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High-performance liquid chromatography of *Pasteurella haemolytica* leukotoxin using anion-exchange perfusion columns

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Abstract

An exotoxin, called leukotoxin (LKT), from *Pasteurella haemolytica*, which had previously proved difficult to purify, was purified by high-performance liquid chromatography using rigid highly hydrophilic microparticulate anion-exchange columns. These anion-exchange stationary phases were employed to overcome difficulties of the relatively hydrophobic LKT interacting with dextran or styrene-based resins. While a short non-porous DEAE column allowed the partial microscale purification of the leukotoxin at pH 7.0, a high capacity strong anion-exchange column of the perfusion chromatography type permitted the purification of LKT on a much larger scale. The purification of the LKT on the large pore strong anion-exchange perfusion column was best achieved when three consecutive linear gradients at increasing NaCl concentration in 20 m*M* Tris buffer, pH 8.0, containing 6.0 *M* urea and 0.25% Tween 20 were used. Under these conditions, a better separation was obtained for the tetrameric and aggregate peaks of LKT from the early eluting contaminant peaks. This separation scheme allowed good recovery of activity and purification of the LKT to near homogeneity. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: *Pasteurella haemolytica*; Perfusion chromatography; Leukotoxin; Toxins; Proteins

1. Introduction

The Gram-negative bacterium, *Pasteurella haemolytica*, produces a protein exotoxin (leukotoxin) which is cytotoxic for leukocytes from ruminant species and is classified in the repeats-in-toxin (RTX) family [1]. These toxins, which were named for tandem amino acid sequence repeats, act by forming transmembrane pores in mammalian cell plasma membranes resulting in increased intracellular Ca^{2+} concentration, dissipation of Na^{+}/K^{+} transmembrane gradients, colloid-osmotic cell swelling and cell lysis [2,3]. Research on the leukotoxin (LKT) mechanism of action and role in pathogenesis and immunity has been hampered by the lack of highly purified LKT. Routine purification schemes have not overcome several difficulties encountered with purification of LKT.

The 1024 amino acid residue LKT protein has a molecular mass of 102 000 with a calculated isoelectric point (p*I*) of 5.9 [4,5]. LKT exhibits amino acid composition and sequence, and protein conformation and configuration features peculiar to the RTX toxin family. LKT is composed of 36.4% non-polar, 38.7%

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polar, 12.1% acidic and 12.8% basic amino acid residues. Although the RTX toxins are relatively large proteins, these toxins lack cysteine, and LKT contains only five methionine residues. The N-terminal half of LKT is more hydrophobic than the C-terminal half and contains six reputed transmembrane spanning domains [4,5]. A post-translationally modified fatty acylated ε-amine lysine is present at residue 554 [6]. The sequence from residues 733 to 786 contains six tandem repeats of a glycine-rich nine amino acid consensus sequence [L–X–G–G–X–G–(N/D)–D–X] from which the RTX toxins derive their family name [5]. The repeats are thought to form a Ca^{2+}-binding motif [7]. The C-terminal sequence is required for toxin secretion across the bacterial inner membrane by a dedicated ATP-binding cassette (ABC) secretion system [8].

The LKT, like other RTX toxins, exists as large aggregates with an estimated molecular mass of >1 000 000 [9,10]. These aggregates are dissociated to tetramers in 6 *M* urea (and other chaotropic agents) and to monomers in 0.1% sodium dodecyl sulfate (SDS) [11]. The large aggregated RTX toxins are not thought to be composed of a single specific-sized multimeric form, but rather are thought to be composed of a dispersion of various-sized aggregates [9].

Purification of the RTX toxins has proved difficult, and the extent of purification of these toxins has not been strictly demonstrated [12]. For LKT, difficulties include low levels of toxin in bacterial culture supernatants (<1 μg/ml), rapid loss of LKT activity and super aggregation in the absence of chaotropic agents or detergents [10,11]. The large LKT aggregates remain a significant impediment to purification because these aggregates contain significant amounts of co-aggregated contaminating proteins and lipopolysaccharides (LPS), which have proved difficult to separate from LKT [13]. One common purification strategy has been immunoaffinity chromatography using anti-LKT monoclonal antibodies [12,14], however, the conditions required for LKT–antibody interaction were not sufficiently stringent to cause dissociation of contaminating proteins from the LKT aggregates.

Another problem encountered with LKT purification is interaction between the LKT and mixed-mode chromatographic resins [13] exhibiting both electrostatic and hydrophobic interactions. In the absence of chaotropic agents or detergents, LKT is usually not recovered on a variety of resins including those based on dextran and styrene beads [10,13]. Thus, the objective of the study present herein was to examine the use of highly hydrophilic anion-exchange chromatography media and conditions for the purification of LKT by high-performance liquid chromatography (HPLC).

2. Experimental

2.1. HPLC instrument and columns

The liquid chromatograph was assembled from an LDC/Milton Roy (Riviera Beach, FL, USA) Model CM4000 solvent delivery pump, a UV interference filter detector Model UV-106 from Linear Instruments (Reno, NV, USA), a sample injector Model 7010 from Rheodyne (Cotati, CA, USA) with a 100-μl sample loop, a fraction collector Model LC 100 from Haake Buchler (Lenex, KS, USA) and a computing integrator Model C-R5A from Shimadzu (Columbia, MD, USA) or a reporting integrator Model 3390A from Hewlett-Packard (Avondale, PA, USA). The wavelength for detection was 280 nm.

Two different columns were examined in this study, a DEAE-NPR TSK gel column of 35×4.6 mm I.D. based on nonporous polymeric sorbent from Tosoh (Tokyo, Japan) and a Poros HQ (quaternized polyethyleneimine) strong anion-exchange column of 10 μm particles having the dimensions 100×4.6 mm I.D. was a gift from PerSeptive Biosystems (Framingham, MA, USA).

2.2. Methods

2.2.1. Preparation of *P. haemolytica leukotoxin*

LKT was produced as described elsewhere [10]. Briefly, a *P. haemolytica* biotype A, serotype 1 strain grown in BHI broth (0.5 l) to an $A_{600\ nm}$ 0.8–1.0 was used to inoculate RPMI 1640 medium (1.0 l) (pH 7.0, 2.2 g/l $NaHCO_3$) to an $A_{600\ nm}$ of 0.25. The RPMI culture was grown at 37°C, and 80 oscillations/min to an $A_{600\ nm}$ of 0.8–1.0, and the culture supernatant was obtained by centrifugation (Sorvall GS3 rotor, DuPont, Wilmington, DE, USA;

8000 *g*, 30 min). This and all subsequent preparation steps were performed at 4°C.

A 40–60% ammonium sulfate fraction of the culture supernatant was prepared by addition of solid ammonium sulfate (0–40% fraction: 226 g/l and 40–60% fraction: 120 g/l) and collection of the precipitate by centrifugation (Sorvall GS3 rotor, 8000 *g*, 45 min). The 40–60% fraction was resuspended in 10 ml of 3 *M* guanidine, 0.1 *M* sodium phosphate, 0.1 *M* NaCl, pH 7.0 buffer, dialyzed against 500 ml of the same buffer overnight, and clarified by centrifugation (Sorvall SS34 rotor, 6000 *g*, 30 min). Buffer was changed to that used for HPLC runs by dialysis and the protein concentration (BCA microprotein assay, Pierce, Rockford, IL, USA) and LKT activity (as described below) measured, and preparations stored at −135°C. LPS was assayed quantitatively by measuring 2-keto-3-deoxy-octosonic acid (KDO) [15].

2.2.2. Application and chromatography of P. haemolytica LKT

One ml of LKT preparations in the initial column buffer were applied to HPLC columns (by injecting into the column 100 μl at a time), and the preparations eluted with gradients specified in Sections 3.1 and 3.2. The column effluent was monitored for proteinaceous analytes at λ=280 nm. The tryptophan, tyrosine and phenylalanine content of LKT are 0.42%, 2.52% and 2.62%, respectively. These figures correspond to 4, 24 and 25 residues per LKT molecule.

2.2.3. Assay of P. haemolytica LKT

P. haemolytica LKT activity was assayed by measuring cytoplasmic lactate dehydrogenase (LDH) released from bovine lymphoma cells (BL3 cells, CRL 8037, American Type Culture Collection, Rockville, MD, USA). These cells were cultured at 37°C and 5% CO_2 in 50% Leibovitz L-5 and 50% Eagle minimal essential medium containing 10% FBS, 2 m*M* L-glutamine, 50 mg/l gentamicin, and 2.2 g/l $NaHCO_3$ as described elsewhere [2]. BL3 cells were exposed to serially diluted LKT preparations or HPLC fractions in 96-well round-bottom microtiter plates at 37°C for 2 h.

Exposure was terminated by centrifugation (5 min at 700 *g*) and the concentration of extracellular LDH was assayed by transfer of 100 μl of incubation supernatant to wells of a flat-bottom 96-well microtiter plate. The plate was warmed to 37°C, 100 μl assay reagent at 37°C was added (LD-L 228-50 ml, Sigma, St. Louis, MO, USA; rehydrated by addition of 25 ml water), and the LDH activity was measured in a thermally controlled kinetic microtiter plate reader (ThermoMax, Molecular Devices, Palo Alto, CA, USA) at 340 nm for 2 min at 37°C. Data were reported as mAu/min. Maximal LDH leakage was determined by replacing leukotoxin with Triton X-100 (final concentration was 0.1%, v/v), and background LDH leakage was determined by replacing LKT with buffer appropriate as control for a particular experiment.

2.2.4. Analysis of HPLC fractions for protein concentration, LKT activity and purity

The volumes of HPLC fractions were measured, and the buffer changed to phosphate-buffered saline (PBS) by dialysis. An aliquot was stored frozen at −135°C, and assayed later for protein concentration, LKT activity and LPS. The protein concentration in dialyzed fractions was determined with the BCA microprotein assay using bovine serum albumin (BSA) as a standard. The amount of LKT activity in fractions measured as described above, and the reciprocal of the dilution causing 50% maximal leakage of LDH determined graphically and used as the relative amount of LKT in the fraction. All assays were conducted in triplicate. LPS was assayed semi-quantitatively in column fractions by a kinetic *Limulus* amebocyte lysate pyrochrome method (LAL Pyrochrome, Associates of Cape Cod, Woods Hole, MA, USA) [16].

The remainder of the dialyzed HPLC fractions were trichloroacetic acid (TCA) precipitated for SDS–polyacrylamide gel electrophoresis (PAGE) and Western blot analysis for LKT protein and relative purification. Precipitation was conducted by mixing of equal volumes of ice-cold fractions and 72% TCA followed by a 30 min incubation. The precipitates were collected by centrifugation (6000 *g*, 30 min, 4°C), washed 2× with acetone (to remove residual TCA), and resuspended in 100 μl SDS–PAGE sample buffer (pH was adjusted using 1 *M* NaOH). Acetone washing was found to extract much of the LPS from TCA precipitated HPLC fractions.

The relative amount of LKT protein in fractions was estimated from the band densities on western blots of the SDS–PAGE using an anti-LKT monoclonal antibody (C6). One μl of 1:5 diluted samples of the TCA precipitated HPLC fractions was run on 10% SDS–PAGE, blotted to nitrocellulose membranes, the membranes blocked with 3% gelatin, reacted with 1:500 diluted C6 mouse ascetic fluid followed by 1:300 biotin-labeled goat anti-mouse IgG (Sigma) and 1:400 streptavidin-conjugated alkaline phosphatase (Bio-Rad Labs., Hercules, CA, USA). The LKT protein bands were then developed with nitroblue tetrazolium insoluble substrate (BCIP/NBT tablets, Sigma), and the relative densities scored. The relative LKT purity of fractions was estimated by running 1–10 μl of undiluted samples of the TCA-precipitated HPLC fractions on 10% SDS–PAGE and staining with a silver stain (Daiichi Silver Stain-II, Daiichi, Tokyo, Japan). The number and intensity of contaminating protein bands were compared with the 102 000 LKT band (identified using Silver Stain SDS–PAGE High Molecular Mass Standards, Sigma).

3. Results and discussion

The protein and LPS-specific sugar KDO content for the starting LKT preparations ranged from 260 to 940 and 2.6 to 8.7 μg/ml, respectively, with a ratio of ≈10 μg KDO/mg protein. Preparations from an LKT deletion mutant *P. haemolytica* strain [17] were used to assess the composition of non-LKT associated proteins in the starting material. The mutant preparations had only ≈70% of the protein and LPS levels of the wildtype LKT-producing strain, suggesting that the LKT and its associated LPS comprised 30% of the starting preparation protein and LPS. As shown in tracing from SDS–PAGE (Fig. 1), the LKT preparation consisted primarily of M_r 102 000 LKT (R_F=0.24) and several smaller size contaminant bands. The most prominent contaminant was M_r≈5000 LPS (R_F=0.99). The silver-stained SDS–PAGE tracing of the culture supernatants from wildtype and LKT-negative mutant demonstrated 14 bands at similar sizes and two bands of M_r 86 000 and 66 000 which were unique to wildtype LKT-producing strain (Fig. 1). In addition, three bands (M_r 102 000, 97 000 and ca. 5000) had greater

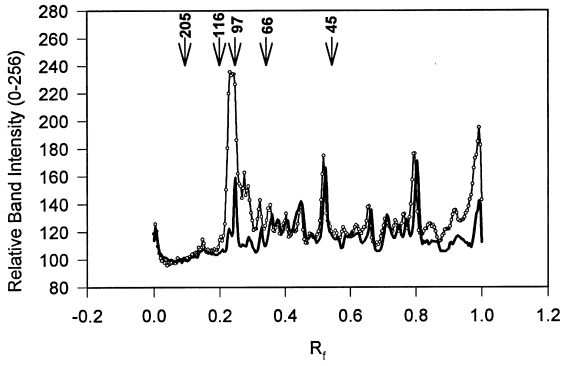

Fig. 1. SDS–PAGE electropherograms comparing the culture supernatants from LKT-producing wildtype and LKT-minus mutant strains of *P. haemolytica*. Applications of culture supernatant contained 4.2 μg protein from a LKT-producing wildtype strain (light solid line with open symbols) and 3.5 μg protein from the isogenic LKT-minus mutant strain (heavy solid line). Arrows indicate R_F of molecular mass markers.

density for the wildtype than the mutant. We conclude that the LKT consists of the M_r 102 000 protein and M_r≈5000 LPS and that the LKT has breakdown forms of M_r 97 000, 86 000 and 66 000.

As discussed in Section 1, LKT poses several problems for purification because it has a relatively large subunit size (M_r 102 000), exists as self aggregates (M_r≈1 000 000) co-aggregated with contaminant proteins and LPS, and adsorbs to chromatographic resins. In addition, the toxic activity is relatively liable. To address the problems of large protein size and adsorption to mixed mode chromatographic sorbents, highly hydrophilic non-porous as well as large pore resins were used. Since LKT has a p*I* of 5.92, it is a weak acidic protein and would be expected to chromatograph readily on anion-exchange columns. To ensure good recovery of LKT during chromatography, columns packed with highly hydrophilic weak and strong anion-exchange sorbents were used at near physiological pH with eluents consisting of aqueous buffers containing guanidine chloride and urea. LKT had been shown previously to retain activity in these chaotropic agents [10].

3.1. Non-porous weak anion-exchange

To address the concerns elaborated above, a non-

porous DEAE-NPR TSK gel column was used with two consecutive linear gradients (i.e., two connected linear gradient segments) as follows: a 15 min linear gradient from 0 to 1.0 *M* sodium chloride in 10 m*M* sodium phosphate, pH 8.0, was followed by a 10 min linear gradient at increasing concentration of urea and guanidine chloride simultaneously from 0 to 6.0 *M* urea and from 0 to 3.0 *M* guanidine chloride, respectively, in 10 m*M* phosphate, pH 8.0. This run yielded a chromatogram with four distinct peaks, see Fig. 2A. Two of these peaks showed the presence of LKT (at 0.39 and 9.69 min) with most of the LKT (ca. 95%) in the first peak or breakthrough peak (see Fig. 3B). LPS co-eluted with the LKT peak under these conditions, and the other peaks contained much lower levels of LPS.

These observations indicate that in the presence or absence of urea and guanidine chloride, the LKT is not retained at pH 8.0 on the DEAE column. Since most of the LKT was eluting at the breakthrough where other unretained contaminants could be eluted, the pH of the mobile phases was decreased to pH 7.0 in an attempt to allow longer retention for the LKT, see Fig. 2B. In fact, changing the pH to 7.0 resulted in retention of most of the LKT in the peak eluting at 9.45 min, and there appeared to be a significant purification of the LKT, although the retained LKT again had the majority of the eluted LPS associated with it (see Fig. 3C). The observation that LPS remained associated with LKT is consistent with the recent observation that LPS is tightly bound to LKT and requires heating in 0.1% SDS and electrophoresis for dissociation of the LPS from LKT [18].

Interestingly, changing the pH from 8.0 to 7.0

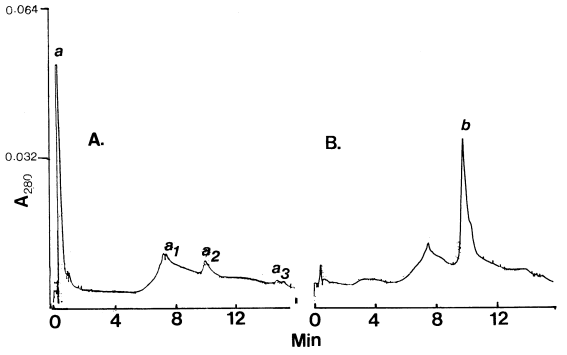

Fig. 2. Typical chromatograms of LKT preparation containing 500 μg protein obtained on non-porous DEAE column. (A) Two consecutive linear gradients: 15 min linear gradient from 0 to 1.0 *M* sodium chloride followed by a 10 min linear gradient at increasing concentration of guanidine chloride and urea simultaneously from 0 to 3.0 *M* and from 0 to 6.0 *M*, respectively. The buffer used in both gradient was 10 m*M* sodium phosphate, pH 8.0. (B) Fifteen min linear gradient at increasing NaCl concentration from 0 to 1.0 *M* in 10.0 m*M* phosphate, pH 7.0. Column, 30×4.6 mm I.D.; flow-rate, 1.0 ml/min; UV detection at 280 nm.

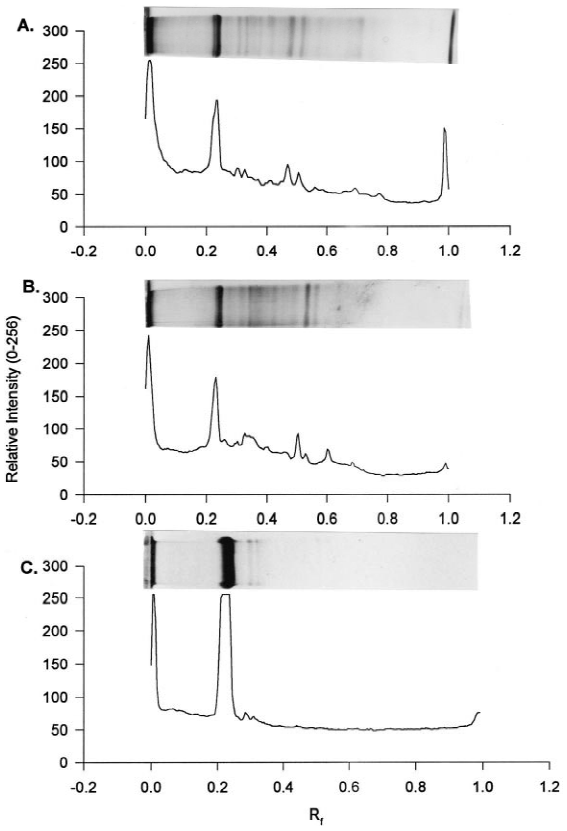

Fig. 3. SDS–PAGE electropherograms of (A) LKT preparation prior to chromatography, (B) HPLC fractions from the breakthrough fraction of Fig. 1A (peak a) and (C) the retained fraction from Fig. 1B (peak b). For (A), the applied LKT preparation contained 2.5 μg protein and 16.6 ng LPS KDO. For (B), the breakthrough peak from the DEAE-NPR TSK column developed in 10 m*M* sodium phosphate, pH 7.0 with a 15 min linear gradient from 0 to 1.0 *M* NaCl followed by a 10 min linear gradient from 0 to 6.0 *M* urea and from 0 to 3.0 *M* guanidine chloride (Fig. 1A) contained 1.6 μg protein. For (C), the retained peak eluting at 9.45 min from the DEAE-NPR TSK column developed in 10 m*M* sodium phosphate, pH 7.0, with a 15 min linear gradient from 0 to 1.0 *M* NaCl (Fig. 1B) contained 4.5 μg protein.

causes the net charge on the LKT to decrease from ca. −12 to ca. −4 as estimated from the ionization of the amino acid residues of the LKT. In this pH range the acidic carboxyl groups of the amino acid residues are fully ionized ($pK_a \approx 3.9$ to 4.1), while histidine imidazole side groups are fully deprotonated ($pK_a = 6.0$) and the other amine residues are fully protonated ($pK_a > 10$). The potential effect of the bound zwitterionic LPS [19] on the overall LKT aggregate charge is difficult to ascertain. The two principle charge groups of LPS, phosphoryl and pyrophosphoryl diesters phosphate anionic and ethanolamine cationic groups are fully ionized at pH 7.0 as well as at pH 8.0, and would have no overall effect on the charge of the LKT aggregate in this range. However, decreasing the running pH from 8.0 to 7.0 does have a significant effect on the protonation of the weak DEAE groups of the sorbent. Therefore, it appears that in the pH 7.0 to 8.0 range, the relative positive charge density on the sorbent is more important than the protonation of the amines in LKT.

Based on our results with LKT chromatography on the non-porous DEAE column, we concluded that at pH 7.0 LKT could be retained on DEAE sorbent and eluted with NaCl with good purification from non-LPS contaminants. Under these conditions, LKT did not interact non-specifically (i.e., hydrophobically) with the hydrophilic resin as had been previously observed with mixed mode resins [10,13]. However, the non-porous column is not suitable for larger scale purification and the weak anion exchanger allowed limited range to optimize the elution buffer pH.

3.2. Strong anion-exchange on perfusion column

Perfusive chromatography media composed of flow through particles [20–22] was chosen because we anticipated that the larger pore size would allow even highly aggregated LKT access to the hydrophilic, positively charged groups of the strong anion-exchange resin while also increasing the chromatographic surface available to the LKT (i.e., high binding capacity) over that of the non-porous gel. A stronger anion exchanger HQ/M was chosen to allow a larger running buffer pH range for optimizing LKT chromatography.

Chromatography of LKT on the perfusion HQ/M column with a 15 min linear gradient from 0 to 2.0 *M* NaCl in 20 m*M* bis-Tris propane (manufacturer does not recommended use of phosphate buffers with this resin), pH 7.0, resulted in a 3-peak elution profile (see Fig. 4A) which is somewhat similar to that obtained with the non-porous DEAE column, except that LKT eluted at 7.14 min, indicating that LKT had eluted at a higher NaCl concentration than it had from the non-porous DEAE column, a fact that is consistent with the stronger anion-exchange nature of the perfusion column.

Addition of urea and detergent were evaluated for the effect of disaggregation and removal of contaminants which might have been co-aggregated with the LKT aggregate. In the absence of dissociating agent, such as urea or Tween 20, LKT exists as large aggregates, whereas in 6.0 *M* urea, LKT exists as a tetramer ($M_r = 408\,000$) [10]. Chromatography of LKT on perfusion column in the presence of 6.0 *M* urea in the mobile phase resulted in shorter retention of the majority of LKT (presumed tetrameric) which eluted at 4.84 min, although some LKT apparently remained aggregated and eluted at near its former retention time of 7 min (compare Fig. 4A and Fig. 4B, and also see Table 1).

Next, it was important to examine the influence of the addition of 0.1% (w/w) Tween 20 (a concentration that is below the critical micellar concentration of Tween 20) at different running buffer pH. The results are summarized in Table 1 in terms of retention times of the LKT using a linear gradient of 15 min at increasing NaCl concentration from 0 to

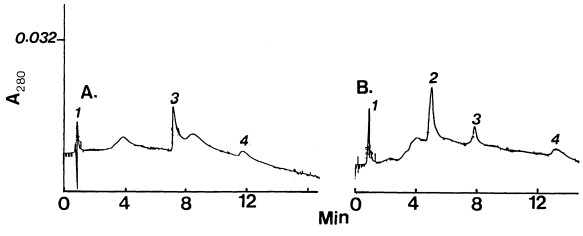

Fig. 4. Typical chromatograms of LKT preparation containing 260 μg protein obtained on strong anion exchanger perfusion chromatography column, Poros HQ/M. (A) Fifteen min linear gradient at increasing NaCl concentration from 0 to 2.0 *M* in 20 m*M* bis-Tris propane, pH 7.00. (B) Gradient as in (A) except that both the initial eluent as well as the gradient former contained 6.0 *M* urea. Column, 100×4.6 mm I.D.; flow-rate, 1.5 ml/min; UV detection at 280 nm.

Table 1
Influence of buffer pH and disaggregating agents on retention time (min) of LKT

Peak	Retention time (min)				
	20 m*M* Bis-Tris propane, pH 7.0			20 m*M* Tris, pH 8.0	20 m*M* ethanolamine, pH 9.0
	None	6.0 *M* urea	6.0 *M* urea, 0.1% (w/w) Tween 20	6.0 *M* urea, 0.1% (w/w) Tween 20	6.0 *M* urea, 0.1% (w/w) Tween 20
1. Breakthrough	0.85 (10)	0.80 (25)	0.70 (48)	0.79 (12)	0.78 (23)
2. Tetrameric LKT	–	4.84 (45)	5.45 (23)	4.42 (38)	4.35 (39)
3. Aggregated LKT	7.14 (70)	7.92 (30)	9.00 (29)	6.35 (28)	5.84 (15)
4. Retained	11.27 (20)	–	–	9.77 (22)	8.59 (23)

Column, strong anion exchanger, Poros HQ/M, 100×4.6 mm I.D.; 15 min linear gradient at increasing NaCl concentration from 0 to 2.0 *M* in the indicated buffers.
The values in parentheses indicate the percent yield of proteins in each peak.

2.0 *M* in the presence of 6.0 *M* urea. First, adding 0.1% (w/w) Tween 20 to the eluents based on 20 m*M* bis-Tris propane, pH 7.0, caused a slightly longer retention of both tetrameric and aggregated LKT (see Table 1). In contrast, increasing the pH decreased the retention time of tetrameric LKT and decreased the separation of tetrameric and aggregated LKT. Using silver-stained SDS–PAGE, the degree of purification for the aggregated LKT (7.14 min peak) run in 20 m*M* bis-Tris propane, pH 7.0, without urea or Tween 20 appeared to be not as good as that from the non-porous DEAE column at the same pH (tracings not shown). However, purification of the tetrameric LKT peaks on HQ/M from the bis-Tris propane (pH 7.0) or 20 m*M* Tris (pH 8.0) in 6.0 *M* urea with 0.1% (w/w) Tween 20 appeared similar to that of the aggregated LKT from the non-porous DEAE at pH 7.0 (see Fig. 5). The tetrameric LKT peak from the HQ/M column developed in 15 min linear gradient from 0 to 2.0 *M* NaCl in 20 m*M* ethanolamine, pH 9.0, with 6.0 *M* urea and 0.1% (w/w) Tween 20 appeared less purified than those from the same HQ/M column in bis-Tris propane (pH 7.0) or 20 m*M* Tris (pH 7.0) with 6.0 *M* urea and 0.1% Tween 20 (traces not shown).

Using the Tris, pH 8.0 as the optimum buffer pH,

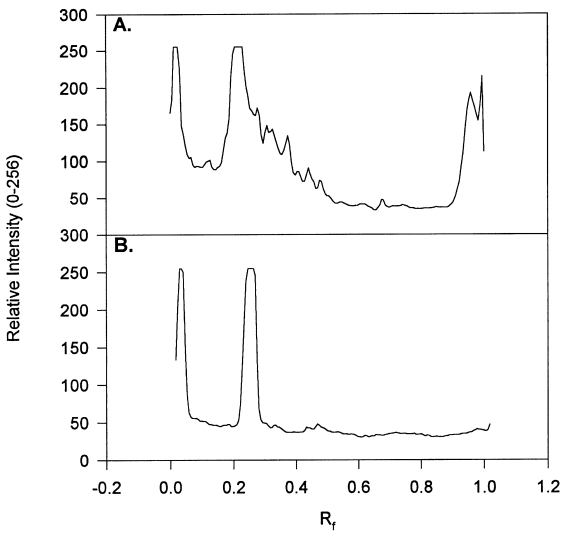

Fig. 5. SDS–PAGE electropherograms of (A) LKT preparation prior to chromatography and (B) the tetrameric LKT fraction (peak 2, Fig. 4B) from the Poros HQ/M column developed in 20 m*M* Tris, pH 8.0, containing 0.1% Tween 20 with a 15 min linear gradient from 0 to 2.0 *M* NaCl. For (A), the applied leukotoxin preparation contained 5.2 μg protein and 23.0 ng LPS KDO. For (B), the applied retained peak eluting at 4.42 min contained 5.0 μg protein.

the effect of increasing Tween 20 concentration from 0.1% to 0.25% (w/w) was examined as well as using another neutral detergent, namely 2 m*M* MEGA 10. Increasing the concentration of Tween 20 resulted in a similar elution time for the tetrameric LKT peak (4.38 min), but the aggregated LKT peak eluted earlier (5.0 min) as compared to the profiles in 0.1% (w/w) Tween 20, and some LKT was detected in the breakthrough fraction. In addition, two early non-LKT containing peaks eluting at 3.23 and 3.86 min were observed. The use of MEGA 10 resulted in 7 peak elution profile with LKT containing peaks eluting at 4.83 and 6.88 min. Judging from silver-stained SDS–PAGE, the degree of LKT purification in MEGA was not as efficient as in 0.25% Tween 20 (tracings not shown). In addition, recovery of LKT activity was better in Tween 20 than in MEGA 10.

Using 20 m*M* Tris, pH 8.0 buffer containing 6.0 *M* urea and 0.25% Tween 20 as the optimal running media, multiple consecutive linear gradients (with an overall increase in NaCl concentration from 0 to 2.0 *M*) were attempted instead of the single 15 min linear gradient in an attempt to further separate the early peaks from the leading side of the tetrameric peak. Contrary to expectations, using consecutive linear gradient from 0 to 0.34 *M* in 1.5 min, 0.34 to 1.0 *M* in 10.0 min and 1.0 to 2.0 *M* in 3.5 min compressed the early peaks back into the tetrameric LKT peak (see Table 2). Adjusting the gradient to 0 to 0.50 *M* in 3.0 min, 0.50 to 0.60 *M* in 5.0 min and 0.60 to 2.0 *M* in 7 min did result in better separation of the early peak from the tetrameric and aggregates peaks. The degree of purification for the tetrameric LKT was good. The level of LPS in the HPLC fractions was too low for detection by quantitative KDO assay, but was detectable by the semi-quantitative endotoxic activity assay. The tetrameric LKT contained less LPS than the aggregated LKT and retained I or II peaks (from Table 2). Low levels of contaminants were detected on the SDS–PAGE of the tetrameric LKT at M_r 38 000, 47 000, 76 000 and 110 000 (and some LPS was also detected, see Fig. 6). Recovery of applied protein and LKT activity ranged from 31 to 76% and 60 to 97%, respectively. The apparent high recovery of LKT activity may be partially caused by the higher activity of the tetrameric LKT in the HPLC fractions as compared to the aggregated LKT in the starting preparations. Although the recovery was somewhat variable, the range of the specific activity of the purified tetrameric LKT was 3.1 to $4.5 \cdot 10^6$ TU/mg LKT as compared to 0.3 to $1.2 \cdot 10^6$ TU/mg protein for the starting preparation. The increase in specific activity from the starting preparation to the purified LKT of 2.5- to 15-fold are compatible with purification to near-homogeneity based on the estimate that LKT

Table 2
Influence of NaCl gradient shape on retention time (min) of LKT

Peak	Retention time (min)		
	0 to 2.0 *M* in 15.0 min	0 to 0.34 *M* in 1.5 min; 0.34 to 1.0 *M* in 10.0 min; 1.0 to 2.0 *M* in 3.5 min	0 to 0.50 *M* in 3.0 min 0.50 to 0.60 *M* in 5.0 min; 0.60 to 2.0 *M* in 7.0 min
1. Breakthrough	0.85 (26)	0.78 (23)	0.84 (80)
1′. Early I	3.23 (11)	2.95 (18)	2.75 (3)
1″. Early II	3.86 (11)	–	–
2. Tetrameric LKT	4.38 (10)	3.83 (14)	4.81 (5)
3. Aggregated LKT	5.00 (30)	5.00 (16)	5.47 (5)
4. Retained I	–	7.00 (19)	–
4′. Retained II	9.84 (12)	13.33 (10)	11.85 (2)

Column, strong anion exchanger, Poros HQ/M, 100×4.6 mm I.D. Single or consecutive linear gradients at increasing NaCl concentration in 20 m*M* Tris, pH 8.0, containing 6.0 *M* urea and 0.25% (w/w) Tween 20.
The values in parentheses indicate the percent yield of proteins in each peak.

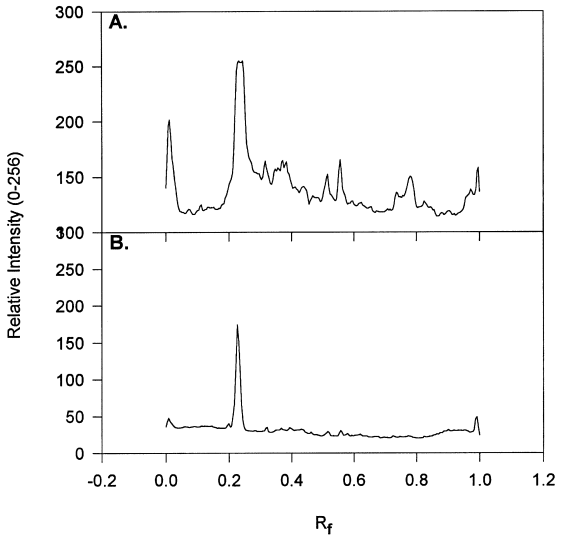

Fig. 6. SDS–PAGE electropherograms of (A) LKT preparation prior to chromatography and (B) the tetrameric LKT fraction from the Poros HQ/M column developed in 20 m*M* Tris, pH 8.0, containing 6.0 *M* urea and 0.25% (w/w) Tween 20 with a 1.5 min linear gradient of 0 to 0.34 *M* NaCl, followed by a 10 min linear gradient from 0.34 to 1.0 *M* NaCl and a 3.5 min linear gradient from 1.0 to 2.0 *M* NaCl (see Table 2). For (A), the applied LKT preparation contained 4.7 μg protein and 43.5 ng LPS KDO. For (B), the applied retained tetrameric LKT peak eluting at 3.83 min contained 1.5 μg protein.

composed ≈30% of the protein in the starting material.

4. Conclusions

As shown above, several problems needed to be addressed in developing a purification strategy for *P. haemolytica* LKT. First, the strategy of using hydrophilic resins as described herein eliminated the problem of LKT hydrophobic interaction with the resin without necessitating the use of chaotropic agents or detergents for this purpose. When using mixed mode resins (e.g., styrene-based resins), LKT was not recovered from the column except under harsh elution conditions, which involved the addition of chaotropic agents and detergents to the eluents [10]. Extensive hydrophobic regions in the N-terminal half of LKT as well as the fatty acetylated ε-amine of lysine residue 554 may mediate LKT hydrophobic binding to dextran or styrene-based resins. The highly hydrophobic lipid A moiety of LPS associated with LKT may also participate in LKT binding to mixed-mode resins.

A second problem is the access of charged moieties of the highly aggregated LKT to the ion-exchange sites on the walls of the pores of the chromatographic resins. This was solved initially by using a non-porous DEAE stationary phase. The aggregated LKT chromatographed well on this type of sorbent in the absence of disaggregating agents. However, the capacity of the non-porous column limited its utility. A perfusion chromatography type column packed with rigid microparticulate sorbent having extra large, flow-through pores was tried with good success. Both the highly aggregated and tetrameric LKT had access to the anion-exchange sites of the perfusion chromatography column.

A third improvement, which was realized in the work described in this report, is the inclusion of urea and Tween 20 in the application and elution buffers. This mobile phase system allowed the separation of contaminants from the hydrophobic, highly aggregated LKT. Based on an estimate that LKT constituted 30% of the starting protein, only 3-fold purification was required for homogeneity. Although LKT composed high percentage of the beginning protein, the contaminating proteins in the starting material were distributed among numerous other protein species. These contaminants were satisfactorily separated from LKT using the optimized chromatographic conditions described above. A problem previously encountered of high levels of an M_r 66 000 band in purified LKT [12,14], was not observed in the LKT purified as described in this article. We concluded that this M_r 66 000 protein was likely a proteolytic fragments of LKT.

A fourth improvement of the optimized separation conditions described here relates to conserving the activity of LKT. This has resulted from a combination of the speed afforded by HPLC and the use of near-physiological pH in our purification scheme. Thermal inactivation of LKT at room temperature has been a problem during the relatively lengthy purification of LKT by low-pressure liquid chromatography. In our purification scheme, the chromatographic step by HPLC at room temperature was achieved relatively fast, and the collected fractions were immediately cooled, and subsequent sample

manipulations were conducted at cold temperature. It should be noted that LKT and other RTX toxins are stable in guanidine chloride or urea, a fact which also contributed to good recovery of activity.

Finally, the problem of contamination of the LKT by LPS was not resolved by the purification scheme employed. The LKT preparation contained high amounts of LPS based on the KDO levels. KDO composes ≈5% of LPS [19], so that our starting material contained ≈0.2 mg LPS/mg protein. Some of this LPS was separated from the LKT during our purification, but the majority of the LPS remained associates with the purified LKT. LKT-associated LPS is apparently tightly bound to LKT. We have recently found that LPS is not removed by gel filtration in 0.1% SDS, but is removed by electrophoresis in 0.1% SDS [18]. Therefore, it appears that the binding of LPS to LKT is sufficiently avid that it would not be expected to be resolved by chromatography under non-denaturing conditions.

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References

[1] R.A. Welch, Mol. Microbiol. 5 (1991) 521.
[2] K.D. Clinkenbeard, D.A. Mosier, A.L. Tinko, A.W. Confer, Am. J. Vet. Res. 50 (1989) 271.
[3] K.D. Clinkenbeard, D. A Mosier, A.W. Confer, Infect Immun. 57 (1989) 420.
[4] C.A. Strathdee, R.Y.C. Lo, Infect. Immun. 55 (1987) 3233.
[5] S.K. Highlander, M. Chidambaram, M.J. Engler, G.M. Weinstock, DNA 8 (1989) 15.
[6] P. Stanley, L.C. Packman, V. Koronakis, Science 266 (1994) 1992.
[7] D.F. Boehm, R.A. Welch, I.S. Snyder, Infect. Immun. 58 (1990) 1959.
[8] M.J. Faith, R. Kolter, Microbiol. Rev. 57 (1993) 995.
[9] H. Ostolaza, B. Martlome, J.L. Serra, F. de la Cruz, F.M. Goffi, Fed. Eur. Biol. Soc. Lett. 280 (1991) 195.
[10] K.D. Clinkenbeard, P.A. Clinkenbeard, B.J. Waurzyniak, Vet. Microbiol. 45 (1995) 201.
[11] K.D. Clinkenbeard, C.R. Clarke, C. Hague, P.A. Clinkenbeard, R.J. Morton, J. Leukocyte Biol. 56 (1994) 644.
[12] M.J. Gentry, S. Srikumaran, Microb. Pathog. 10 (1991) 411.
[13] B.J. Waurzyniak, K.D. Clinkenbeard, A.W. Confer, S. Srikumaran, Am. J. Vet. Res. 55 (1994) 3233.
[14] O. Ortiz-Carranza, C.J. Czuprynski, J. Leukocyte Biol. 52 (1992) 558.
[15] A.D. Karkhanis, Anal. Biochem. 85 (1987) 595.
[16] G.K. Lindsay, P.F. Roslansky, T. Novitsky, J. Clin. Microbiol. 27 (1989) 947.
[17] G.K. Murphy, L.C. Whitworth, K.D. Clinkenbeard, P.A. Clinkenbeard, Infect. Immun. 63 (1995) 3209.
[18] J. Li, K.D. Clinkenbeard and A. Confer, Proceedings of the 78th Conference of Research Workers in Animal Diseases, Chicago, IL, 1997, Abstract 182.
[19] O. Lüderitz, M.A. Freudenberg, C. Galanos, V. Lehmann, E.T. Rietschel and D.H. Shaw, in F. Bromer and A. Kleinzeller (Editors), Current Topics in Membranes and Transport, Academic Press, New York, 1982, pp. 79–151.
[20] F.E. Regnier, Nature 350 (1991) 634.
[21] N.B. Afeyan, N.F. Gordon, I. Mazsaroff, J. Chromatogr. 519 (1990) 1.
[22] N.B. Afeyan, S.P. Fulton, F.E. Regnier, J. Chromatogr. 544 (1991) 267.