Abstract: Melamine (2,4,6-triamino-1,3,5-triazine) has been used on numerous occasions by individuals as a food adulterant to boost the apparent nitrogen content of food. Together with cyanuric acid, melamine causes acute kidney failure. In the recent infant formula incident, 150,000 children were hospitalized and several died. The WHO recently requested the development of a rapid method to quantitatively detect melamine and related compounds in foods. Our laboratories at the University of Minnesota have collaborated with BIOO Scientific to develop and market an easy-to-use melamine test kit for foods (www.bti.umn.edu/gateway/melamine.html). The test kit is based on the enzyme melamine deaminase that was discovered at the University. Melamine deaminase liberates ammonia from melamine, which be detected via a simple color test. The toxicity of melamine has been shown in cats to be due to co-crystallization with cyanuric acid resulting in blockage of kidney tubules. There is now heightened awareness that melamine and cyanuric acid together in foods are highly toxic, and their co-occurrence in foods will cause deaths, disruption in food distribution systems, and a world-wide economic disaster. Thus, a rapid method to detect cyanuric acid in foods is urgently needed. Here we propose to develop a rapid test kit for detecting cyanuric acid in foods by purifying and stabilizing two enzymes, cyanuric acid hydrolase and biuret hydrolase, that act sequentially to liberate ammonia from cyanuric acid. If successful, we will collaborate with BIOO Scientific, which builds on our experience with enzymes involved in s-triazine metabolism and BIOO's expertise in food testing methodologies and marketing. We have previously purified cyanuric acid hydrolases so we have experience with this class of enzyme. Our laboratories have recently identified a highly stable cyanuric acid hydrolase that will be studied here to determine if it is suitable for use in a test kit. The gene encoding biuret hydrolase has been cloned into several different bacteria for testing. In the proposed studies, we will express biuret hydrolase in a recombinant E. coil bacterium to produce large quantities of enzyme. An alternative enzyme that has already been cloned and expressed, allophanate hydrolase, has been shown by us to hydrolyze biuret. Since the reaction rate is currently too slow for practical use, experiments will be conducted to increase the biuret hydrolysis rate of allophanate hydrolase. An advantage of using allophanate hydrolase is that it produces three molecules from one biuret molecule, resulting in increased sensitivity of the cyanuric acid test kit.

End User Engagement:

- Academic Community
- DHS Labs
- Food and Agriculture Industries
- Food and Drug Administration
- State and Local Food and Agriculture Labs
Executive Summary (2011): Melamine-Cyanuric Acid Detection System for Purposely Adulterated Foods

PI: Lawrence Wackett, University of Minnesota
Co-PI: Michael Sadowsky, University of Minnesota

October, 2009 - April 30, 2011

Research Aims
The research proposed here is designed to develop new enzyme technology that can be used in a second generation test kit for detecting cyanuric acid, alone or in combination with melamine, in foods and food products. Melamine and cyanuric acid together are exquisitely nephrotoxic. This combination of chemicals will likely be an adulterant of choice to artificially increase apparent nitrogen content of foods, for future attacks on people, or for causing widespread and intentional economic disruption in the food distribution system. The World Health Organization has called for a global effort to develop rapid testing methods for cyanuric acid.

Methodology
We have purified five different cyanuric acid hydrolases, three of them specifically for this project. Each enzyme was derived from a different bacterial strain, Pseudomonas, Acidovorax, Bradyrhizobium, Rhizobium, and Moorella. The genes encoding each of the enzymes has been cloned in E. coli and heterologously expressed with an N-terminal polyhistidine tail. These enzymes have each been purified in a single step via Ni-column chromatography. The purified proteins have been subjected to steady-state enzyme kinetic analysis and substrate specificity studies. Previously, no one had been successful in purifying a biuret hydrolase to homogeneity and obtaining an active enzyme. To this end, we took a bioinformatics approach. We then purified a biuret hydrolase. We conducted steady-state kinetic experiments. We conducted 13C-NMR experiments to show that we could measure cyanuric acid to allophanate and ammonia by the combined action of cyanuric acid hydrolase and biuret hydrolase.

Final Results, Accomplishments and Deliverables

To determine which cyanuric acid hydrolases was best for our purposes, kinetic constants were determined for each. For comparative purposes, the AtzD from P. ADP and TrzD were also purified, and kinetic values determined. Stability studies with the P. ADP enzyme found that when the enzyme was frozen a substantial loss in activity was observed. Storage of the enzyme at 4°C resulted in stable activities for over a month. For this reason, all enzymes were stored at these new conditions and activity monitored throughout the study to ensure no loss of activity. The kcat from the P. ADP enzyme stored in this new fashion yielded a ten fold increase over previously published results (Fruchey, et al, 2002). The TrzD enzyme, on the other hand, had kcat values that were ten fold reduced from those previously published [Karns, 1999]. This could in part be due to differences in assay temperatures, though data from Li, et al. suggests that this may not be the sole reason [Li, 2009]. The kcat and Km values for the newly cloned cyanuric acid hydrolases are within an order of magnitude of each other. The kcat values ranged from 5-73 s⁻¹ and the Km from 23-130. Based on kinetic parameters and stability, the enzyme from Moorella was used for studies (Li, et al, 2009). We also filed a patent on this enzyme for use in potential test kit applications.

Peer-reviewed journal articles produced from this project

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Patents Awarded

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