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EFFECT OF A PROTEASE INHIBITOR ON ADENYLATE CYCLASE OF *BORDETELLA PERTUSSIS*

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The heterogeneity of adenylate cyclase (AC) of *Bordetella pertussis* reported in the literature has been revealed by SDS-PAGE and immunoblotting. This signifies that AC might be susceptible to endogenous proteases. The multiplicity of the bands appeared in SDS-PAGE and immunoblotting were found to be reduced when a protease inhibitor, phenyl methyl sulphonyl flouride (PMSF) was used during AC preparation. This study indicates that AC does not exist physiologically as multiple peptides.

Key words: Protease, *Bordetella pertussis,* Adenylate cyclase.

Introduction

B. pertussis produces an array of toxins among which AC is one of the recently recognized toxins. *B. pertussis* AC has been indentified in different forms. These include polypeptides of various molecular masses ranging from 43 to 660 kDa [1]. The structural-functional relationship became clearer when Ladant *et al.* [2] isolated and purified these species of molecular mass 43, 45 and 50 kDa and found them to be structurally related. Furthermore, purified AC from concentrated bacterial extracts revealed two forms of AC of 200 kDa and 47 kDa [3]. These forms showed immunologically related domains [3]. It was concluded that the low molecular mass form was probably formed by the proteolytic cleavage of the high molecular mass, 200 kDa form of AC.

The heterogeneity of AC indicates that it might be susceptible to degradation by endogenous proteolytic enzymes and suggests that at least some low molecular mass peptides are degraded forms of AC.

Materials and Methods

Culture of organism and preparation of AC. Bordetella pertussis strain BP348 (pRMB1) (AC+) was grown at 37°C in modified SS medium [4]. AC was extracted by adapting and modifying urea extraction technique described by Brownlie *et al.* [5] briefly, the culture was grown until an OD_{650} of 2. The cells were harvested at 6000xg for 10 min. (Sorvall RC5B) at 4°C.Immediately after harvest, the palette was resuspended in a solution of 0.5m M PMSF (Sigma) in distilled water. The suspension was stored overnight at - 20°C and then crystalline urea (BDH) was added to a final concentration of 4M and stirred at 4°C for 10 min. The suspension was centrifuged at 8000xg for 20 min. at 4°C and the supernate was ultra-centrifuged at 160,000xg for 1hr. at 4°C in an OTD-Combi/A148561 machine. The resultant supernate was collected and stored at -20°C as the urea extract. SDS-PAGE was performed according to the method of Laemmli [6] and immuno blott-ing was performed as described by Towbin *et al.* [7] in a Bio-Rad Transblot transfer apparatus. A sandwich of nitrocellulose memberane (Schleicher and Schuell) and gel was prepared between two papers of Whatman paper 1 (3mm),with one scotch bright pad on each side. This sandwich was loaded into a sandwich assembly and placed in a tank of transer buffer (tris 3g, glycine 14 g, methanol 1000 ml) with the nitrocellulose facing the anode. Protein was transferred at 30V across the sandwich for 18hr. followed by 50V for lhr. in order to transfer the higher molecular weight proteins. Then, the nitrocellulose membrane was stained with 0.5% Ponceau S in 0.1% acetic acid for 5 min. and de-stained with distilled water and MW standard bands were marked. A complete de-staining was then performed with PBS pH 7.3 (Dulbecco A). Blots were probed with monoclonal antibody 1H6 for lhr. under shaking condition. After probing, the membrane was washed two times, for 15min. each, with PBS and then incubated with anti-immunoglobulin serum conjugated to horseradish peroxide (Scottish antibody production unit SAPU). Subsequent washing was carried out two times for 15 min. each in PBS. Colour development was performed with diaminobenzidine (DAB, Aldrich) substrate solution (3,3"DAB 0.5g., 1%$CoC1_2$ in H_2O 2ml., PBS 98 ml 30% H_2O_2). The reaction was stopped exactly after 3 min. with distilled water.

Results and Discussion

Addition of PMSF immediately after the harvest of the *B. pertussis* cells to reduce degradation as determined by SDS-PAGE and immunoblotting with monoclonal antibody

1H6. Fig. 1 A and B shows the SDS-PAGE and immunoblot profiles of PMSF-treated and untreated urea extracts of *B. pertussis* BP348(pRMB1). It is evident from the profiles that PMSF-treated urea extracts of *B. pertussis* showed less degradation than PMSF-untreated sample. However, the degradation was not completely prevented by the use of PMSF. Although there was a marked reduction in proteolytic cleavage, this partial prevention suggests that other protease inhibitors may be necessary and perhaps used as a cocktail to prevent the proteolytic cleavage during storage and other manipulative procedures.

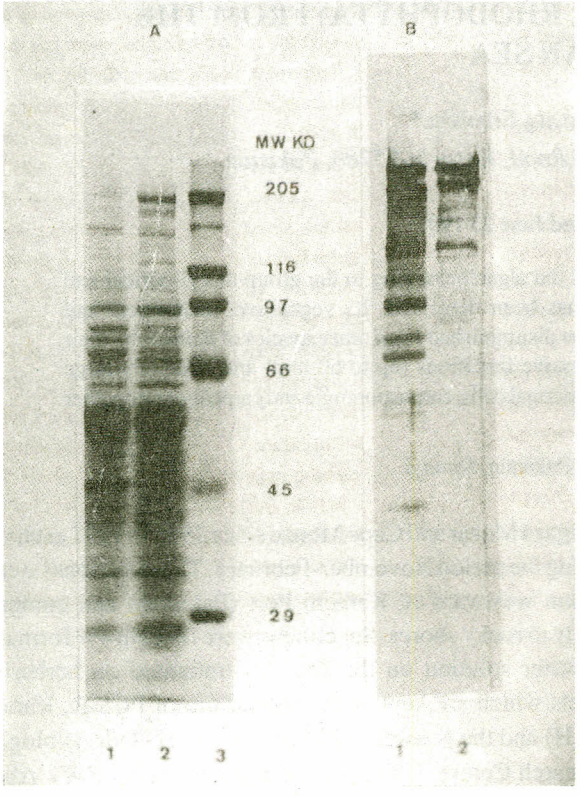

Fig.1AB: Effect of Protease Inhibitor (PMSF) on AC.
Crude preparations of AC (Urea extracts) were used to investigate the effect of protease inhibitor on AC; Urea extracts containing 0.5 mM PMSF were kept at 4°C for 48 hr. A control of untreated sample was also included in this study.

(A) Coomassie blue-stained gel of PMSF-treated and untreated urea extracts. (1) = *B. pertussis* BP348 (pRMB1) without PMSF; (2) = *B. pertussis* BP348 (pRMB1) with PMSF; (3) = Molecular weight standard (SDS-6H, Sigma)

(B) Immunoblot of PMSF-treated and untreated urea extracts.
The blots were probed with anti-AC monoclonal at a 1:1000 dilution. (1) = *B. pertussis* BP348 (pRMB1) without PMSF; (2) = *B. pertussis* BP348 (pRMB1) with PMSF.

A similar study on degradation of FHA (filamentous haemagglutinin) of *B. pertussis* has been reported by Irons *et al.* [8] in which they found that the degradation was completely abolished by the presence of PMSF in the solution. These data support the view that the other bands observed in the urea extracts or other crude preparations of AC by other investigators were mainly the degradation products of the high molecular mass AC. This also suggests that the major cell-associated AC is the 200 - 210 kDa molecule.

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