Isolation of a Toxic Phospholipase D From *Corynebacterium pseudotuberculosis*

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Introduction

Corynebacterium pseudotuberculosis causes caseous lymphadenitus (thin ewe syndrome) in sheep and goats, chronic abscesses in horses, and occasionally, infections in other mammals, including man (Lipsky et al. 1982). The organism usually invades its host through breaks in the skin and is carried by phagocytes to the draining lymph nodes where it multiplies, disseminates to visceral organs, and causes death of the host.

All known isolates of *C. pseudotuberculosis* secrete a toxic phospholipase D (PLD). PLD hydrolyzes lysophosphatidylcholine into lysophosphatidic acid and choline, and sphingomyelin into N-acylsphingosylphosphates and choline (Soucek et al. 1967). The role of PLD in pathogenesis remains unclear. It is thought to function as an aggressin, facilitating the spread and survival of *C. pseudotuberculosis* by allowing the organism to multiply, and to avoid the usual effects of the immune system. The bacteria are known to survive in host phagocytes and can cause phagocytolysis (Tashjian and Campbell 1983). PLD also activates complement by the alternate pathway.

PLD is a 30–40 kD protein, with a pI of about 9.8 (Linder and Bernheimer 1978). It requires calcium or magnesium ions for activity and has a pH optimum of 8.5 for its substrates. Activity is sensitive to extremes of pH. In a single rapid step, preparative IEF with the Rotofor® cell was used to obtain PLD of sufficient quantity and purity for both in vitro and in vivo studies. In addition, PLD purified from cell culture was used for the production of monoclonal antibodies.

Methods

Bacteria and Culture Conditions

C. pseudotuberculosis biovar *equi* isolate 155 was cultivated in 500 ml of brain heart infusion broth. Bacterial cells were removed by centrifugation and the supernatant fluid was passed through a Millipore filter (22 µm pore diameter).

Treatment of the Supernatant for Preparative IEF on the Rotofor Cell

Crude supernatant (200 ml) was dialyzed overnight against 100 volumes of HPLC grade water and lyophilized. The lyophilized material was brought to a volume of 51 ml with 2% (w/v) 3–10 ampholytes and 10% (v/v) glycerol.

The Rotofor focusing chamber was cleaned by prerunning it with HPLC grade water until a reading of 2.0 mA and 2,000 V was obtained. The focusing chamber was emptied and dried under a stream of nitrogen gas.

Running Conditions

Focusing on the Rotofor cell required 4 hr at 12 W constant power at 4°C. The initial conditions were 343 V and 62 mA. At equilibrium the values were 691 V and 18 mA. The electrolytes in the anode and cathode chambers were 0.1 M H_3PO_4 and 0.1 M NaOH, respectively.

Twenty fractions were collected and their pH values measured, and aliquots were analyzed on silver stained and Coomassie Blue R-250 stained gels. A pH 2.73–12.13 gradient was formed, with a linear region from pH 3.43 to 10.19 in fractions 2–18 (Figure 1).

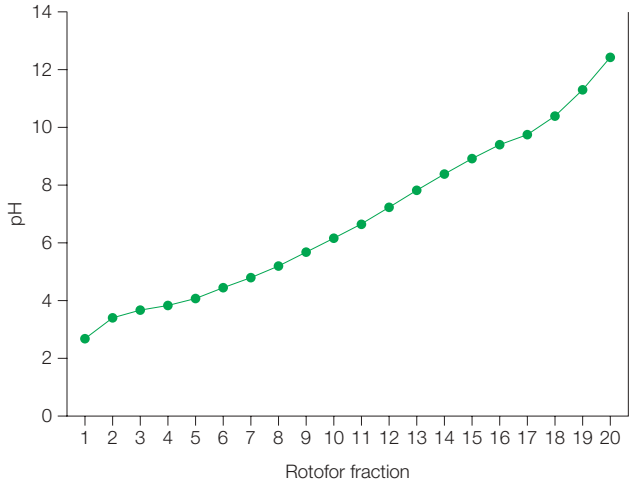

Fig. 1. pH of Rotofor fractions.

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Removal of Ampholytes

Removal of ampholytes was accomplished by dialyzing fractions overnight against 100 volumes of 0.1 M Tris, pH 9.0, containing 0.25 mM NaCl and 0.5 mM $MgCl_2$.

Activity and Protein Assays

Enzyme activity in the Rotofor fractions, as well as in the original, dialyzed, and concentrated supernatant fluids was determined by measuring the release of ^{14}C-choline from labeled sphingomyelin (Linder and Bernheimer 1978). A 10 µl aliquot of each fraction was incubated for 30 min at 37°C with labeled sphingomyelin in assay buffer. Liberated choline was separated from sphingomyelin by extraction with chloroform-methanol (Kates 1986). The aqueous phase was counted and enzyme activity was expressed as nmol sphingomyelin hydrolyzed per 30 min.

Protein concentrations were calculated from absorbance measurements (A_{280}) using BSA fraction V as a standard.

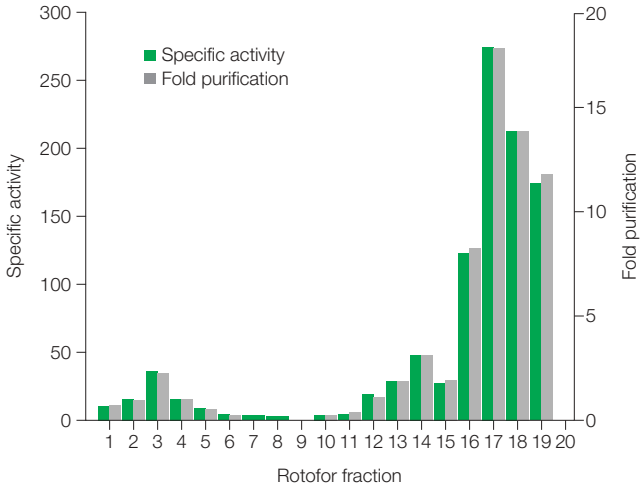

Fig. 2. Specific activity (nmol sphingomyelin hydrolyzed per 30 min per mg protein) and fold purification of PLD in Rotofor fractions.

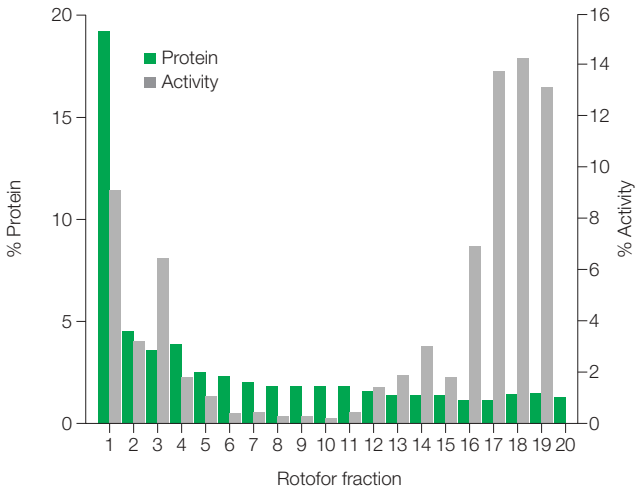

Fig. 3. Percent total protein recovered and percent of recovered PLD activity (nmol sphingomyelin hydrolyzed per 30 min) in Rotofor fractions. Based on total protein (2.4 g) and activity (4.5 x 10^4 U) loaded into the Rotofor cell.

SDS-PAGE Gel Analysis

A 40 µl aliquot of fraction 17 was concentrated 15-fold by vacuum centrifugation and electrophoresed through a 15% gel as described by Smith (1984). Gels were fixed and stained using both the color-producing silver method of Sammons et al. (1981) and Coomassie Blue R-250 stain.

Results

Sample preparation resulted in a negligible fold purification with substantial loss of both total protein and activity. After dialysis, the recovery of total protein and activity was 26% and 31%, respectively. Lyophilization of the dialyzed fluids resulted in no additional losses. The concentrated material, which was loaded into the Rotofor cell, contained a total of 2.4 g of protein with 4.5 x 10^4 units of activity. The purest fraction, fraction 17 (Figure 2), contained 23 mg of protein with a total activity of 6.2 x 10^3 units.

Purification in the Rotofor cell yielded 54% of the protein and 78% of the total activity found in the concentrated supernatant. Precipitation occurred in fraction 1. The PLD was focused in fractions 16–19 (Figure 3) within a pH range of 9.22 to 11.09 (Figure 1). These four fractions contained 8.6% of the total protein and 61% of the activity remaining after focusing. The fold purifications of the fractions were 8.3, 18.1, 14.3, and 11.6, respectively (Figure 2). A single 31 kD band was visible on a Coomassie Blue R-250 stained gel. Silver stained gels (Figure 4) revealed three bands (including a 31 kD band), all of which have molecular weights consistent with values reported for PLD (Goel and Singh 1972).

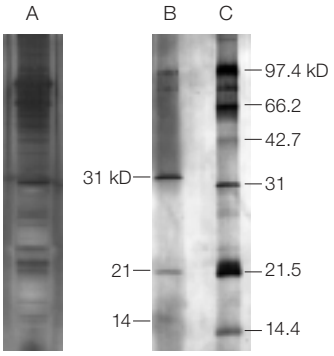

Fig. 4. SDS-PAGE analysis. Lane A, culture supernatant prior to purification on Rotofor cell; lane B, Rotofor fraction 17; lane C, molecular weight markers. The approximate molecular weights of the proteins recovered in fraction 17 are 31, 21, and 14.

Conclusions

PLD, which has a pI of 9.8, was effectively separated from the primarily acidic contaminating proteins in supernatant fluids of *C. pseudotuberculosis* cultures using the Rotofor cell. Previously reported methods of purification have been laborious, involving ammonium sulfate precipitation and chromatography on several types of matrices. In a single step, the Rotofor cell provides PLD of comparable purity (Egen et al. 1989).

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