Manganese Dependence in *B. japonicum*

Sarah Popadowski and Dr. Mark O'Brian

Department of Biochemistry, SUNY at Buffalo, Amherst, New York 14214

Introduction

Maintaining metal homeostasis is critical to survival for any organism. Manganese is a useful metal necessary for many biological processes. Manganese also serves to protect the organism from oxidative stress. If the concentration of manganese becomes too high, it can be toxic. In *B. japonicum*, manganese homeostasis is maintained primarily through a homologue of the bacterial mntH protein. It has been found that *mntH* expression is regulated by manganese and that mntH is the major manganese transporter in *B. japonicum*.

Pyruvate kinase (Pyk) is critical for the production of energy from glycolysis as it catalyzes the production of pyruvate from Phosphoenolpyruvate (PEP). Pyruvate kinase requires a divalent metal, usually magnesium, for this process.

Superoxides are a class of free radicals produced by the reduction of O_2, as in respiration. These molecules can damage iron sulfur proteins and have additional toxic effects. Compounds like superoxide dismutase (SOD) exist to dismutate the superoxide into oxygen and hydrogen peroxide. We overexpressed a SOD protein from *B. japonicum* in order to study this process in more detail.

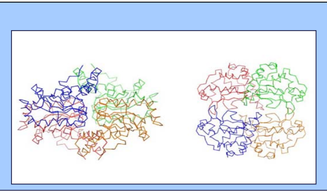


Fig 1. Crystal structure of human Mn SOD (180° view)
University of Florida
http://msg.mbi.ufl.edu/res-mnsod.html

Results

I. Overexpression of PykM

B. japonicum genomic DNA was amplified using specifically designed primers via PCR or polymerase chain reaction. The resulting DNA fragment was ligated into PSK. The construct was inserted into pET 14b and then transformed into chemically competent cells. The cells were cultured and added to 1.0 L of 2xYT media. The cells are grown at 37° for several hours until an OD595 of about 0.45 was reached. The cells were induced with 0.5 mM IPTG. This causes the promoter to turn on, producing the protein of interest.

II. Protein Purification

The cells were then lysed using a french press, and then spun down. The resulting fractions were run on a gel to see whether the protein was present in the soluble or insoluble fraction. The fractions were run on a Ni-NTA column, washed 5 times, then eluted with 0.5 M imidazole. The imidazole was then removed from the purified protein through a size exclusion column.

Figure 2. PykM Purification

A small portion of the soluble fraction was incubated with Nickel containing beads so that the protein then bound to them. Five washes were done to remove any excess protein as well as anything that didn't bind to the beads. The protein was then eluted off the beads with six washes with elution buffer containing 0.5 M imidazole. The samples were collected and run on a protein gel. The result is seen in figure 2. Looking at the gel, there is a large band at the expected size of PykM (55 kDa) corresponding to elution #2. Figure 2 contains samples of the unwashed protein, first and last wash and each of the six elutions.

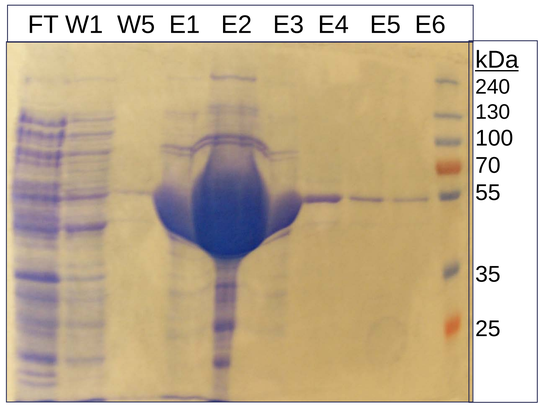
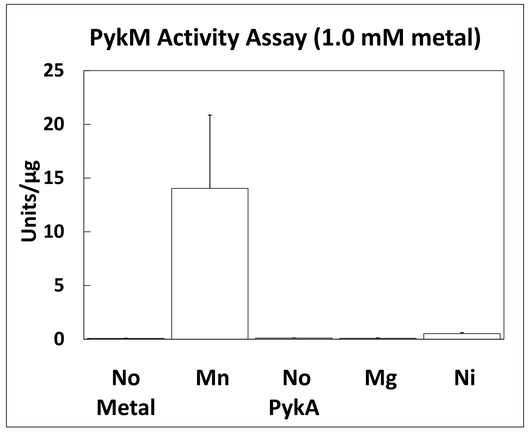


Figure 3. Activity assay for PykM enzyme

The purified protein was then assayed for protein content and activity. In the assay, PEP is used as a substrate to form pyruvate which forms lactate. When the lactate dehydrogenase reacts with lactate, it forms NAD from NADH. This change is measured by the shift in OD340 per minute. From this, the number of moles of NADH that is reduced per minute can be calculated per microgram of protein. This becomes the units of activity per µg of protein. The results of the activity assay are displayed in figure #3. Activity can only be seen in the presence of 1.0 mM manganese.



III. Overexpression of SodM

B. japonicum genomic DNA was amplified with primers using PCR, or polymerase chain reaction. The resulting DNA fragment was then ligated into blunt ended PSK. The construct was inserted into pET 14b and then transformed into chemically competent cells. The cells were cultured and added to a 1.0 L culture of 2xYT media. The cells are grown at 37° for several hours until an OD595 of about 0.45 was reached. The cells were induced with 0.5 mM IPTG. This causes the promoter to turn on, producing the protein of interest. After french pressing, the fractions were run on a gel (figure 4) and it was found that the protein was present in the insoluble fraction.

Figure 4. SodM Protein after French Press

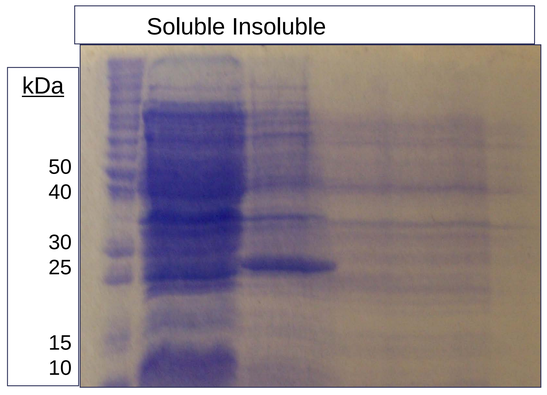
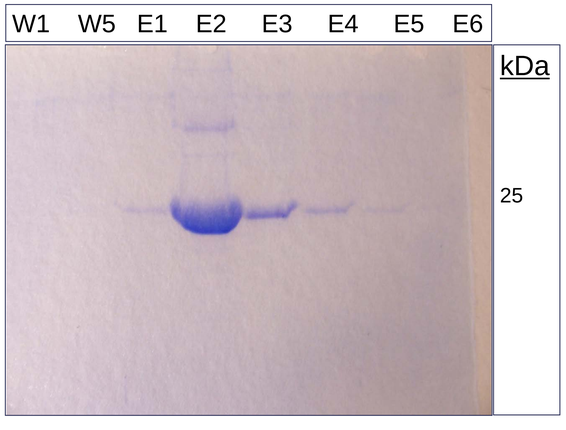


Figure 5. SodM Purification

The purification was similar to that of PykM. However, it was the insoluble fraction which was incubated with the Nickel beads. The entire purification was done at 20° instead of 4° The protein was washed a total of five times and then eluted six times. As with PykM, the fractions were collected and run on a protein gel. The results are shown in figure 5. Below are samples of the first and last wash and each of the six elutions. A band approximately corresponding to the size of SodM (25 kDa) can be seen in the band corresponding to elution #2.



IV. Protein Dialysis

Reconstituting the SodM protein proved to be more challenging . 500 µL of the protein was dialyzed in the presence of iron, manganese, and no metal. But upon analysis of the samples it was discovered that the protein had been lost during the dialysis. A small scale buffer exchange dialysis was attempted using filters and elution #3 from Fig 5. Finally after diluting elution #2 and dialyzing it in the presence of manganese, the protein was reconstituted. It showed up on a protein gel and was found to have a concentration of 0.177 µg/µL.

V. SodM Activity Assay

Two different techniques were utilized to assay the SodM protein for function. In the in-gel assay, a protein gel lacking SDS is run in the cold room for 2 hours, then stained with Nitro blue Tetrazolieum, then with soaking buffer containing riboflavin. The riboflavin reacts with the light, producing superoxides, which react with the NBT to turn the gel purple. If the protein that was loaded into the gel has SOD activity, it will compete with the NBT for the superoxides, leaving a clear band in the gel where the protein was loaded. So far all of these assays have been inconclusive.

In the liquid assay, xanthine and xanthine oxidase are added to produce O_2^-. When cytochrome c reacts with the O_2^-, the reaction proceeds at a rate of 0.015-0.020 ΔA550/min. When Sod is added, it competes with the cytochrome c for the superoxide radical, it slows down the reaction to about half of the original rate. From this, the strength of the SOD can be determined by using an *E. coli* control SOD. It was calculated that the overexpressed protein had an activity of 0.682 units per microgram of protein.

Conclusions

- PykM requires manganese, not magnesium to function
- SodM has sod activity. However this is slightly less than would be expected when compared to extracts.
- Future work will include doing more functionality assays. We are also interested to see if iron can be utilized to reconstitute SodM.

Acknowledgments

I'd like to thank Mark O'Brian and Thomas Hohle for all the time, advice, and patience they gave throughout my time in the lab. And a big thanks to all my lab mates, Siva, Rosalba, and Sid.

For further information

Please contact Mark O'Brian at mrobrian@buffalo.edu