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Recovery of AC enzymic activity of CyaA of *Bordetella pertussis* in the absence of 8 M urea with new purification methods

Sharifi A., Khoramrooz S. S., Ielami O., Rabani M. R., Mohseni R. and Khosravani S. A. M.*

Cellular and molecular research center,Yasuj University of Medical Sciences, Yasuj, Iran
**Coressponding Email: khosravani2us@yahoo.com*

ABSTRACT

Adenylate cyclase toxin (CyaA) toxin is an important virulence factor of Bordetella pertussis, the causative agent of whooping cough, and a potential component of acellular pertussis vaccine. The aims of this study were to remove the urea, normally used to stabilize the protein, and to determine the stability of the enzymic and cytotoxic activities. The work was designed to produce the native enzymatically-active, invasive toxin (CyaA) as recombinant proteins. The AC enzymic activity was assayed by a conductimetric method. The toxins were purified by column chromatography using Q and Butyl Sepharose as new method. Produced CyaA was formulated as protein-coated microcrystals (PCMCs) on the surface of microcrystals of DL-valine. CyaA coprecipitated with different combination of CaM, BSA, $CaCl_2$ or ATP and crystals were dissolved in different buffers at various pHs. The CyaA in the new formulation was shown not to be readily soluble in aqueous buffers, but could be solubilised in urea buffers and retained high AC and cytotoxic activity. Many different types of PCMC formulation were prepared in order to increase solubility of PCMCs. The most promising results were obtained when PCMCs were dissolved in 100mM Bicine (pH 8). It was clear that the combination of these two were a more suitable method for CyaA purification. The results indicated that CyaA-CaM-BSA-PCMCs offer a promising way to preserve the activity and antigenicity of CyaA in non-aqueous formulation. Such production could have application for presentations of protein antigens that normally require cold storage for stability.

Keywords: Adenylate cyclase toxin, *B. pertussis*, PCMCs, AC activity

INTRODUCTION

Adenylate cyclase toxin (*CyaA*) of *Bordetella* species is unique among RTX toxins as it has both AC and haemolytic activity. The protein has ability to enter into eukaryotic cells and deliver a part of its catalytic domain into the cell [1]. where, upon activation by endogenous calmodulin, it catalyses formation and production of unregulated cAMP levels. CyaA intoxication leads to evident toxic effects on such cells as neutrophils and macrophages. It is able to release a catalytically-active adenylate cyclase (AC) 45 kDa fragment by proteolysis [2]. The gene for CyaA toxin was cloned and sequenced by Glaser *et al.*, (1988). Calmodulin binds with high affinity to the enzyme and stimulates its activity up to 1000-fold. It shows 25% similarity with *E. coli* HlyA and 22% similarity with *M. haemolytica* LktA and is responsible for the haemolytic phenotype of *B. pertussis* [3].

CyaA of *B. pertussis* penetrates target cells by binding to a specific receptor (CD11b/CD18). CyaA displays a selective cytotoxicity towards cells that possess the integrin receptor CD11b/CD18 indicating that its interaction with CD11b is required for translocation of the catalytic domain and the subsequent increase of intracellular cAMP concentration and cell death [4]. These authors also noted that Ca^{2+} ions were required for translocation of the catalytic domain into cells suggesting that Ca^{2+} binding to CyaA is necessary for interaction of CyaA with CD11b.

A recent study demonstrated that the acylation of CyaA is required for interaction of the toxin with cells expressing CD11b. It was shown that pro-CyaA is still able to bind the CD11b receptor, but this interaction is weak and does not allow membrane penetration of the pro-toxin. Hence, toxin acylation may be needed to confer a conformation on CyaA that is required for the transmembrane delivery of the catalytic domain to the cell cytosol where it can catalyze the conversion of ATP to cAMP [5]. Protein-coated microcrystals (PCMCs) are a new advance in vaccine formulation and have the potential to by-pass the cold chain. Initially developed to stabilize enzymes for industrial applications [6-8]. PCMCs are formed by rapid co-precipitation of protein(s) with an amino acid or sugar, producing particles with an inert core microcrystal coated with protein(s). Vaccine antigens, loaded onto PCMCs, exhibited much higher resistance to heat stress compared to native antigens. These reports used PCMC formulations which were instantly soluble in aqueous [9, 10]. In this study the stability of the proteins contained in the PCMC dry powder preparations (with regard to maintenance of a protective immune response) were determined at different temperatures and humidity, to monitor the high potential AC enzymatic and cytotoxicity activity. Combinations of different pertussis proteins were formulated as PCMC to determine which combination provides the most effective stability, AC activity and cytotoxicity effect.

MATERIALS AND METHODS

Bacterial strains and plasmids

The strain used for CyaA expression was *E. coli* BL21/DE3. This strain contains chromosomally-located λDNA expressing the gene encoding T7 RNA polymerase under the control of the inducible *lac* UV5 promoter. The addition of isopropyl-1-thio-β-D-galactoside (IPTG) to a growing culture induces the RNA polymerase that in turn transcribes the target DNA in the plasmid from a T7 promoter. *E.coli* BL21/DE3 lacks the *Lon* protease and outer membrane proteins (OMP) that can degrade some recombinant proteins. The enzymatically-active, acylated form of CyaA was produced from separate compatible plasmids, pGW44 and pGW54, each under the control of the inducible T7 RNA polymerase promoter [11, 12].

Preparation of crude urea extract

Crude urea extracts from *E. coli* BL21/DE3 were firstly purified by Q Sepharose column chromatography and step-wise elution with increasing concentrations of NaCl and then followed by Butyl Sepharose and different washing steps. Q Sepharose column chromatography was most effective in removing contaminating proteins and LPS. After Q Sepharose chromatography, indicating that most of the LPS was removed by the washing steps. Using silver staining, LPS is visualised, indicating that the crude urea extract contained a large amount of LPS, comparable to that in the control LPS sample from *E. coli* BL21/DE3. Western blotting result of the final purified CyaA preparations with anti-CyaA antibody, a rabbit polyclonal antiserum raised against the 200 kDa CyaA protein. It was clear that the combination of these two chromatographies (Q Sepharose and Butyl Sepharose) were a more suitable method for CyaA purification.

PCMC preparation:

1 ml of crude urea extract of CyaA containing 3.4 mg protein//ml in 8 M urea, 20 mM histidine, pH 6.0 was added to 1 ml of saturated DL-valine solution (60 mg/ml in distilled H_2O) and mixed for approximately 30 s. The aqueous protein solution was then added dropwise, with rapid mixing or stirring, to 40 ml of absolute ethanol saturated with DL-valine. Stirring (1500 rpm) was continued for a set time (15-20 mins). Crystals became visible gradually. The resulting, crystals were filtered onto filter paper (Millipore Ltd., UK, 0.45 µm HV) and washed with 40 ml of absolute ethanol. The filter paper was placed in a petri dish, and covered with parafilm; the parafilm was pierced to allow air to circulate in the dish and the PCMCs were left to dry overnight at room temperature.

CyaA-CaM-BSA-PCMCs preparation:

A volume of 4 ml of purified CyaA (290 µg protein/ml), 200 µl of calmodulin (1.8 mg/ml) and 12 ml of sodium phosphate buffer containing 3480 µg of BSA was added to 2 ml of saturated DL-valine to give a ratio of 1: 3: 10 molar (60 mg/ml dH_2O) and vortexed for 30 s.

Adenylate cyclase enzymic activity

AC enzymic activity was evaluated by measuring the change in conductance using an enzymometer (conductimetry assay) which was developed by Lawrence *et al*, [13]. The device consists of 8 glass cells (tubes) each containing platinum electrodes, which have been fused to the bottom of the tube. The sampling period is one second per cell, cycling through from one to eight glass cells. The apparatus is linked to computer software which processes the

conductimetric readings and uses software to create curve slopes and total changes, and the export of standard format data files. Buffer containing 10mM bicine, 1.5 mM magnesium acetate pH 8.0 was degassed by heating for 1-2 min >90 °C and placed under the vaccume pressure for 30 sec. After allowing the buffer to cool to room temperature, ATP was added to a final concentration of 0.5 mM. A volume of 2 ml of buffer, 1 µl of calmodulin (1.8 mg/ml, kindly provided by Dr. A. Lawrence, Glasgow University) and 1 µl pyrophosphatase (0.5 IU, Sigma) were added to each cell. Balancing of the cells to the same base line was allowed to occur before addition of CyaA samples. The reaction was started by addition of 2 µl of CyaA sample. Curve slopes and total changes in conductance were measured on screen whilst reactions were proceeding. One especially important feature is the ability to subtract the reading of one cell from all of the others so that test samples can be standardized to a blank loading. There is no change in conductance unless the $(PPi\ Mg)^{2-}$ product is cleaved, freeing the Mg^{2+} ion from chelation. It is the release of the independently mobile ions, $2Pi^{2-}$ and Mg^{2+} that produce the change in conductance that is measured by the assay.

$$(ATP.\ Mg)^{2-} + Bicine^{-} \rightarrow cAMP^{-} + (BicineH) + (PPi\ Mg)^{2-} \rightarrow 2Pi^{2-} + Mg^{2+}$$

Finally the total conductance changes were measured by gradient of the conductance curves.

Cytotoxicity activity of CyaA:

MTT Dye reduction assay (Cytotoxicity assay)

Cytotoxic activity was determined by the MTT assay as described originally by Mosmann [14]. using the CellTiter 96 ™ assay kit (Promega). This assay is based on the reduction of a yellow tetrazolium dye {3-(4,5-dimethylthiazol-2-3yl) –2-5-diphenyl tetrazoliumbromide, MTT} into insoluble purple formazan by dehydrogenases active in mitochondria of living cells. The activity of toxin on cells inhibits this reaction. J774.2 mouse macrophage-derived cells and other cell lines were prepared in appropriate medium (Section 2-12) to a concentration of 5 x 10^5 cells/ml. A 50 µl volume of cells was diluted in 10 µl trypan blue 0.25% and 40 µl water and transferred to a Neubauer chamber (haemocytometer). This was placed on a horizontal surface to allow the cells to settle for 1 min. Under the X 10 objective of a light microscope, the average number of cells in four squares were counted and then multiplied by two as a factor of dilution to give total cell number. Uptake of trypan blue reflects the lack of integrity of the plasma membrane and cells were examined to ensure >90% live cells. Aliquots of 50 µl of cells in duplicate were transferred to wells of a 96-well microtiter plate (Corning) and incubated at 37°C for 1 hour. A CyaA sample was diluted in cell culture medium in serial 2-fold dilutions starting at a protein concentration of 5 µg/ml. Then, volumes of 50 µl of each dilution were added in duplicate to wells of 96-well plate. The plate was incubated at 37°C for 2 hours in a humidified 5% CO_2 atmosphere before the addition of 15 µl of MTT dye (Promega) to each well and incubation continued as above for 4 h. The stop (solubilization) solution (100 µl/well, Promega) was added and incubation continued at 37°C overnight. The OD_{540nm} of the wells was measured using an ELISA reader (Life Science Int, Uk) against a positive (cells + 1% Triton X-100) and a negative (Cell + medium) control. 100% killing was taken as the mean absorbance value for the positive control. The percentage killing of cells was calculated using the following formula:

$$\text{Percentage killing} = 1 - \left(\frac{\text{test sample} - \text{positive control}}{\text{negative control} - \text{positive control}} \right) \times 100$$

RESULTS

Adenylate cyclase assay

AC enzymic activity of purified CyaA preparations from *E. coli* BL21/DE3 (pGW44, pGW54) and (pGW44-188, pGW54)] were determined by conductimetric assay. CyaA showed high enzymatic activity (Fig 1). In order to calculate the AC enzymic activity in international units (IU) per mg protein, the total conductance change should be known. A total conductance change in arbitrary units for a substrate concentration of 1 mM of ATP was 2200 and this value was used for determination of AC enzymic activity [13]. The conductance of CyaA preparation and level of enzymic activity of the CyaA preparation were routinely in the range of 660-880 µmol cAMP/mg protein/min. In

order to calculate the AC enzymic activity in international units (IU) per mg of protein the stock preparation of CyaA (300 µg protein/ml) was used at 1 in 5 dilution and 2 µl were added to a final volume of 2 ml buffer.

Initial reaction rate = tangent of reaction (y) containing 0.06 µg protein/ml (units/min)/ total change value (x) (arbitrary units).

For example: 86 (y)/ 2200 (x) = 0.039 µmoles/ml/min

To convert µmoles/ml/min to µmoles/min/µg protein
= 0.039 µmoles/ml/min/ 0.06 µg protein/ml= 0.65 µmoles/min/µg protein (650)

Cytotoxic effect of purified CyaA preparations

The effect of CyaA preparations on cell killing of mouse macrophage-derived (J774.2) cells grown in RPMI 1640 medium was determined by the MTT dye reaction assay using the CellTiter 96™ assay kit (Promega). This assay is based on the inhibition of the ability of active mitochondria in living cells to reduce tetrazolium dye solution. CyaA demonstrated a high level of killing effect that is in line with its enzymic and invasive activities. Some cytotoxicity effect was detected with CyaA* at the higher concentrations of toxin. As both preparations were prepared and solubilized in the same amount of urea buffer (starting at 8 M) it is possible that this low level of cytotoxicity may have been due to an effect of urea at the higher concentrations of toxin (7.19, 21.2 and 5.5 µg/well for CyaA and CyaA*, respectively. It should be noted that all CyaA dilutions were made in RPMI 1640 medium without urea, which means that lower concentrations of toxin contained lower concentrations of urea. The results with the different CyaA preparations against J774.2 mouse macrophage cells using the MTT assay showed the importance of both the AC enzymic activity and the acylation of CyaA for cytotoxicity. Similar experiments were performed on different mammalian cells, including undifferentiated U937 human monoblastic cells and U937 cells differentiated by treatment with phorbol myristate acetate (PMA) and IFNγ as a stimulator. However, whereas J774.2 cells were killed by CyaA at low concentrations of toxin (e.g 50% killing at 0.025 µg protein/ml), RBL-2H3 (rat basophil leukemia) cells, Vero (African green monkey kidney) cells, and mast cells (sheep bone marrow) showed less than 50% killing at all toxin concentrations tested even though these cells were cultured in DMEM medium which contain 5 mM of $CaCl_2$. Similarly, a maximum 30% killing was seen in undifferentiated U937 cells grown in RPMI 1640 medium but up to 60% cytotoxicity was seen with CyaA at much lower levels by differentiating the cells by adding IFNγ (500 units/ml) or 0.020 µg/ml PMA final concentration (Figure 3.5.2.3). It should be noted that U937 is an immature human monoblastic cell line. To differentiate these cells to monocytic, macrophage-like cells, they needed to be stimulated by adding PMA or IFNγ. Such cells are known to have a greater capacity to generate superoxide and express higher levels of CD11b, the receptor for CyaA [15].

Table1 Characterisation of purified CyaA preparation

Sample	Total volume ml	Protein µg/ml	LPS levels IU/mg protein	Cytotoxicity (50% killing of macrophages) µg protein/ml	AC enzymic activity µmol cAMP/mg protein/min
CyaA	35	290	128	0.025 ± 0.01	674 ± 9.19

CyaA was prepared and purified by Q and Butyl Sepharose chromatography. The AC enzymic and cytotoxicity activities for J774.2 macrophage-like cells were determined by conductimetry and MTT assays, respectively. LAL assay was used to measure the LPS levels.

Urea concentration in CyaA-PCMC

The removal of urea was a major aim in this project. The CyaA-PCMCs were determined by the urease assay, the % urea was found to be 0.00051g/100 ml. The concentration of urea in the CyaA stocking material in 8 M was 48 g/100 ml therefore; the ratio of urea after and befor PCMCs is 0.00001. The result shows that during the process majority of urea has been removed.

Table 2 Urea concentration in CyaA-PCMC

Sample	Meter reading Ammonium concentration (mg/L)	Urea concentration (mg/L)	% Urea (g/100 ml)
PCMC	0.42	0.51	0.051

Experiments with purified CyaA-PCMCs

Stability of AC enzymic activity of CyaA-PCMCs

Based on previous experiments where CyaA-PCMCs were made with crude CyaA preparations, CyaA-PCMCs were prepared with purified CyaA for further investigation. Prepared crystals were kept in different conditions for two weeks (i.e. 37, 20, 4, -20° C). 10 mg of each of two preparations (prepared from CyaA in the presence of 2 M or 8 M urea) were dissolved in 1 ml of 20 mM histidine, 8 M urea (pH 6.0) and AC enzymic activity determined. In parallel, 100 µl of original CyaA in 8 M urea was stored at the above conditions for two weeks (Table 4). As Table 2 shows, the PCMCs prepared from CyaA in 8 M displayed more stable and higher activity were obtained at a temperature of 4° C and above. The results from the original CyaA preparation in 8 M urea stored for 2 weeks at different temperatures showed that AC enzymic activity was retained well even at room temperature but, at 37° C, only 13.5 % of the original activity was retained (Table 5). It can be concluded that CyaA-PCMCs prepared from CyaA in 8 M urea pH 6.0 retained AC enzymic activity at approximately 90 % even at 37° C which is in contrast to original CyaA kept in 8 M urea at 37 ° C when 80-90 % of activity was lost. PCMCs have the potential, therefore, to be stored at ambient temperature and still retain anzymic activity.

Table 3 Characterisation of CyaA-PCMCs from crude CyaA (batche A)

CyaA Sample	Buffer used to dissolve crystals	Protein concentration µg/ml	AC enzymic activity µmol/mg protein/min	Cytotoxicity 50% killing of J774.2 cells µg/ml protein	LPS content visualized on gel by silver stain
Crude urea extract A*	8 M urea, 20 mM histidine pH 6.0	2000	375	0.033	++
CyaA-PCMCs A1#	8 M urea,20 mM histidine pH 6.0	76	159	0.069	-
CyaA-PCMCs A2♦	8 M urea, 20 mM histidine pH 6.0	97	240	0.035	-

** A, refer to original crude CyaA preps used for PCMCs.*
 # A1 refer to PCMCs made by shaking
♦ A2, refer to PCMCs made by stirring

Table 4 Stability of purified original CyaA

Samples	AC enzymic activity (µmol/min/mg protein)
CyaA at 37 °C	109 ± 14 (13.5 %)
CyaA at room temp	600 ± 19 (84 %)
CyaA at 4 °C	750 ± 16 (92 %)
CyaA at –20 °C	812 ± 11 (100 %)

Purified CyaA in 8M urea was examined for stability of toxin activity. 200 µl of sample was stored in different conditions (37, 20, 4 ,-20°C) for two weeks. The enzymic activity was determined by conductimetry assay. Results are the mean values ± standard deviation of 2 determinations.

CyaA-PCMCs were prepared from purified CyaA in 8Murea to examine stability of the toxin in the PCMCs format. 10 mg of each sample was dissolved in 1 ml of 8 M urea, 20mM histidine (pH 6.0) after storage at different temperatures (37, 20, 4 ,-20°C) for two weeks. The enzymic activity was determined by conductimetry assay. The sample (CyaA-PCMCs) prepared from purified CyaA in 8 M urea. Results are the mean values ± standard deviation of 2 determinations.

Table 5: Stability of purified CyaA-PCMCs

Assay →	AC enzymic activity µmol/min/mg protein				
	Befor storage	After storage two weeks			
Sample ↓		-20°C	4°C	20°C	37°C
CyaA-PCMCs in 8 M urea	480 ±13 (100 %)	480 ±13 (100 %)	440 ±15 (92 %)	403 ± 4 (84 %)	402 ± 12 (84 %)

AC enzymic and cytotoxic properties of CyaA-PCMCs made with different components

It was decided to make different CyaA-PCMCs which included a number of other components (e.g. CaM, BSA, ATP and $CaCl_2$) in an attempt to identify any extra component that might have an effect on CyaA-PCMCs properties. It is known that folding of protein in the bacterial cell is dependent on the assistance of chaperone proteins such as GroEL [16]. so it was thought that addition of other protein components with CyaA during PCMC formulation may aid solubility. As no AC activity was detected when CyaA-PCMCs were dissolved in histidine buffer, the CyaA may have formed aggregates on the crystal surface or aggregated when reconstituted in aqueous buffer. The toxin is known to be prone to aggregation in aqueous solution due to its hydrophobic nature and is the reason why CyaA preparations are stored in 8 M urea. Thus, introducing other biomolecules into the PCMC procedure may help to separate CyaA molecules and prevent aggregation.

CyaA-CaM-PCMCs and CyaA-CaM-BSA-PCMCs were made of CyaA plus CaM and BSA in molar ratio of 1: 3 and 1: 10, respectively. 10 mg of each PCMC sample was dissolved in 1 ml of 8 M urea, 20 mM histidine (pH 6.0) to measure the total protein concentration (Bradford assay) in order to calculate the amount of protein incorporated into the PCMCs. In addition, the weight of PCMCs was obtained and this provided the theoretical amount of protein in the PCMCs by dividing the total protein added to make the PCMCs by the total weight of the PCMCs. The measured protein content of the PCMCs would allow calculation of the actual total protein in the PCMCs. In order to calculate the CyaA content of the different PCMCs, an assumption was made that each protein would coat the valine crystals equally, by weight (Table 5 and 6).

10 mg of each type of crystal was dissolved in 1 ml of either 8 M urea, 20 mM histidine pH 6.0 or in 100 mM Bicine buffer alone at various pHs (Bicine buffer 10 mM pH 8.0 is the normal diluent for the conductimetry assay). AC enzymic and cytotoxicity activities of these samples were assayed and compared to that of CyaA-PCMCs dissolved in 8 M urea in 20 mM histidine pH 6.0.

Table 6 AC enzymic and cytotoxicity activities of different PCMC formulations dissolved in buffer at various pHs

Sample	AC enzymic activity µmol/min/mg protein and % activity	Cytotoxicity 50 % killing of J774.2 macrophages Protein µg/ml
CyaA-PCMCs in 8 M urea, 20 mM histidine pH6.0	670 ± 14 (100)	0.12 ± 0.021 (100)
CyaA-PCMCs in histidine (pH 6.0)	N/D	N/D
CyaA-CaM-PCMCs in Bicine pH 7.7	174 ± 6 (26)	0.51 ± 0.028 (23)
CyaA-CaM-PCMCs in Bicine pH 8.1	159 ± 5 (24)	0.38 ± 0.035 (31)
CyaA-CaM-PCMCs in Bicine pH 8.5	151 ± 9 (22)	>1
CyaA-CaM-BSA-PCMCs in Bicine pH 7.7	781 ± 12 (116)	>1
CyaA-CaM-BSA-PCMCs in Bicine pH 8.1	610 ± 11 (91)	>1
CyaA-CaM-BSA-PCMC in Bicine pH 8.0 + $CaCl_2$*	595 ± 10 (89)	>2
CyaA-CaM-BSA-PCMCs in Bicine pH 8.5	720 ± 30 (107)	0.51± 0.042 (23)
CyaA-CaM-BSA-ATP-PCMCs in Bicine pH 8.0	490 (73)	>2

The results for CyaA-CaM-PCMCs showed AC enzymic and cytotoxic activities at ~ 23% of that found in 8 M urea at pH 7.7 (Table 6). Cytotoxicity improved at pH.8.1 but AC enzymic was worse at pH 8.5. In-corporation of BSA with CaM, CyaA allowed almost full recovery of AC activity at all pH values but only low recovery of cytotoxicity which was best at pH 8.5. Some of the CyaA-PCMCs from batch number 1 were dissolved in 100 mM Bicine (pH.8.0) in presence of 200 µl of 5 mM $CaCl_2$ and then assayed. The results indicated that in the presence of $CaCl_2$, most of the AC activity was retained but the cytotoxicity was increased (Table 6). In another experiment, AC enzymic activities of different types of crystals were compared in 8 M urea, 20 mM histidine (pH.6.0) and 100 mM Bicine (pH 8.0) and compared with the original CyaA preparation (Table 5). The most significant finding here is

that enzymatic activity was recovered from CyaA-BSA. Again, the highest level of activity was seen in CyaA-PCMCS prepared in cooperation with CaM and BSA.

CyaA-PCMCs, CyaA-CaM-PCMCs and CyaA-CaM-BSA-PCMCs were prepared and 10 mg of each was dissolved in Bicine buffer of three different pH values. AC enzymic and cytotoxicity activities were compared with those obtained with CyaA-PCMCs dissolved in 8 M urea, 20 mM histidine (pH 6.0) as a positive control. Results are the means of 2 determinations. (± S.D)

CaCl2 added to Bicine not to crystals
N/D not detected

DISCUSSION

Adenylate cyclase enzymic activity

Lawrence *et al* [17]. developed a conductimetric assay for determination of adenylate cyclase activity of CyaA. This method offers a large number of practical advantages in comparison with the more usual radiochemical method. The benefits of the new conductimetry method are in terms of cost, ease of managing data and in particular, the procedure is rapid. In this study, the conductimetric assay was used to measure the specific enzymatic activity of purified CyaA preparations and after different treatments, such as PCMC preparation. Both CyaA pro-toxin and CyaA were enzymatically-active, but the CyaA pro-toxin displayed a higher specific activity than the acylated form [Table 3). These results are similar to the results reported by Westrop *et al*. [10] and Hormozi *et al* [18], who, using the conventional αP-ATP assay. In the present investigation, the specific activities of CyaA was 660 µmol/min/mg protein compared with 626 and 217 µmol/min/mgprotein reported by Hormozi *et al*. and Westrop *et al* , respectively. These differences may be a reflection of the accuracy of the method used, or measurement of the protein concentrations or differences in the methods of preparation.

CyaA protein- coated microcrystals (PCMCs)

PCMCs represent a novel method for the formulation of a wide range of biomolecules, including proteins, peptides, DNA, and vaccines. PCMCs consist of a core crystalline material, such as a sugar, amino acid or salt on which the biomolecules are immobilised [**7**]. The preparation of PCMCs is straightforward and applicable in any laboratory. The process involves dissolution of the appropriate crystal-forming carrier together with the given biomolecule in aqueous solution. Immediately, dehydration of the two components is facilitated by the addition of the aqueous solution to a water-miscible organic solvent, resulting in the rapid formation of the PCMCs with the biomolecule immobilised on the surface of the crystalline core carrier (via a crystal-lattice mediated self-assembly process). These PCMCs may be stored as a suspension or filter-dried to form a free-flowing powder [19]

The aim of current work was to prepare CyaA-coated microcrystals free of urea and to improve stability of CyaA with a view to preparing a novel vaccine formulation. This would provide, on redissolving in an appropriate aqueous solution, a sample of CyaA, in the absence of urea, as a model antigen to be used *in vitro* and *in vivo*. Purified CyaA preparations are routinely stored in 8 M urea because, in aqueous solution at high concentration, CyaA tends to aggregate due to the hydrophobic nature of the protein. The crystalline core carrier material DL-valine was chosen with CyaA at an initial concentration of 300 µg protein/ml added to a saturated solution of 60 mg/ml DL-valine. This aqueous solution was slowly added into 40 ml of valine-saturated ethanol solution under vigorous stirring. This resulted in the formation of CyaA-protein coated microcrystals. The solvent was then removed from the crystals using a Millipore filtration system (Millipore Ltd., UK) to yield upon drying, a free-flowing crystalline powder. Control samples of valine-PCMCs or other PCMCs without the addition of CyaA were also produced by this method to determine the effect of CyaA inclusion on crystal size and morphology. The yield of CyaA-PCMC crystals recovered varied (50-90%) from the theoretical amount. The actual loading of protein on the PCMC was determined by Bradford assay. The recovered yield and actual protein loading of CyaA onto the DL-valine crystals could probably be improved by optimisation of the formulation strategy. The CyaA-PCMCs properties were then determined by dissolving PCMCs in 8 M urea buffer or other buffers to assess activities by conductimetry and cytotoxicity assay.

Improvements to the process were designed to obtain a good cytotoxicity and AC activity, suggesting that the antigenicity of the CyaA molecule would be preserved and would be suitable for vaccine applications.

PCMC coated-microcrystal as different types of protein formulation and stability
AC enzymic and cytotoxicity activities of different types PCMCs
It was shown that all of the ceude CyaA-PCMC preparations tested had both cytotoxic and enzymatic activities when crystals made from crude or pure CyaA were dissolved in 2-8M urea (data not shown). This is evidence that the process used to make the PCMCs was working well to preserve the toxin activities. The problem that was encountered early on was that the CyaA could not be recoverd when crystals were dissolve in aqueous buffer (20 mM histidine alone). A urea concentration more than 1 M was required for the complete dissolution of the crystals and recovery of CyaA, which indicated that further investigation into methods of solubilising the PCMCs was required. Purified CyaA toxin was used for these experiments. A large range of different detergents (non-ionic and zwitter ionic) was used in attempts to solubilise the CyaA-PCMCs, but little AC activity was detected. At this stage in the investigation, urea was still the best reagent for solubilisation of CyaA-PCMC preparations.

Following the encouraging results on CyaA-PCMCs allowing for recovery of CyaA activities when PCMCs were dissolved in 2-5 M urea buffer, further investigation into methods of CyaA-PCMCs preparation were carried. A range of compounds (BSA, CaM, $CaCl_2$ and ATP) which might act as chaperones to prevent aggregation of CyaA, either on the PCMCs as they formed or upon resolubilation, were selected CaM and BSA were added to DL-valine to prepare different types of CyaA-PCMCs for testing the CyaA properties in different aqueous buffers and at various pH values. BSA had been used previously as a stabiliser for CyaA. Bellalou *et al* [1]. reported that when *B. pertussis* was grown in a medium containing high levels of BSA, both the adenylate cyclase and haemolysin functions were maintained in the toxin whereas, without PBS, the CyaA toxin was cleaved to produce a 45 kDa protein with AC activity. Calmodulin is a key calcium ion sensor and versatile intracelullar second messenger that can interact with targets via N- and C-terminal domains. Calcium binding to CaM induces conformational changes, from a mainly hydrophobic closed state to an open conformation, exposing a large hydrophobic binding pocket [20]. Previous work showed that the AC enzymic activity of CyaA is stimulated up to 1000-fold by binding of CaM to the AC domain. ATP and Ca^{2+} also bind to CyaA. The results obtained in this study showed that incorporating BSA with CyaA in the PCMCs allowed retention of AC enzymic and cytotoxic activities, particularly when combined with CaM, when the PCMCs were subsequently dissolved in aqueous buffer (Table 5). The AC enzymic activity in histidine buffer was stable for at least 7 days (Table 5). The data indicated that incorporating CaM into PCMC increased AC enzymic activity of CyaA but not its cytotoxicity activity, upon resolubilisation. The results showed that incorporation of ATP and Ca^{2+} had no obvious effect on the properties of CyaA-PCMCs.

These results are consistent with previous studies presented by Hormozi *et al* [18]. and MacDonald-Fyall *et al*. [21]. who reported that only CyaA with same activity and cytoxicity was highly immunogenic in mice and was able to stimulate serum anti-CyaA IgG antibody responses.

PCMC offer a number of advantages for the preparation of biomolecules for vaccine or drug delivery that is straightforward to apply and does not require specialist laboratory equipment and, importanly, particles show good stability towards stress conditions of high temperature and humidity. The overall conclusion from the present study was that PCMCs of CyaA co-precipitated with CaM and BSA could be used for immunisation to induce specific antibody responses to the toxin. Based on work carried out in this study it can be concluded that encouraging results have been obtained. The process by which the PCMCs are made has proven to be successful in coating the CyaA onto the valine crystals and, in addition, it seems as though most of the urea is removed when the CyaA coats the crystals. This is necessary if the PCMC preparations are to be used as a vaccine and hence the fact that there seems to be very little urea present in PCMC preparations is encouraging. Further work needs to be conducted with a range of CyaA-PCMCs, prepared with other components such as the inclusion of vaccine adjuants, and a more detailed parallel investigation should be made into their immunological properties so as to provide a deeper understanding of this promising technique.

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