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Large scale purification of *Clostridium perfringens* toxins: a review

Maria Taciana Holanda Cavalcanti[1,2], Tatiana Porto[1,2], Ana Lúcia Figueiredo Porto[3], Igor Viana Brandi[4] , José Luiz de Lima Filho[1], Adalberto Pessoa Junior[2]*

[1]Laboratório de Imunopatologia Keizo-Asami (LIKA), Universidade Federal de Pernambuco [2]Departamento de Tecnologia Bioquímico-Farmacêutica, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, [3]Departamento de Morfologia e Fisiologia Animal, Universidade Federal Rural de Pernambuco, [4]Instituto de Ciências Biomédicas, Universidade de São Paulo.

Clostridium perfringens*, a Gram-positive anaerobic bacterium, is widespread in the environment and commonly found in the intestines of animals, including humans.* C. perfringens *strains are classified into five toxinotypes (A, B, C, D and E) based on the production of four major toxins (α, β, ε, ι). However the toxins (theta, delta, lambda and enterotoxin) are also synthesized by* C. perfringens *strain. Many attempts to purify the toxins produced by C. perfringens have been proposed. In this review we discuss the purification methods used to isolate toxins from* C. perfringens *reported in last four decades.*

Uniterms
- *Clostridium perfringens*
- Purification
- Toxin
- Downstream

***Correspondence:**
A. Pessoa-Jr
Biochemical and Pharmaceutical Technology Department
FCF/USP
P.O. Box 66083
05315-970 - São Paulo-SP – Brazil
E-mail: pessoajr@usp.br

INTRODUCTION

Recent advances in genetic engineering, DNA recombinant technology, cellular fusion technology, and biotechnology in general, make possible the commercial production of new active products as pharmaceuticals, vaccines and hormones. However, the general purification technology for these products has been developed slowly mainly as compared to the production technology. Purification is troublesome because of system complexity and the need to retain biological activity. Usually, biological material has been purified by precipitation with salts or organic solvents, and by using various chromatographic techniques, all of them having enormous difficulties in large-scale applications (Alves *et al.*, 2000). Besides, according to Diamond and Hsu (1992), 50-90% of the biological products production costs are determined by the purification strategy.

Many attempts to purify toxins from *Clostridium perfringens* have been reported (Krug, Kent, 1984; Moreau, Jolivet-Reynaud, Alouf, 1986; Stephen, 1961; Ito, 1968; Smyth, Arbuthnott., 1974; Mitsui, Mitsui, Hase, 1973; Möllby, Wadström, 1973; Bird, Low, Stephen, 1974; Takahashi, Sugahara, Ohsaka, 1974; Yamakawa, Ohsaka, 1977; Jolivet-Reynaud, Moreau, Alouf, 1988) However the separation is rather difficult since the variety of toxins produced from this microorganism is high (12 toxins are known) (Hirata *et al*. 1995). Generally, the main methods used for protein purification are carried out by chromatography as ion exchange, affinity, hydrophobic interaction, and gel filtration. These different types of processes provide the choice for selective fractionation and concentration of macromolecular commodities and the new expanding portfolio of bioproducts (Lyddiatt, 2002).

C. perfringens is a Gram-positive anaerobic bacterium, and able to form spores. It is widespread in the environment, commonly found in the intestines of animals and humans, and can be pathogenic. In humans, it can

cause gangrene and gastrointestinal diseases (for instance: food poisoning and necrotic enteritis), whereas in other animals, gastrointestinal and enterotoxemic diseases occur more frequently (Petit, Gibert, Popoff, 1999; Gkiourtzidis *et al*., 2001).

C. perfringens strains are classified into five toxinotypes (A, B, C, D and E) based on the production of four major toxins (α, β, ε, ι) (Table I) (Petit, Gibert, Popoff, 1999). *C. perfringens* type A is the most common toxinotype in the environment, is ubiquitous, and responsible for gangrene in humans, mediated primarily by α-toxin, secondarily by θ-toxin and hydrolytic enzymes. This strain is also associated with food poisoning in humans, because synthesizes an enterotoxin (CPE) that is responsible for the gastrointestinal symptoms. Unlike other toxins, CPE is only produced during sporulation (McClane, 1996). The enterotoxin (CPE) is also produced by toxinotype B, C, D and E (Table II) (McClane, 1996). *C. perfringens* types B produce α-toxin, β-toxin and ε-toxin, but the higher production is β-toxin, which causes enterotoxemia and necrotic enteritis in lambs, piglets and calves (Table I). In humans, the β-toxin is a known cause of necrotic enteritis (Steinthorsdottir *et al*.,1995). *C. perfringens* types C produce α-toxin and β-toxin (Table I). *C. perfringens* type D produces α-toxin and ε-toxin, however its higher production is of ε-toxin (Table I). The *C. perfringens* type E produces ι-toxin, which has been implicated in fatal calf, lamb, and guinea pig enterotoxemia (Table I) (Bosworth, 1943; Madden, Horton, McCullough, 1970). *C. perfringens* type B, E and some by type D, produce λ-toxin, which cause enteritis and enterotoxemia in domestic animals (Table II) (Bidwell, 1950; Hatheway, 1990; Rood, Cole, 1991). This λ-toxin may contribute to the pathogenesis by activating other potent toxins, such as the ε- and ι-toxins produced by these type strains (Jin *et al*., 1996).

C. perfringens type C and some by type B, produce δ-toxin, which is one of the three extracellular hemolytic toxins that can be released, along with many other exotoxins and exoenzymes (Brooks, Stern, Warrack, 1957; Glenny *et al*., 1933; Oakley, Warrack, 1953; Orlans, Jones, 1958; Smith, 1979; Sterne, Warrack, 1964).

In this review, the purification methods of toxins, produced by *C. perfrigens*, are presented and discussed. The information here presented can help the production of antigens aiming industrial application, and the comprehension of their structures or effects.

Alpha-toxin (α)

Alpha toxin is one of the most important lethal and dermonecrotic toxins produced by *C. perfringens,* known as phospholipase C (PLC). It is produced in different amounts by all types (A, B, C, D, E) of *C. perfringens* and is considered as a primary virulence factor involved in clostridial myonecrosis (Williamson, Titball, 1993; Award *et al*., 1995). In *C. perfringens* type A, the alpha-toxin is

TABLE I – Diversity of *Clostridium perfringens* toxinotypes and associated diseases (Petit, Gibert, Popoff, 1999)

Toxinotype	Major Toxins				Minor Toxins				Associated diseases	
	α	β	ε	ι	CPE	λ	θ	δ	Humans	Animals
A	++	–	–	–	+	–	+	–	Gangrene Gastrointestinal diseases	Diarrhea (foals, pigs…) Necrotic enteritis in fowl
B	+	+	+	–	+	+	–	+	–	Dysentery in newborn lambs Hemorragic enteritis in neonatal calves and foals Enterotoxemia in sheep
C	+	+	–	–	+	–	–	+	Necrotic enteritis	Necrotic enteritis in piglets, lambs, calves and foals Enterotoxemia in sheep
D	+	–	+	–	+	+	–	–	–	Enterotoxemia in lambs, sheep, calves and goats
E	+	–	–	+	+	+	–	–	–	Enterotoxemia in calves

– – no detected toxin production; + – detected toxin production; ++ – highest toxin producer

TABLE II – Mode of action and biological activity of *Clostridium perfringens* toxins

Toxin	Mode of action	Homology[9]	Biological activity
α	Phospholipase C / sphingomyelinase	α toxin *C. novyi* (60.8%) α toxin *C. bifermentans* (50.8%) phospolipase *C Bacillus cereus* (26.5%)	Cytolytic, hemolytic, dermonecrotic, lethal[1]
β1, β2	Pore-forming activity Cell membrane disruption?	γ-hemolysin (30%) α toxin (27.4%) leukocidin components D, E, F (21.1-29.6%)	Cytolytic, dermonecrotic, lethal hemorragic necrosis of intestinal mucosa[2]
ε	Alteration of cell membrane permeability	Mtx3, *Bacillus sphaericus* (26.7%) C53, *B. thuringiensis* (26.5%) Mtx2, *Bacillus sphaericus* (20.2%)	Edema in various organs: liver, kidney and central nervous system, dermonecrotic, lethal[3]
ιa, ιb	ADP-ribosylation of actin for Ia	*C. spiroforme* toxin (78.6-80.7%) CDT *C. difficile* (80.4-80%) C2 *C. botulinum* (31.1-40%)	Disruption of actin cytoskeleton, disruption of cell barrier integrity dermonecrotic, lethal[4]
δ	Hemolysin, specific GM2		Additional virulence factors[5]
λ	Protease	Protease, *B. thuringiensis*(43.2%) Protease, *Lactobacillus* sp. (42.5%)	Additional virulence factors[6]
θ	Hemolysin, specific to cholesterol	Alveolisin, *B. alvei* (71.4%) Hemolisin, *B. cereus* (66.9%) Streptolisin O, *Streptococcus pyogenes* (63.5%)	Additional virulence factors[7]
(CPE)	Pore-forming activity	*C. botulinum* C (24%)	Cytotoxic, erythematous, lethal leakage of water and ions by enterocytes, diarrhea[8]

1-Buting *et al*., 1997; Saint-Joanis, Garnier, Cole, 1989.; **2**-Gibert, Jolivet-Reynaud, Popoff, 1997; Hunter *et al*. 1993; Sakurai, Fujii, 1987; **3**-Petit, Gibert, Popoff, 1997; Hunter *et al.*, 1992; **4**-Perelle *et al*., 1993; Perelle *et al*., 1995; Vandekerckhove *et al*., 1987; **5**-Alouf, Jolivet-Reynaud, 1981; **6**-Jin *et al*., 1996; **7**-Tweten, 1988; **8**-Williamson, Titball, 1993; Award *et al*., 1995; **9**-Petit, Gibert, Popoff, 1999.

the unique lethal protein produced during vegetative growth. Owing to its role in gas gangrene disease, food poisoning and animal enterotoxemia, *C. perfringens* type A strains, particularly the alpha-toxin, have been the subject of intense investigations over the past 60 years (McDonel, 1986). Alpha-toxin, produced by *C. perfringens*, is a metalloenzyme with molecular weight of 43 kDa (Takahashi, Sugahara, Ohsaka, 1974; Hale, Stiles, 1999) and LD_{50} of 40 ng/mL^{-1} (Naylor, Martin, Barker, 1997), and catalyses the hydrolysis of lecithin and phospholipids (Saint-Joanis, Garnier, Cole, 1989; Hale, Stiles, 1999).

C. perfringens produces many different biologically active proteins, thus complicating the characterization of any one specific toxin without laborious purification procedures (Macchia, Bates, Pastan, 1967). Various methods of purification have been proposed for alpha-toxin (Mitsui, Mitsui, Hase, 1973a; Bangham, Dawson, 1962; Macchia, Bates, Pastan, 1967; Shemanova *et al*., 1968; Diner, 1970; Sugahara, Ohsaka, 1970; Ispolatovskaya, 1971; Casu *et al*., 1971; Stahl, 1973). They include conventional or classical procedures such as precipitation with salts or organic solvents, electrophoresis, gel filtration, and adsorption or ion exchange chromatography. The first purification of alpha-toxin was achieved by precipitation with mixture sodium sulfate and ammonium sulfate (MacFarlane, Knight, 1941), and subsequently by precipitation with ammonium sulfate (Saint-Joanis, Garnier, Cole, 1989). The crude toxin was precipitated with different concentrations of ammonium sulfate, until to

achieve the desired degree of ammonium sulfate saturation. After, one the table was elaborated to determine the exact concentrations of ammonium sulfate (McDonel, 1980; Saint-Joanis, Garnier, Cole, 1989). The ammonium sulfate precipitation was often used to prepare a crude toxin for application to gel filtration chromatography (Ito, 1968; Mitsui, Mitsui, Hase, 1973a; Diner, 1970; Ikezawa, Yamamoto, Murata, 1964; Katsaras, Hartwigk, 1979) or electrofocusing (Smyth, Arbuthnott, 1974). These purification steps provided poor recovery yields, such as 54% (van Heyningen, 1941), 56% (Bangham, Dawson, 1962), 14% (Roth, Pillemer, 1953), and 56.6% (Smyth, Arbuthnott, 1974) (Table III). During the ammonium sulfate precipitation, the alpha-toxin suffers considerable biochemical modifications, with possible denaturation of the molecular structure (Odendaal, 1987).

Stephen (1961) was one of the first author to employ high-pressure ultrafiltration to obtain alpha-toxin in high concentration, and the method used to purify was zone electrophoresis and immunoelectrophoresis (Method 1) (Table III). The efficiency of high-pressure ultrafiltration for purification of alpha-toxin was evaluated by Odendaal (1987), and its use was compared in 4 different purification methods (Table III). The results obtained with 3 different ultrafiltration membranes followed by gel filtration showed that by using the membranes Millipore™ PSED OHV10 and Amicon™ XM-100 filter (method 7), a three-hundred-and-fivefold purification could be achieved as against a twelvefold increase obtained with ammonium sulphate/acetone precipitation (method 5). The ultrafiltration process is less time-consuming, easier to perform and less laborious than ammonium sulfate and acetone precipitations. Method 5 was easy and relative fast, but showed low purification factor (12.7-fold); method 6 showed to be easy to perform, less laborious, less time-consuming, provided high purification factor (200-fold), but was of high cost. Method 8 showed to be easy to perform, less laborious, less time-consuming, but showed low purification factor (24-fold) (Table III).

Alpha-toxin has been successfully purified through chromatography column, specifically ion exchange. It was purified by the method 2, in batchwise, and reached a 200-fold increase in specific activity (Möllby, Wadström, 1973) (Table III). Yamakawa and Ohsaka (1977) purified alpha toxin by using method 4; the final product was purified in approximately 15.5-fold, and 17% the toxin was recovered (Table III). In comparison with the others methods described, this method was inefficient of the point of view of purification factor and recovery.

C. perfringens alpha-toxin was also purified by affinity chromatography described for method 3 (Table III). The purification factor encountered was 200-fold, with a recovery yield of about 60% of enzymatic, lethal or hemolytic activity. The purified toxin was homogenous in polyacrylamide gel electrophoresis, ultra-centrifugation and immunodiffusion. By isoelectric focusing of the purified toxin, three major molecular forms were encountered (Takahashi, Sugahara, Ohsaka, 1974) (Table III).

The alpha-toxin from recombinant *Bacillus subtilis* cells was purified after three steps: ammonium sulfate precipitation (60% saturation), which was followed by affinity chromatography (Sepharose 4B-linked egg yolk lipoprotein column), and then by chelating chromatography (Sepharose Fast Flow gel column). The three purification steps yielded increasingly pure toxin. The final step resulted in a homogeneous product that was purified approximately 130-fold, and 23% of the toxin was recovered (Hirata *et al*.,1995).

Comparing all described aforementioned methods, it can be observed that the use of chromatography methods (size exclusion molecular, affinity chromatography, ion exchange) is very important to obtain pure toxins. The combined utilization of size exclusion chromatography and utlrafiltration membranes provided higher purification factor, but this procedure its of high cost.

Beta-toxin (β)

Beta-toxin is produced only by *C. perfringens* types B and C (Table I), and causes enterotoxemia and necrotic enteritis in lambs, piglets and calves. In humans, this toxin causes necrotic enteritis. In spite of the importance of beta-toxin in veterinary medicine, the biological activity of this protein is poorly defined (Steinthorsdottir *et al*., 1995; Gkiourtzidis *et al*., 2001). Its molecular weight is 35 kDa (Hsieh *et al*., 1998), LD_{50} is 310 ng/kg^{-1} (Jin *et al.*, 1996).

According to Hauschild (1971) the combined effects of beta- and epsilon-toxins are responsible for the diseases caused by *C. perfringens* type B, such as lamb dysentery and enterotoxemia of foals, goats, sheep and calves.

Beta-toxin is responsible for the diseases caused by *C. perfringens* type C which including Struck of sheep and enterotoxemia of lambs, calves and piglets and necrotic enteritis of man and fowls (Hauschild, 1971). Most of the conditions in which beta-toxin plays the major role are characterized by ulceration of the intestines or acute hemorrhage enteritis (Worthington, Mülders, 1975). The purification of this toxin is necessary to study the mechanism of action.

The beta-toxin was purified after four steps (method 1), as presented in Table IV, and the final purification factor was of 28-fold (Worthington, Mülders, 1975). The

TABLE III – Purification methods used for a-toxin of *C. perfringens*

Method	Procedure	Purification factor (-fold)	Advantages	Disadvantages	Ref.
1	High pressure ultrafiltration, electrophoresis and immunoelectrophoresis	–	Good purity	Not for large-scale purification	1
2	DEAE-Sephadex A25, Sephadex G75 and isoeletric focusing	200	Good purity	Long time-consuming particularly for large-scale purification	2
3	Affinity chromatography, sephadex G100, isoelectric focusing	200	Good purity	Long time-consuming particularly for large-scale purification	3
4	Ultrafiltration, calcium acetate precipitation, CM-Sephadex, DEAE-Sephadex, sephadex G-100	15.5		Not for large-scale purification, laborious	4
5	Ammonium sulfate/ acetone precipitation, gel filtration	12.7	Easy, and relatively fast	Low purification factor	5
6	Ultrafiltration with Amicon XM-50, gel filtration	200	Easy to perform, less laborious, and less time-consuming	High cost	5
7	Ultrafiltration with Millipore PSED OHV 10 and Amicon XM-100, gel filtration	305	Easy to perform, less laborious, less time-consuming	High cost	5
8	Ultrafiltration XM-100, gel filtration	24	Easy to perform, less laborious, and less time-consuming	Low purification factor	5

1-Stephen, 1961; 2-Möllby, Wadström, 1973; 3-Takahashi, Sugahara, Ohsaka, 1974; 4-Yamakawa, Ohsaka, 1977; 5-Odendaal, 1987.

2nd purification method of beta-toxin involved immunoaffinity chromatography (Table IV). The toxin was purified about 340-fold from the culture supernatant of *C. perfringens* type C with a yield of about 24%, in terms of biologically active beta-toxin. Purity of the toxin was checked by polyacrylamide gel electrophoresis; a purified toxin gave a single band (Sakurai, Duncan, 1977).

The comparison between the aforementioned methods indicates that immunoaffinity chromatography to purify beta-toxin should be utilized, since the purification factor was of 340-fold and the yield of 24%.

Epsilon-toxin (ε)

Epsilon-toxin produced by *C. perfringens* type B and D is the third most potent clostridial toxin, after botulinum and tetanus toxins. This toxin is produced as a relatively inactive prototoxin with molecular weight of

TABLE IV - Purification methods used for β-toxin from *C. perfringens*

Method	Procedure	Purification factor (-fold)	Disadvantages
1*	ammonium sulfate precipitation, gel filtration chromatography (Sephadex G50, Sephadex G100) and ion exchange chromatography (DEAE cellulose).	28	Multiple steps, long time-consuming
2**	ammonium sulfate fractionation, gel filtration (Sephadex G-100), isoelectrofocusing in a pH 3 to 6 gradient, and immunoaffinity chromatography	340	Multiple steps, not for large-scale purification

*Worthington, Mülders, 1975; **Sakurai, Duncan, 1977

~33 kDa (Payne, Oyston, 1997; Subramanyam *et al*., 2001; Parreiras *et al*., 2002), and as an active toxin with molecular weight of 32 kDa (Payne, Oyston, 1997) their LD_{50} is 70 ng/kg^{-1} (Miyamoto et al., 2000). It is responsible for the pathogenesis of fatal enterotoxemia in domestic animals. This toxin exhibits toxicity toward neuronal cells via the glutamatergic system (Miyamoto *et al*., 1998; Miyamoto *et al*., 2000) or extravasation in the brain (Finnie, Blumbergs, Manauis, 1999). It has been suggested that is a pore-forming toxin based on the following observations: (i) ε-toxin can form a large complex in the membrane of Madin-Darby canine kidney cells, and permeabilizes them (Petit *et al*. 1997; Nagahama, Ochi, Sakurai, 1998); (ii) the large complex formed by ε-toxin is not dissociated by SDS-treatment, which is a common feature of pore-forming toxins (Petit *et al*. 1997); and (iii) the CD spectrum of ε-toxin shows that it mainly consists of β-sheets (Habeeb, Lee, Atassi, 1973), as observed for pore-forming β-barrel toxins. A characteristic feature of ε-toxin is its potent neurotoxicity, which is not observed for other structurally well-defined pore-forming toxins (Miyata *et al*., 2001). Another characteristic of ε-toxin is the activation of the inactive precursor (ε-protoxin) by proteases such as trypsin, chymotrypsin (Hunter *et al*., 1992), and λ-protease produced by *C. perfringens*. This activation is accompanied by removal of both N- and C-terminal peptides (Jin, *et al*. 1996; Minami *et al*., 1997).

Epsilon-protoxin has been purified by methanol precipitation (Verwoerd, 1960), and by ion exchange chromatography (Thomson, 1963; Orlans, Richards, Jones, 1960; Hauschild, 1965; Habeeb, 1969). Protoxin prepared by Habeeb (1969) presented higher toxicity than protoxin prepared by other workers above but was not electrophoretically homogeneous.

Highly purified *C. perfringens* type D epsilon-protoxin was prepared from culture filtrate of *C. perfringens* by ammonium sulfate precipitation and ion exchange chromatography (DEAE-cellulose). The purification factor was approximately 77-fold (Worthington, Mülders, Van Rensburg, 1973).

Theta-Toxin (θ)

Theta(θ)-toxin is one of the toxins produced by *Clostridium perfringens* type A, a causative pathogen for gas gangrene (Ispolatovskaya, 1971). The toxin is cytolytic (hemolytic) and lethal, belonging to a group of oxygen-labile or SH-dependent hemolysin (Bernheimer, 1974; Bernheimer, 1976). In the same group are found streptolysin O, cereolysin, tetanolysin and listeriolysin produced by *Streptococcus pyogenes*, *Bacillus cereus*, *Clostridium tetani* and *Listeria monocytogenes*, respectively. These hemolysins share common properties (Bernheimer, 1974; Bernheimer, 1976).

Many workers have attempted to purify theta-toxin from culture filtrate of *Clostridium perfringens* but have failed to obtain the toxin in good yield because of its instability (Roth, Pillemer, 1955; Mitsui, Mitsui, Hase, 1973b; Smyth, 1975; Soda, Ito, Yamamoto, 1976), probably because this toxin is formed by isoforms (Smith, 1975) and due the presence of reduced form and oxidized form toxins (Yamakawa, Ito, Sato, 1977).

The theta-toxin of *C. perfringens* was purified by a series of methanol precipitation and subsequent high-speed centrifugation. The purified preparation was free of kappa (κ)-toxin and hyaluronidase but contained traces of alpha(α)-toxin. Electrophoretic patterns indicated that the (θ)-toxin constituted about 80–90% of the total protein of purified preparation (Roth, Pillemer, 1955). Authors as Habermann,

1959 and Habermann, 1960, purified theta-toxin by methanol precipitation, starch-gel electrophoresis, and ion exchange chromatography (DEAE-cellulose). The purified preparations were free of alpha and kappa-toxins, and hyaluronidase. This purified preparation produced a single spot and single precipitin line in membrane electrophoresis and in immunoelectrophoresis, respectively.

By purification method using ammonium sulfate precipitation and electrofocusing (method 1 – Table V), four isoforms of theta-toxin were found. The overall recovery was 86.8% and the purification factor varied, depending of isoform, of 1060 to 1800-fold (Smyth, 1975).

This toxin was purified by Hauschild, Lecroisey, Alouf, (1973) by ammonium sulfate precipitation and successive chromatography steps, described method 2 (Table V). The total recovered theta-toxin was about 20% of the crude extract. The toxin losses, during the four purification steps were about 30, 20, 25, and 5%, respectively. This purification method was developed to produce pure theta-toxin on large scale. The authors did not relate purification factor, but tests realized indicated that the toxin is pure.

Yamakawa, Ito, Sato, (1977) purified theta-toxin 3300 fold from culture filtrate of culture *C. perfringens* by initial ion exchange chromatography (DEAE-Sephadex A-50), and gel filtration chromatography (Sephadex G-150) (method 3 – Table V). The specific activity was of 105,000 U/mg, with recovery of 50.4%. The electrophoresis of the solution containing the purified toxin showed two distinct bands, and indicated the presence of two forms of toxin.

Iota-Toxin (ι)

The iota-toxin is produced only by *C. perfringens* type E and has been implicated in fatal calf, lamb and guinea pig enterotoxemias (Bosworth, 1943; Madden, Horton, McCullough, 1970). Its biological activity is dermonecrotic and lethal. Studies suggested that iota-toxin is a binary toxin dependent on two non-linked proteins. The molecular weight of iota-toxin is 47.5 kDa and of iota b is 71.5 kDa (Stiles, Wilkins, 1986).

The iota-toxin was purified by ammonium sulfate precipitation followed by ionic exchange chromatography (DEAE-Sepharose CL-6B), isoeletric focusing, and gel filtration chromatography (Sephadex G-100). In the first chromatography, the recoveries were of 88 and 74% for $Iota_a$ e $Iota_b$, respectively (Stiles, Wilkins, 1986).

Delta-toxin (δ)

C. perfringens delta-toxin is one of the three extracellular hemolytic toxins released by a number of type C strains and also possibly by type B strains (Brooks, Stern, Warrack, 1957; Glenny *et al*., 1933; Oakley, Warrack, 1953; Orlans, Jones, 1958; Smith, 1979; Sterne, Warrack, 1964). The molecular weight of delta-toxin is 42

TABLE V - Purification methods used for θ-toxin of *C. perfringens*

Method	Procedure	Purification factor (-fold)	Recovery	Advantages	Ref.
1	Ammonium sulfate precipitation and electrofocusing	1060 to 1800	86.8%	High purification factor and recovery	1
2	Ammonium sulfate precipitation, successive chromatography: DEAE-cellulose, Sephadex G-100 QAE-Sephadex	-	20%	Large-scale purification	2
3	Anionic exchanger chromatography (DEAE-Sephadex A-50), gel filtration chromatography (Sephadex G-150)	3300	50.4%	High purification factor, easy to perform	3

1- Smyth, 1975; 2- Hauschild, Lecroisey, Alouf, 1973; 3- Yamakawa, Ito, Sato, 1977

KDa (Alouf, Jolivet-Reynaud, 1981). Previous investigations established that this toxin was immunogenic and lytic for the erythrocytes of even-toed ungulates (sheep, cattle, goats and swine) but not for the erythrocytes of other species such as humans, rabbits and horses (Brooks, Stern, Warrack, 1957; Oakley, Warrack, 1953; Willis, 1970). This difference can be owing to the low sensitivity or number of the specific receptors (possibly G_{M2} ganglioside). Besides, the receptors can be hidden, as suggested by experiments performed with binded radiolabeled toxin onto cells. The cells might also differ in their relative distributions around the lipids and proteins of the membrane (Alouf, Jolivet-Reynaud, 1981).

Delta-toxin was purified from culture supernatant by ammonium sulfate precipitation, thiol-Sepharose gel chromatography, isoelectric focusing, and gel filtration (Sephadex G-75). 200-fold purification was achieved by using a four-step process descriptive above with recovery of 16%. Some denaturation occurred during the isoelectric focusing (Alouf, Jolivet-Reynaud, 1981). The purified preparation showed a specific activity of 320,000 hemolytic units per mg of protein.

Lambda-toxin (λ)

The lambda-toxin is produced by most type B and E strains and some type D strains of *C. perfringens*. It causes enteritis and enterotoxemia in domestic animals (Madden, Horton, McCullough, 1970; Bidwell, 1950; Hatheway, 1990). Studies indicated that this toxin is a zinc metalloprotease with molecular weight 36 kDa (Jin *et al*., 1996) and contributes to the pathogenicity by degrading

TABLE VI – Purification methods of toxins produced by *Clostridium perfringens*

Toxin	Procedure	Purification factor (-fold)	Advantages	Disadvantages
α	Ultrafiltration with Millipore PSED OHV 10 and Amicon XM-100, gel filtration	305	Easy to perform, less laborious, less time-consuming, good purity	High cost
β	Ammonium sulfate fractionation, Sephadex G-100, isoelectrofocusing in a pH 3 to 6 gradient, and immunoaffinity chromatography	340	Good purity	Long time-consuming, not large-scale purification
ε	Ammonium sulfate precipitation, chromatography DEAE-cellulose	77	Good purity	-
θ	Chromatography DEAE-Sephadex A-50, and Sephadex G-150	3300	High purification factor, easy to perform	Not tested for large-scale purification
ι	Ammonium sulfate precipitation, chromatography DEAE-Sepharose CL-6B, isoelectric focusing, chromatography Sephadex G-100	-	-	Long time-consuming, laborious
δ	Ammonium sulfate precipitation, thiol-Sepharose gel chromatography, isoelectric focusing, Sephadex G-75	200	Good purity	Long time-consuming, laborious
λ	Ammonium sulfate precipitation, size exclusion, anion-exchange, and hydrofobic interaction chromatography	87.7	Good purity	Laborious
CPE	Ammonium sulfate precipitation, chromatography Sephacryl S-200	5.5	Easy to perform, less laborious, less time-consuming	Low purification factor

certain protein components of host cells. Alternatively, the toxin may contribute to the pathogenesis by activating other potent toxins, such as the epsilon- and iota-toxins, produced by these type strains (Rood, Cole, 1991). Lambda-toxin affects the vascular permeability, the same mechanism of action shown by the epsilon-toxin (Jin *et al*., 1996).

The lambda-toxin can be purified by ammonium sulfate precipitation, followed by size exclusion, anion-exchange, and hydrofobic interaction chromatography. By using this sequence of purification the enrichment factor and recovery yield of the lambda-toxin were estimated to be 87-fold and 30.5%, respectively (Jin *et al*., 1996).

Enterotoxin (CPE)

C. perfringens produces an enterotoxin that is responsible for human food poisoning in which diarrhea and abdominal cramps are associated with the ingestion of food contaminated. The enterotoxin is a single polypeptide of 35 kDa that is produced and accumulated intracellularly during sporulation of many strains of this microorganism, and is released upon sporangial lysis (Labbe, 1989).

Due to the importance of this protein, several methods have been developed to purify the enterotoxin (Bartholomew, Stringer, 1983; McDonel, McClane, 1988; Barnhart *et al*., 1976; Enders, Duncan, 1978; Granum, Whitaker, 1980; Stark, Duncan, 1972; Uemura *et al*., 1985). They include chromatography, such as affinity chromatography, size exclusion molecular and high performance liquid chromatography, polyacrylamide gel electrophoresis. The most commonly employed enterotoxin purification method involves a two-step ammonium sulfate precipitation followed by gel filtration on Sephadex G-100 (Granum, Whitaker, 1980).

The enterotoxin was also purified by using ammonium sulfate precipitation followed by gel filtration chromatography (Sephacryl S-200). The purification factor was 5.5-fold, and the recovery yield was 50%. The time (less than 48 h) and effort required by this purification method are far less than any previously reported procedure. Besides, the method may be useful for those laboratories needing to produce significant amounts of purified enterotoxin (Heredia, Garcia-Alvorado, Labbé, 1994).

CONCLUSIONS

Purification is troublesome because of system complexity and the need to retain biological activity. The purification procedures of different toxins produced by *Clostridium perfringens,* and presented in this review, involve multiple steps of the classical methodologies, such as chromatography and precipitation with salts or organic solvents. Comparing the results reported in the literature, and presented in the tables of this paper, generally, the purification factors were satisfactory for laboratory and industrial scale, but the results, obviously, depend on the type of toxin. The clear differences between purification factors of the same or different toxins, is owing to the characteristics of each individual protein. Besides, minor changes in upstream processes may require changes in the purification method to be applied. It is clearly observed that the main objective of the downstream processing of toxins is to attain high purification factors and recoveries, at low cost. Therefore, depending on the target toxin, it is necessary to include some adaptations of the current purification methods.

RESUMO

Purificação de toxinas produzidas por *Clostridium perfringens*: uma revisão

Clostridium perfringens *é uma bactéria anaeróbia Gram-positiva, amplamente distribuída no meio ambiente e comumente encontrada no intestino de animais, incluindo o homem. As espécies de* C. perfringens *estão classificadas em cinco tipos toxigênicos (A, B, C, D, E) em função da produção de quatro toxinas (α, β, ε, ι). Entretanto, as toxinas teta, delta, lambda e enterotoxina são também sintetizadas por outras espécies dessa bactéria. Muitas metodologias para purificação das toxinas produzidas por* C. perfringens *têm sido propostas e, portanto, nesta revisão foram apresentados e discutidos os métodos e resultados de purificação dessas toxinas relatados nas últimas quatro décadas.*

UNITERMOS: Clostridium perfringens. *Purificação. Toxina. Processos.*

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