ABSTRACT: Isatin (Indole-2,3) is involved in diverse functions in both plants, bacteria and mammals including humans. It has been found in different mammalian tissue types and body fluids. It is a product in the degradation of tryptophan but, the metabolic pathway of isatin is yet largely unknown. Stressed rodents have shown increased levels of isatin[1,2,3]. In humans the normal level of isatin is often found in the range of 1-3µM but increased levels of isatin have been correlated with stress and Parkinson’s disease, whereas decreased levels are correlated to anxiety[4,5]. Because of the diverse functions of isatin, a precise, fast and well-functioning diagnostic tool was of high relevance both as a mean to study animal models in relation to human disease and as a diagnostic tool in Parkinson’s disease as well as a maker for stress levels. Here we present an assay, based on the purified recombinant enzyme, isatin hydrolase, from Stappia aggregata[6]. This enzyme catalyse the reaction that convert isatin into isatinoic acid. Isatinoic acid can be further decarboxylated into anthranilic acid by incubation with H2O2. Anthranilic acid is a fluorophore.

Standard and proof of concept: Pig serum was used to test whether increasing amount of isatin in this sample can be converted into antracnic acid and detected based on its fluorescence properties. Samples were selected at 315 nm and emission measured at 365 nm.

Graph 1: This standard is measured in 25% dilution and suggest that this serum contains 2.0±.66.4µM isatin. Isatin should be present in 0.1-1.1µM in tissue this observation falls within this range. The error on these measurements are quite large, and we are currently working on implementation of steps in the assay which will increase the signal to noise ratio.

Liniarity: In a fluorescence experiment, the intensity of the emitted light, F follows the relation: F = a* (1-e-c) (1), a is the quantum efficiency, meaning the percentage of molecules in an excited state from which fluorescence is possible, L is the inherent radiant power, e is the photolysis, b is the path length of the cell, and c is the molar concentration of the fluorescent dye. For diluted concentrations, where the factors ebc < 0.05, equation (1) can be rewritten as: F = kabc (2), meaning there is a linear correlation between the concentration of the sample and the emitted fluorescence. Graph 1 is made under conditions where ebc < 0.05[6].

Graph 2 (left-above): Raw data showing that increasing [isatin] results in increased fluorescent signal. The toppoint is slightly shifted in +H2O2 compared to -H2O2, maybe due to minute changes in pH. Intensity at 401nm is pulled out and plotted for further analysis.

Graph 3 (left-below): Shows a linear correlation between fluorescent isatin and concentration with an R2-value of 0.9931 in +H2O2 samples, This can be used as an internal standard, to calculate the isatin concentration in this serum, from the difference between +/−H2O2-samples with no isatin added. In this sample data suggest [isatin]=950nM in serum. The current status is that measurements have a large error margin, and because of this the values can not be trusted yet. to get statistical well founded data, protocol optimization is needed.

Protocol for Isatin detection in blood samples

1. Heated serum preparation
   Blood clotted for 45min. Blooded clot is spun for 15 min. @ 4k x G to remove red blood cells. 0,4 g/1mL NaCl was added to the serum and diluted to double the volume. The serum was heated to 80°C and spun at 45k rpm. The supernatant is frozen for isatin detection[7].

2. Sample preparation
   a. Adjust vol. to 1mL
   b. Add 50µL H2O2 to each sample
   c. Add 5µL catalase to each sample
   d. Incubate the sample at 37°C in a sonicator bath to remove interfering oxygen species

3. Sample measurement
   On a Jasco FP2200
   Each sample is measured twice to check for intra-sample differences, each spectra is an accumulation of three measurements. ex. bandwidth=2.5 nm, em.bandwidth=5 nm. Ex. wavelength=320 nm, scan interval=310-450 nm, sens.=medium, scan speed=200 nm/min. Xe lightsource, response 1 sec.

Graph 1: Fluorescence as a function of isatin concentration

Graph 2 (left-above): Fluorescence as a function of isatin concentration in serum

Graph 3 (left-below): Fluorescence as a function of isatin concentration in serum

CONCLUSION

Through the use of H2O2 an isatin hydrolase, a functional assay for detection of isatin in blood has been established.

The serum from different pigs have been isolated and heated to examine differences between individual animals. Preliminary results suggest that the isatin concentration is within the range typically observed for humans.

Detection of isatin in urine samples is possible, but due to high fluorescent background the signal to noise ratio makes detection complicated.

PROSPECTS

To improve the current signal to noise ratio, especially the H2O2 concentration in the samples is critical. To overcome this issue, either another oxidation agent is needed, lower concentration of H2O2 should be added or more effective removal of residual H2O2 by a catalyst could be implemented.

When the assay is fully functional in pig serum, we wish to test our assay in humans and especially in Parkinson’s patients or stressed individuals.

REFERENCES: