HPLC Method to Assay the ADP-Ribosyltransferase Activity of Cholera Toxin

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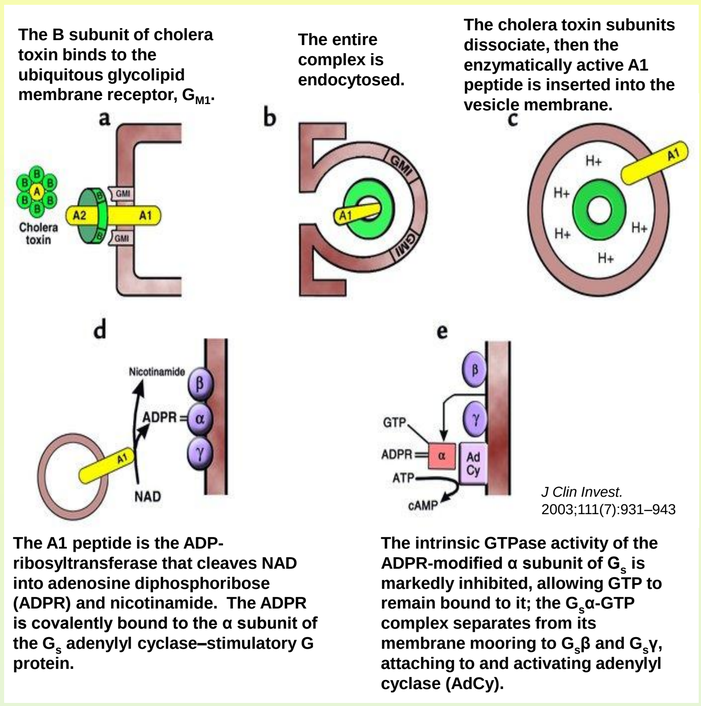
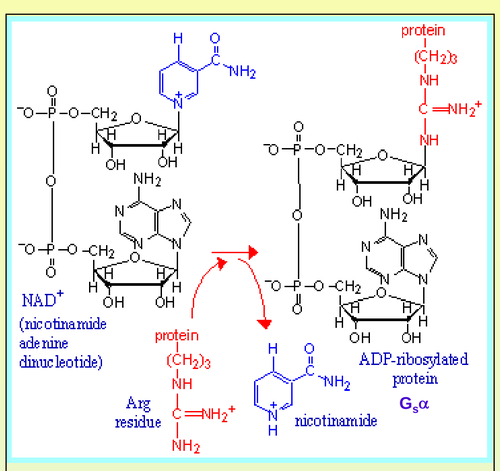
INTRODUCTION

ADP-ribosyltransferases constitute a class of enzymes which are responsible for the toxicity of a number of bacterial pathogens including cholera toxin, pertussis toxin, diphtheria toxin, and exotoxin A from *Pseudomonas aeruginosa*. The present study is focused on cholera toxin (CT) and the development of a sensitive, reliable assay method to measure ADP-ribosyltransferase activity. Cholera toxin is a 85,620 kD heterohexameric protein consisting of a single 27, 234 kD A subunit (CTA) and five identical 11,677 kD B subunits (CTB). The B-pentamer binds specifically to the ganglioside, G_{M1}, on the surface of human intestinal epithelial cells. The A subunit is the ADP-ribosylating enzyme. The transferase activity is dependent on proteolytic cleavage (nicking) of the A-subunit which gives rise to two domains, A1 and A2, connected by a single disulfide bond. Reduction of the disulfide occurs in the endoplasmic reticulum, activating the A1 domain. A1 crosses the ER membrane, enters the cytosol, and catalyzes the transfer of an ADP ribosyl group from NAD to an arginine of a specific protein in the cell stimulatory signaling pathway, Gsα (see representations below). This signaling pathway is permanently activated resulting in elevated levels of cyclic AMP and massive loss of fluids.



J Clin Invest. 2003;111(7):931–943



Diwan, J. (n.d.). Biochemistry of Metabolism. *Signal Transduction Cascades* . Retrieved March 22, 2011, from http://www.dentistry.leeds.ac.uk/biochem/MBWeb/mb1/part2/signals.htm

Schematic representation of the ADP-ribosylation reaction catalyzed by the A1 subunit of cholera toxin. The ADP ribosyl group of NAD is bound to Arg residue of the α subunit of G_s.

MATERIALS and METHODS

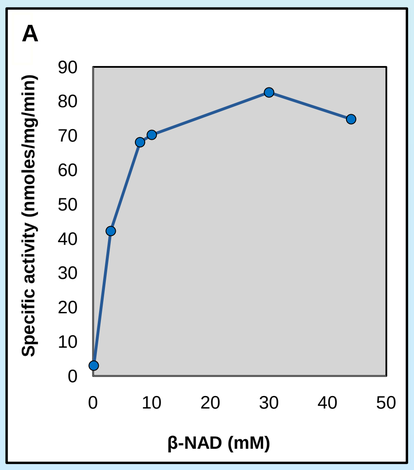
Materials: Cholera Toxin (Prod # 100B) and CTB (Prod # 104) are products of List Biological Laboratories, Inc. Agmatine, used as the ADP-ribose acceptor, and β-NAD were purchased from Sigma-Aldrich. Nicotinamide was from Supelco and dithiothreitol (DDT) was from Pierce.

Assay Sample Prep: Assay mixture consisted of 30 mM agmatine, 7 mM β-NAD, 20 mM dithiothreitol, in 30 mM sodium phosphate, pH 7.0. The ADP-ribosylation was initiated by adding 5, 10, or 15 µg/ml of cholera toxin. Incubation was at 30°C or 37°C for 90 minutes. Deviations in this procedure are detailed in the figure legends.

HPLC Method: The increase in the nicotinamide peak as ADP ribosylation by CT occurs was monitored using HPLC. The HPLC was performed using a Zorbax Eclipse Plus, C18 reverse phase column, (4.6 x 150mm) (Agilent Technologies, Santa Clara, CA) attached to a Varian ProStar HPLC system (Varian, Walnut Creek, CA). Solvent A was 25 mM potassium phosphate, pH 5.8 containing 2% acetonitrile, and solvent B was 100% acetonitrile. The column gradient was as follows: 0%B for 6 minutes, 0-72%B in 2 minutes, 72%B for 6 minutes, and column re-equilibration for 9 minutes at a flow rate of 1 ml/min. The column effluent was monitored at 260 nm to detect the nicotinamide and ADP-ribosyl-agmatine. The injection volume was 20 µl. Further details of the experiments are described in the figure legends.

RESULTS

A. Rate of ADP-Ribosylation as a Function of NAD and Agmatine Concentration.



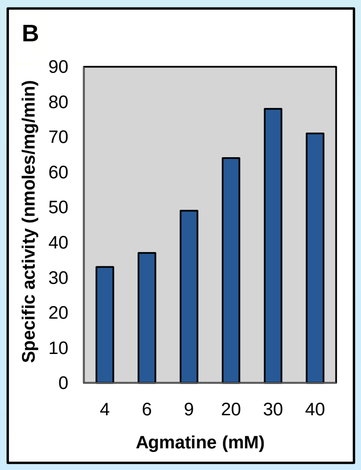


Figure 1: Effect of β-NAD and agmatine substrate concentrations on CT ADP-ribosyltransferase activity. ADP-ribosylation assays were conducted at 37°C in 30 mM sodium phosphate buffer, pH 7.0 containing 20 mM dithiothreitol, and β-NAD and agmatine as indicated. The reaction was initiated by addition of 20 µg/ml CT and monitored after 90 minutes by HPLC. (**A**) Plot of the specific activity as a function of β-NAD concentration. Agmatine concentration was 20 mM. (**B**) Plot of the specific activity as a function of agmatine concentration. β-NAD concentration was 8 mM. A Lineweaver-Burk plot of the 1/velocity versus 1/[substrate] indicated an approximate K_m value of β-NAD is 7 mM. **Figure 1B** demonstrates that the optimum concentration of agmatine for the reaction is 30 mM.

RESULTS (continued)

B. Nicotinamide Standard Curve.

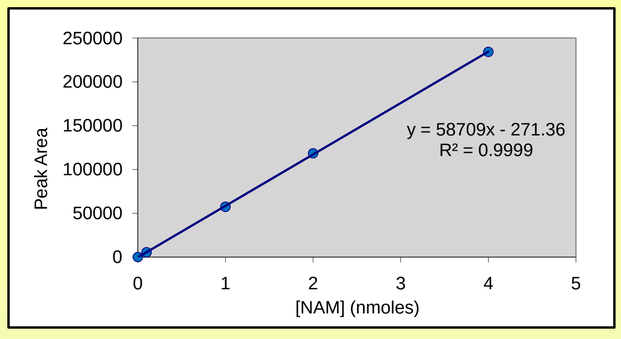


Figure 2: Nicotinamide standard curve.

A standard curve of the ADP-ribosylation product was created from 0-4 nmoles nicotinamide, in 30 mM sodium phosphate, pH 7.0 buffer containing 20 mM dithiothreitol and incubated at 30°C for 90 minutes. The standard curve showed a linear response (R^2 = 0.9999) between nicotinamide intensities detected versus concentration. This standard curve was used to determine the amount of nicotinamide produced from the ADP-ribosylation reactions in the assay samples.

C. ADP-Ribosylation of Agmatine as a Function of CT Concentration.

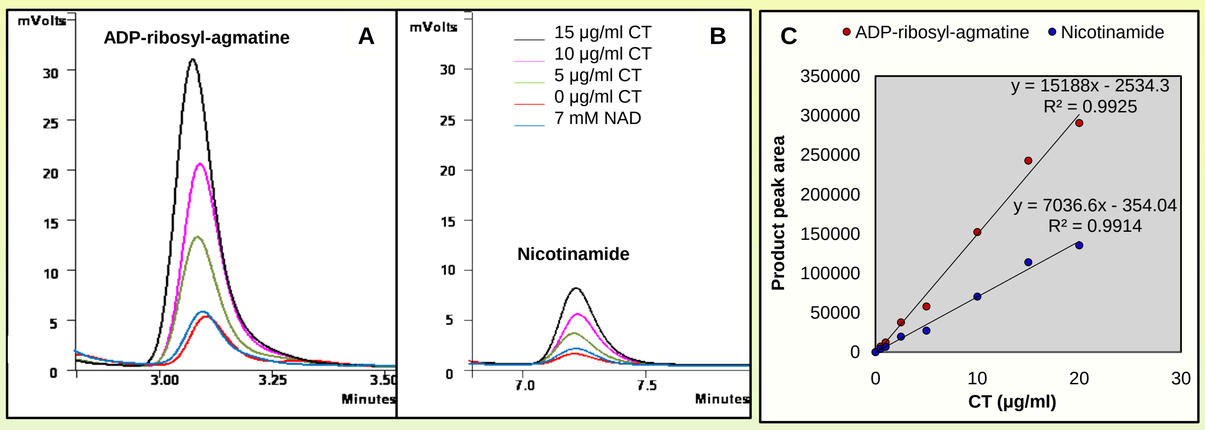


Figure 3: HPLC chromatograms demonstrating the ADP-ribosylation activity of CT. Two peaks increased in intensity as a function of CT concentration, nicotinamide at a retention time of 7.2 min (**B**), and a peak at 3.1 min (**A**) tentatively assigned to ADP-ribosyl-agmatine, based on absorbance at 260 nm. Chromatograms were obtained after 90 min incubation in 30 mM sodium phosphate, pH 7.0 buffer and 20 mM dithiothreitol for: 7 mM β-NAD (blue), 7 mM β-NAD and 30 mM agmatine (red), 7 mM β-NAD and 30 mM agmatine and 5 µg/ml (green), 10 µg/ml (pink), and 15 µg/ml (black) CT. In the chromatogram (red and blue) there are trace intensities for nicotinamide and ADP-ribosyl-agmatine due to small amount of autocatalysis of 7 mm NAD to ADP-ribose and nicotinamide over time. No significant change is observed after addition of agmatine until addition of enzyme CT. The linear response observed for both reaction products is shown in the graph of the peak areas obtained as a function of CT concentration (**C**).

D. Specific Activity.

The values obtained for the specific activity of CT at 30°C are given in the table below. Six vials of toxin were tested at 5, 10, and 15 µg/ml CT. Each test was done in duplicate. The average specific activity of all 36 tests is 46 ± 3.4 nmoles/mg/min. The data indicate low vial to vial variability.

CT (µg/ml)	Specific activity (nmoles/mg/min) in vial #						Specific activity (nmoles/mg/min)		
	1	2	3	4	5	6	Average	STD	%CV
5	50	39	47	44	33	46	44	5.2	12%
	49	40	47	44	39	49			
10	45	43	48	40	43	51	44	3.7	8%
	45	41	48	40	41	49			
15	47	43	52	44	50	51	48	3.3	7%
	48	51	48	45	47	53			

Specific activity of CT from 6 vials (nmoles/mg/min)	
Average	46
STD	3.4
% CV	7%

E. Limit of Detection (LOD).

The limit of detection (LOD) is the minimum concentration of CT that can be measured with 99% confidence that the concentration of CT present is greater than zero under our conditions using this HPLC assay. A calibration curve was generated by measuring the nicotinamide peak area as a function of enzyme concentration. At 30°C, twelve replicates of 5 µg/ml CT from 6 individual vials were performed, see table in **D**. Each analysis was calculated as µg/ml using the calibration curve. The detection limit was calculated as 2.681 (Student's t-Distribution with 99% confidence and 12 degrees of freedom) times the standard deviation of the 12 replicates. At 37°C, seven replicates of 5 µg/ml CT from a single vial were analyzed. The LOD was calculated as 2.998 (Student's t-Distribution with 99% confidence and 7 degrees of freedom) times the standard deviation of the 7 replicates. For both temperatures, the LOD was determined as 1.4 µg/ml CT.

CONCLUSIONS

The method described an assay for the specific activity of cholera toxin provides consistent results over the range from 5-15 µg/ml cholera toxin analyzed. The linear response indicates that the β-NAD and agmatine concentrations, 7 mM and 30 mM, respectively, are appropriate. The reaction is only dependent on the cholera toxin concentration. The assay is performed at concentration significantly above the LOD. The data indicate good precision.