The development of a quantitative ammonia assay for nicotinamidase

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Abstract

As the primary treatment of Lyme disease is antibiotics, alternative treatments for Lyme disease could help prevent the growing problem of antibiotic resistant bacteria. Nicotinamidase is an enzyme critical to the function of *Borrelia burgdoferi,* the bacteria that causes Lyme disease, but is not found in humans. This makes it a good target for an inhibitor. The activity of nicotinamidase has been qualitatively measured but this is not an ideal method to test the effectiveness of new inhibitors. An ammonia assay can be used to quantitatively determine the activity of the enzyme during purification. The nicotinamidase purification steps from *Saccharomycies cereviscia* were modified to remove the ammonium sulfate precipitation step, so the ammonia assay could be used to investigate novel inhibitors.

Introduction

Nicotinamidase is a vital enzyme for the bacteria *Borrelia burgdoferi*, which causes Lyme disease.[1] Finding an inhibitor for this enzyme could offer an alternative treatment for Lyme disease. The goal of this experiment was to quantitatively test enzyme activity with an ammonia assay and to simplify the purification process for nicotinamidase by removing the ammonium sulfate precipitation step.

Nicotinamidase catalyzes the hydrolysis reaction that transforms nicotinamide to ammonia and nicotinic acid (Fig 1).[2] This allows the activity of the enzyme to be quantitively measured by measuring the amount of ammonia produced.

Figure 1. Nicotinamide and water is converted to ammonia and nicotinic acid by nicotinamidase.

An ammonia assay kit from Millipore Sigma was used to analyze ammonia production and can measure ammonia concentrations as low as 0.2 µg/ml (Fig. 2).[6]

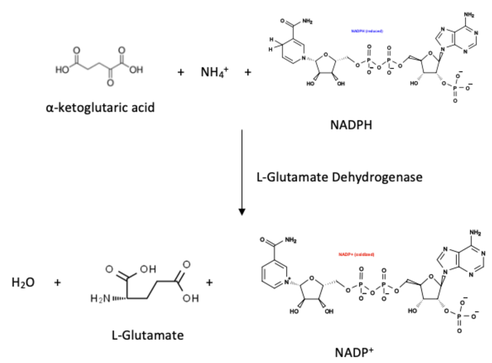

Figure 2. The ammonia assay kit uses the enzymatic reaction shown above to detect ammonia through monitoring the production of $NADP^+$ via spectroscopy.[7,8,9]

Quantitative Ammonia Assay Development

To develop the ammonia assay, the standard solution was tested multiple times for consistency. Standard solution (50 µl) was mixed with 1 mL of ammonia assay reagent using a vortex and incubated at 37°C for 5 minutes. Absorbance data was collected at 340 nm. L-Glutamate Dehydrogenase (10 µl) was added and the solution was mixed with a vortex and incubated 5 min at 37°C. Final absorbance was collected and using the equation from the assay kit, the amount of ammonia was calculated (Table 1).

Table 1. Results from the consistency testing of standard samples during the development of the ammonia assay procedure.

mg of NH_3/ mL of standard	Change in procedure	Number of runs in set
Results were negative		2
0.040 ± 0.005	Cleaning method Reaction in the cuvette Ultra-filtered DI water	4
0.052 ± 0.005	1.5 mL disposable cuvette Acetone to ethanol for cleaning procedure	8
0.049 ± 0.000	Vortex rather than invert to mix Pipette calibration Finished drying after cleaning in incubator for 20 min.	4
0.056 ± 0.005		8
0.054 ± 0.006		4
0.054 ± 0.007		8

Nicotinamidase Purification Procedure

The purification procedure of nicotinamidase from yeast (*Saccharomyces cerevisiae*) from Calbreath and Joshi was modified because its ammonium sulfate precipitation would interfere with the ammonia assay measurement (Table 2).[4]

Table 2. The original purification procedure compared to the modified procedure used in this experiment.

Calbreath and Joshi Procedure	Modified Procedure
Lyse yeast	Lyse yeast
Centrifuge 1	Centrifuge 1 (Supernatant 1)
Centrifuge 2	Centrifuge 2 (Supernatant 2)
Ammonium sulfate precipitation	
Acetone precipitation in Dry Ice- ethanol bath	Acetone precipitation in $N_{2(l)}$ -ethyl acetate bath
Centrifuge 3	Centrifuge 3 (Supernatant 3 and Pellet 3)

Nicotinamide (500 µl of 10mM) and supernatant, pellet, or water (100 µl) were mixed and incubated at 37°C for 30 minutes for the nicotinamidase assay. For the ammonia assay, each of the Nicotinamidase solutions (100 µl) was mixed with 1 mL of ammonia assay reagent and procedure described above was followed.

References

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Results and Discussion

The initial data showed that ammonia standard values were inconsistent. Several improvements to the method were made to develop the assay including reacting the samples in the cuvettes, using 1.5 mL disposable cuvettes, and using a vortex to mix the samples (Table 1). In addition, cuvettes were thoroughly cleaned with:

1. 50% acetic acid for 10 min
2. 6 rinses with ultra-filtered deionized water
3. An ethanol rinse
4. Dried in an incubator at 37 °C for 20 min

With these changes, the ammonia assay was able to produce consistent standards (Table 1).

In the enzyme assays, Supernatant 1 showed the most activity and Pellet 3 showed the least amount of activity (Table 3). This means that the ammonia assay successfully determined the ammonia concentration after incubation of nicotinamidase with nicotinamide. While not the ultimate goal, it is a huge forward step since this assay is quantitative.

Unfortunately, without having the total protein concentration of each sample, the specific activity of each sample could not be calculated. Thus, it is hard to determine which yeast purification step fraction is the most concentrated in nicotinamidase.

Table 3. The average ammonia concentration in each sample and the rate of production.

Sample Name	Avg. mg of NH_3/ mL of original sample	Rate of Activity (µg/(mL*min))
Supernatant 1	0.258	8.60
Supernatant 2	0.122	4.07
Supernatant 3	0.166	5.53
Pellet 3	0.0877	2.92
Water	0.0439	1.46

Future Work

Future work should focus on finding total protein so specific activity can be calculated and used to compare the purity of the samples. When the purification process of nicotinamidase has improved, inhibitors for nicotinamidase could be assayed and analyzed using the ammonia assay developed in this work. Once inhibitors are found they could be developed into drugs for Lyme disease treatment and the efficacy of those drugs could be compared to the efficacy of antibiotics currently used to treat Lyme Disease.