510 mℓ was packed with 34 g of Sephadex G100[11] after equilibration in 1 ℓ of 0,01 M borate buffer, pH 7,1. Gel filtration was performed on the material obtained by ammonium sulphate and acetone fractionation, and ultrafiltration with each of the XM-50, XM-100 and XM-100/PSED OHV 10 filter membranes. The fraction volume ranged betwee 5 and 7 mℓ, the hydrostatic pressure was 45 cm, and the flow rate was approximately 48 mℓ per h. In every case, 100 fractions were collected and tested for the presence of phospholipase C. This procedure was performed at 4 °C. The protein concentrations and optical densities were determined of those fractions that showed phospholipase C activity.

[3] Senior Medical Services, Johannesburg

[4] Gallenkamp Modular Fermenter, supplied by Merck Chemicals, Johannesburg

[5] Sorvall RC3B, Du Pont Instruments, distributed by Zeiss (West Germany), Johannesburg

[6] Hitachi Perkin Elmer UV-VIS, Coleman 139, distributed by Protea Laboratories, Johannesburg

[7] PL Biochemicals Inc., Wisconsin USA, distributed by Weil Organization, Johannesburg

[8] Sigma Chemicals, supplied by Labretoria BK/CC, Posbus 20295, Alkantrant, Pretoria

[9] Amicon Diaflo, distributed by Laboratory and Scientific Equipment, Johannesburg

[10] Pellicon Cassette System, Millipore, (SA) Johannesburg

[11] Pharmacia, Protea PNI, Johannesburg

TABLE 1 The optical activity, phospholipase C and protein concentrations of fractions 42–46 of the purified alpha toxin after ammonium sulphate/acetone fractionation and Sephadex gel chromatography

Fraction	Optical density (280 nm)	Protein (mg/mℓ)	Total protein/fraction (mg)	Phospholipase C (μg/mℓ)	Total phospholipase C per fraction (mg)	mg phospholipase C/mg protein
42	0,105	0,04	0,28	167	1,2024	4,29
43	0,1	0,036	0,25	167	1,2024	4,29
44	0,095	0,062	0,44	155	1,116	2,5
45	0,09	0,022	0,15	130	0,936	6,24
46	0,08	0,019	0,13	111	0,7992	6,14

RESULTS

Production of a toxic culture supernatant

The procedure used was selected after testing 122 different *C. perfringens* type A strains in 4 different media and at 2 different variations in pH and temperature (Odendaal, 1983).

Preliminary comparison of various culture media and conditions showed that modified Jayko & Lichstein medium gave the best toxin yield. Toxin was obtained by growing *C. perfringens* type A strain 142, in 1 ℓ of Jayko & Lichstein medium in a fermenter with an automatically regulated pH of 7,0 and a temperature of 37 °C. It contained 0,34 mg protein/mℓ; 0,49 mg phospholipase C/mg protein and 167 μg/mℓ phospholipase C activity.

Ammonium sulphate and acetone precipitation followed by gel filtration

The purification of culture supernatant by ammonium sulphate and acetone precipitation, followed by Sephadex gel filtration chromatography, resulted in only a twelvefold concentration of the alpha toxin, as judged by the amount of phospholipase C per mg protein (Table 1).

The first phospholipase C activity appeared in fraction 25, reached a peak in fractions 42 and 43 and disappeared completely in fraction 53. The mg phospholipase C per mg protein ratio differed slightly in its fraction distribution in that the peak values occurred in fractions 45 and 46. With this method, the phospholipase C was purified only twelvefold from 0,49 mg phospholipase C per mg protein to 6,24 mg phospholipase C per mg protein. The optical density measured at 280 nm showed no increased activity in the eluted toxic fractions, although some activity was noted in the region of fraction 20 and fractions 68–72. An unexpected high value of 0,44 mg protein which occurred for fraction 44 resulted in a decreased mg phospholipase C/mg protein ratio of 2,5. No feasible explanation can be given.

TABLE 2 The volume, protein concentration and phospholipase C activity of the culture supernatant (SUP) before filtration and the retentate (RET) and filtrate (FIL) after ultrafiltration with XM-50 membrane

Fraction	Volume (mℓ)	Protein (mg/mℓ)	Phospholipase C (μg/mℓ)	mg phospholipase C/mg protein
Sup	477,5	18,2	75,4	0,0044
Ret	46,16	24,2	114,15	0,0045
Fil	423,33	16,86	6,4	0,0012

TABLE 3 The phospholipase C activity and protein concentration of fractions 39–41 after gel filtration of XM-50 retentate on Sephadex G100

Fraction	Phospholipase C		Protein		mg phospholipase C/mg protein
	μg/mℓ	Total mg	mg/mℓ	Total mg	
39	138	0,828	0,174	1,044	0,79
40	139	0,834	0,154	0,924	0,9
41	124	0,744	0,2	1,2	0,62

TABLE 4 The phospholipase C activity and protein concentration of the culture supernatant (SUP), the retentate (RET 1) and filtrate (FIL 1) after filtration through PSED OHV 10 membrane, with retentate (RET 2) and filtrate (FIL 2) after filtration through XM-100 membrane

Fraction	Volume (mℓ)		Protein mg/mℓ	Phospholipase C μg/mℓ	mg phospholipase C/mg protein
	Pre-	Post-			
Sup	900		13,2	125	0,009
Ret 1	900	150	22,4	117	0,005
Fil 1	900	750	3,6	0	0
Ret 2	150	27	42,25	113	0,002
Fil 2	150	120	23,2	100	0,0043

TABLE 5 The phospholipase C activity and protein concentration of fractions 42–44 after completion of gel filtration of RET 2 on Sephadex G100

Fraction	Phospholipase C μg/mℓ	Protein mg/mℓ	mg phospholipase C/mg protein
42	33	0,012	2,75
43	38,5	0,022	1,75
44	38	0,022	1,75

TABLE 6 Protein and phospholipase C concentration of the supernatant (SUP) before the retentate (RET) and filtrate (FIL) after filtration with a XM-100 filter membrane

Fraction	Volume (mℓ)		Protein mg/mℓ	Phospholipase C μg/mℓ	mg phospholipase C/mg protein
	Pre-	Post-			
Sup	500		0,92	150	0,163
Ret	500	50	0,66	167	0,253
Fil	500	450	0,87	146	0,167

TABLE 7 Phospholipase C and protein concentrations of fractions 39–41 after elution of filtrate (XM-100 membrane) on Sephadex G100

Fraction	Phospholipase C μg/mℓ	Protein mg/mℓ	mg phospholipase C/mg protein
39	129	0,032	4,03
40	144	0,036	4,00
41	147	0,05	2,94

A control run with pure medium, applying the same procedures as for a toxic supernatant, produced no signs of phospholipase C.

Ultrafiltration and gel filtration chromatography

Amicon Diaflo XM-50. The volume, protein concentration and phospholipase C activity were measured from the various fractions before and after ultrafiltration (Table 3). The mg phospholipase C per mg protein in the culture supernatant and the retentate after filtration did not differ significantly, which meant that there was still a large quantity of contaminating protein or polypeptide present.

TABLE 8 A summary of the results obtained for each of the 4 methods used to purify the phospholipase C in mg phospholipase C per mg protein

Method	Sup	Ret	Fil	GF*	Purification factor
1	0,49	—	—	6,24	12,7
2	0,0044	0,0045	—	0,9	200
3	0,009	—	0,043	2,75	305
4	0,163	—	0,167	4,03	24

* The eluted fraction with the highest mg phospholipase C/mg protein ratio

Method 1—Ammonium sulphate/acetone precipitation with gel filtration

Method 2—Ultrafiltration with XM-50 membrane with gel filtration

Method 3—Ultrafiltration with PSED OHV 10 and XM-100 membrane with gel filtration

Method 4—Ultrafiltration with XM-100 and gel filtration

After gel filtration, the phospholipase C activity occurred only in fractions 32–47. Fraction 40, containing the most phospholipase C activity of 139 μg/mℓ with a protein content of 0,154 mg/mℓ, gave a mg phospholipase C per mg protein ratio of 0,9 (Table 3). A two-hundredfold increase in purity was achieved. The optical density was not measured.

Millipore PSED OHV 10 and Amicon Diaflo XM-100. The phospholipase C activity and protein concentration of toxic culture supernatant prepared for ultrafiltration was 125 μg/mℓ and 13,2 mg/mℓ, respectively. After the first filtration through the PSED OHV 10 ultra membrane with a molecular cut off of 25 000 Dalton units, there was a slight decrease in the phospholipase C activity to 117 μg/mℓ and an increase in the protein content to 22,4 mg/mℓ (Table 4). After elution of the RET 2 fraction, through Sephadex G100, the protein content declined dramatically to 0,012 mg/mℓ and 0,022 mg/mℓ for fractions 42 and 43, respectively (Table 5). This led to an increased mg phospholipase C per mg protein ratio of 2,75 and 1,75 for fractions 42 and 43, respectively. For fraction 42, a three-hundred-and-fivefold purification was achieved.

Amicon Diaflo XM-100. The mg phospholipase C per mg protein ratio for the crude toxin in culture supernatant of 0,163 did not differ greatly from that of the final product (0,167) in the filtrate (Table 6). The retentate also contained a substantial amount of phospholipase C activity and did not really fit into the picture. The filtrate was freeze-dried to be reconstituted for later use. After elution on the Sephadex column, fractions 39–41 gave the highest ratios mg phospholipase C per mg protein of 4,03; 4,00 and 2,94, respectively. The protein concentrations decreased from 0,92 to 0,032 mg/mℓ (Table 7, fraction 39), with a slight concomitant decrease in phospholipase C from 150 to 129 mg/mℓ. A twenty-fourfold purification was achieved.

A summary of the results obtained with all 4 methods is given in Table 8. Methods 2 and 3 provided a product which was purified 200 and 305 times, respectively.

DISCUSSION

The molecular mass of the alpha toxin, as determined by various authors quoted by Smyth & Arbuthnott (1974), ranges from 26 300–106 000. Though Mitsui *et al.* (1973) calculated its molecular mass as 49 000 by means of Sephadex gel filtration, Smyth & Arbuthnott's (1974) calculation with SDS polyacrylamide disk-gel electrophoresis was 53 800. During ultrafiltration, both these molecular masses were used as guide-lines as a subjective indicator to the toxic activity of each fraction. This was well illustrated after ultrafiltration with the XM-50 membrane with a cut-off level of 50 000 Dalton units. The retentate, which included most of the large molecules, contained most of the phospholipase C activity, whereas the filtrate, which included all the molecules with a molecular mass less than 50 000, contained much less activity. This would imply that the molecular mass of the alpha toxin was greater than 50 000 Dalton units. After ultrafiltration with the Millipore PSED OHV 10 filter membrane, it was quite clear that the alpha toxin was not less than 25 000 Dalton units (Table 4), but after filtration through the XM-100 filter membrane, phospholipase C activity appeared in the retentate fraction as well as in the filtrate. The alpha toxin was expected only in the filtrate (FIL 2, Table 4). One possible explanation for this phenomenon is that the alpha toxin formed aggregates with a molecular mass exceeding 100 000 Dalton units.

Compared with the single membrane system, the combined ultrafiltration system gave the best results (Table 8). From a practical point of view it is evident that ultrafiltration is less time-consuming, easier to perform and less laborious than ammonium sulphate and acetone precipitation. It is not feasible to use it alone, because it cannot replace the other more sensitive and technically more involved techniques, but, if used purely in conjunction with gel filtration chromatography, it is effective as a method for concentrating the alpha toxin activity.

REFERENCES

BANGHAM, A. D. & DAWSON, R. M. C., 1962. Electrokinetic requirements for the reaction between *C. perfringens* alpha-toxin (phospholipase C) and phospholipid substrates. *Biochemica et Biophysica Acta*, 59, 103.

CARTER, G. R., 1973. Diagnostic procedures in veterinary microbiology. (2nd edition). Springfield, Illinois: Charles C. Thomas.

CLARKE, G. M., 1977. Statistics and experimental design. Edward Arnold, 25 Hill Street, London.

COX, J. C., LIEFMAN, C. E., PREMIER, R. R., CHANDLER, H. M., HERRINGTON, R. W., MIDDLETON, H. D. & HURRELL, J. G. R., 1984. Immune response and reactions to various dose regimens for raising hyperimmune antisera in sheep. *Veterinary Immunology and Immunopathology*, 7, 65–72.

DINER, B. A., 1970. Purification and properties of *Clostridium welchii* phospholipase C. *Biochemica et Biophysica Acta*, 198, 514–522.

DIXON, M., 1953. A nomogram for ammonium sulphate solutions. *Biochemical Journal*, 54, 457–458.

DOLBY, E. E. & MACFARLANE, M. G., 1956. Variations in the toxicity of lecithinases (Alpha Toxin) from different strains of *Clostridium welchii*. *British Journal of Experimental Pathology*, 37, 324–332.

GARVEY, J. S., CREMER, N. E. & SUSSDORF, D. H., 1977. Methods in immunology. A laboratory text for instruction and research. (3rd edition). W. A. Benjamin Inc.: Reading, Massachusetts.

IKEZAWA, H., YAMAMOTO, A. & MURATA, R., 1964. Gelfiltration of *Clostridium perfringens* alpha toxin (Phospholipase C). *Journal of Biochemistry*, 56, 480.

ITO, A., 1968. Alpha toxoid of *Clostridium perfringens*. I. Purification and toxoiding of alpha toxin of *C. perfringens*. *Japanese Journal of Medical Science and Biology*, 21, 379–391.

IZUMI, N., KONDO, H., OHISHI, I & SAKAGUCHI, G., 1983. Purification and characterization of alpha toxin of *Clostridium oedematiens* type A. *Japanese Journal of Medical Science and Biology*, 36, 135–145.

JAYKO, L. G. & LICHSTEIN, H. C., 1959. Nutritional factors concerned with growth and lecithinase production by *Cl. perfringens*. *Journal of Infectious Diseases*, 104, 142–151.

KATSARAS, K. & HARTWIGK, H., 1979. Reinigung von Toxinen des *Clostridium perfringens* Typ A, Mittels DEAI Ionen Austauscher und Gelfiltration. *Zentralblatt für Veterinär Medizin*, B, 26, 623–633.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. L., 1951. Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, 103, 265–274.

MACFARLANE, M. G. & KNIGHT, B. C. J. G., 1941. The biochemistry of bacterial toxins. The lecithinase activity of *Clostridium welchii*. *Biochemical Journal*, 35, 884–901.

MAHONEY, D. E., 1977. Stable L-forms of *Cl. perfringens:* Growth, toxin production and pathogenicity. *Infection and Immunity*, 15, 19–25.

MITSUI, K., MITSUI, N. & HASE, J., 1973. *Cl. perfringens* exotoxins. 1. Purification and properties of alpha toxin. *Japanese Journal of Experimental Medicine*, 43, 65–80.

MÖLLBY, R., HOLME, T., NORD, C. E., SMYTH, D. J. & WADSTRÖM, T., 1976. Production of phospholipase C (alpha toxin), haemolysins and lethal toxins by *Cl. perfringens* types A to D. *Journal of General Microbiology*, 96, 137–144.

MURATA, R., YAMAMOTO, A., SODA, S. & ITO, A., 1965. Nutritional requirements of *Cl. perfringens* PB 6K for alpha toxin production. *Japanese Journal of Medical Science and Biology*, 18, 189–202.

NAKAMURA, M. & CROSS, W. R., 1968. The lecithinase (alpha toxin) activity of strains of *Cl. perfringens*. *Procedures of the Society of Experimental Biology and Medicine*, 127, 719–722.

NAKAMURA, M., COOK, J. A. & CROSS, W. R., 1968. Lecithinase production *Cl. perfringens* in chemically defined media. *Applied Microbiology*, 16, 9, 1420–1421.

NAKAMURA, M., SCHULTZE, J. A. & CROSS, W. R., 1969. Factors affecting lecithinase activity and production in *Cl. welchii*. *Journal of Hygiene*, 67, 153–162.

NORD, C. E., MÖLLBY, R., SMYTH, C. & WADSTRÖM, T., 1974. Formation of phospholipase C and theta haemolysin in pre-reduced media in batch and continuous culture of *Cl. perfringens* type A. *Journal of General Microbiology*, 84, 117–127.

ODENDAAL, M. W., 1983. Die alpha toksien van *Clostridium perfringens* tipe A. Thesis submitted to the Faculty of Veterinary Science, University of Pretoria, in partial fulfilment for the degree M.Med. Vet. (Bact).

PIVNICK, H., HABEEB, A. F. S. A., GORENSTEIN, B., STUART, P. F. & HAUSCHILD, A. H. W., 1964. Effect of pH on toxinogenesis by *Cl. perfringens* type C. *Canadian Journal of Microbiology*, 10, 329–344.

PIVNICK, H., HAUSCHILD, A. H. W., GORENSTEIN, B. & HABEEB, A. F. S. A., 1965. Effect of controlled pH on toxigenesis by *Cl. perfringens* type D. *Canadian Journal of Microbiology*, 11, 45–55.

ROTH, F. B. & PILLEMER, L., 1953. The separation of alpha toxin from filtrates of *Clostridium welchii*. *Journal of Immunology*, 70, 5, 533–537.

SCHULTZE, J. A. & NAKAMURA, M., 1968. Lecithinase activity of mutant and wild strains of *Cl. perfringens*. *Applied Microbiology*, 16, 1619–1620.

SMITH, L. D. S., 1975. The pathogenic anaerobic bacteria. 2nd ed. Springfield, Illinois USA: Charles C. Thomas.

SMYTH, C. J. & ARBUTHNOTT, J. P., 1974. Properties of *Cl. perfringens* type A alpha toxin (phospholipase C) purified by electrofocusing. *Journal of Medical Microbiology*, 7, 41–66.

STEPHEN, J., 1961. The isolation of the alpha toxin of *Cl. welchii* type A by zone electrophoresis in vertical columns. *Biochemical Journal*, 80, 578–584.

STERNE, M. & BATTY, I., 1975. Pathogenic *Clostridia*. London and Boston: Butterworths.

SUTTER, V. L., VARGO, V. L. & FINEGOLD, S. M., 1975. Wadsworth anaerobic bacteriology manual, Second Edition. From the Anaerobic Bacteriology Laboratory, Wadsworth Hospital Center, Veterans Administration, Wilshire and Sawtelle Bluds, Los Angeles, Ca 90073 and the Department of Medicine UCLA School of Medicine, Los Angeles, Ca 90024.

VAN HEYNINGEN, W. E., 1941. The biochemistry of the gas gangrene toxins. 2. Partial purification of the toxins of *C. welchii* type A. *Biochemical Journal*, 35, 1246–1256.

WEISS, K. F. & STRONG, D. H., 1967. Some properties of heat resistant and heat sensitive strains of *C. perfringens*. *Journal of Bacteriology*, 93, 21–26.

ZWAAL, R. F. A., ROELOFSEN, B., COMFURIUS, P. & VAN DEENEN, L. L. M., 1971. Complete purification and some properties of phospholipase C from *Bacillus cereus*. *Biochemica et Biophysica Acta*, 233, 474–479.