Fluorescent Assays for the Detection of beta-Amyloid and Tau Modification Enzymes: Pin1 and Glutaminyl Cyclase

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Introduction

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases that leads to dementia. The hallmark of AD is the presence of senile plaques and neurofibrillary tangles in the affected brain. It is believed that senile plaques originate due to the aggregation of beta-Amyloid (A β) peptides while neurofibrillary tangles are caused by the accumulation of hyperphosphorylated Tau proteins. Both Aβ and Tau, can undergo different modifications during cellular trafficking that may affect their aggregation properties. Thus, it is important to identify what enzymes can modify these two AD biomarkers and study these modifying agents in more detail. Glutaminyl cyclase, also known as Glutaminyl-peptide cyclotransferase (QPCT), and Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1) are such enzymes which modify Aβ and Tau, respectively.

Pin1 catalyzes cis/trans isomerization of the phospho-Serine/Threonine-Proline peptide bond of Tau protein. This leads to Tau dephosphorylation and prevents it from forming neurophibrillary tangles. On the contrary, Glutaminyl cyclase can catalyze production of pyroglutamic beta-amyloid peptides that aggregate much faster compared to the unmodified ones and may produce senile plaques at a higher rate. In order to investigate properties of these two protein modifying enzymes, reliable assays for Pin1 and Glutaminyl cyclase activities have to be developed.

We have designed two assays to detect the activity of Pin1 and Glutaminyl cyclase enzymes using fluorescent based peptide substrates. Both assays use a long-wavelength green fluorophore that is released after enzyme and developer action and can be monitored at Ex/Em=490/520 nm. Pin1 catalyzes a change of the fluorescent substrate into the *trans* form that is cleaved by the substrate developer and releases the fluorophore much faster compared to the unmodified *cis* substrate. Thus, the intensity of the fluorescent signal can be correlated with Pin1 activity in a sample. For the Glutaminyl cyclase assay, the substrate is converted into the pyroglutamate form that is readily cleaved by the assay developer and generates green fluorescence.

Both assays have sensitivity in the nanogram range. Glutaminyl cyclase assay was validated with human saliva and cerebrospinal fluid. Inhibitors for both assays were selected to ensure that they act on enzymes and not on the assay developer.

Materials and Methods

- **SensoLyte® Green Glutaminyl Cyclase Activity Assay (AnaSpec, CA):**
  - √ Purified human glutaminyl cyclase (50 µl/well) was mixed with fluorogenic substrate (50 µl/well) in 96-well black opaque plate and allowed to react for 30 min; developer was added (50 µl/well) and allowed to react for 30 min; fluorescent signal was read at Ex/Em=490/520 nm
- **SensoLyte® Pin1 Activity Assay (AnaSpec, CA):**
  - √ Purified human Pin1 and developer were mixed together (50 µl/well final volume) in 96-well black opaque plate with fluorogenic substrate (50 µl/well); fluorescent signal was read at Ex/Em=490/520 nm
- **Human Samples Tests:**
  - √ Human Cerebrospinal Fluid (CSF) (Bioreclamation LLC, NY), Human Saliva (donation)
  - √ Human CSF and saliva were diluted 1:2,5 ratio in the glutaminyl cyclase assay buffer with and without inhibitor and were tested for glutaminyl cyclase activity
- **Glutaminyl Cyclase and Pin1 Inhibitors:**
  - √ 1-Benzylimidazole: glutaminyl cyclase inhibitor (Fisher Scientific, PA)
  - √ 5-Hydroxy-1,4-naphthoquinone (Juglone): Pin1 inhibitor (Sigma, MO)
  - √ Tannic Acid and 3-Nitrophenol (Fisher Scientific, PA) 1,10-Phenanthroline (Sigma, MO)
- **Fluorescent measurements:**
  - √ Flex Station 384II (Molecular Devices, Sunnyvale, CA) FLx800 (BioTek Instruments, Winooski, VT)

Assay Principle

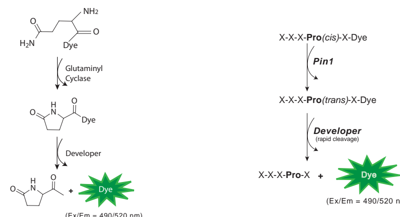

Figure 1. Glutaminyl cyclase assay principle. Glutamine substrate is converted into the pyroglutamic form by human Glutaminyl cyclase enzyme; pyroglutamate is removed by the assay developer which releases the fuorophore. The activity of the Glutaminyl cyclase is directly proportional to the fluorescent signal at Ex/Em=490/520 nm.

Figure 2. Pin1 assay principle. Pin1 converts *cis* substrate into *trans* form that is more readily cleaved by the developer compared to the *cis* isoform. Pin1 activity is directly proportional to the fluorescent signal at Ex/Em=490/520 nm.

Glutaminyl Cyclase Assay

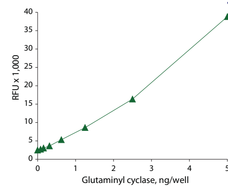

Figure 3. Glutaminyl cyclase assay sensitivity. Assay sensitivity reaches as low as 156 pg/ml of active human glutaminyl cyclase enzyme.

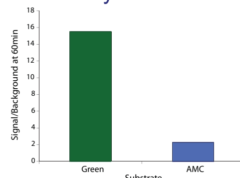

Figure 4. Glutaminyl cyclase assay: Green vs AMC substrate. The same peptide sequence substrate was labeled with green or 7-amino-4-methylcoumarin (AMC) fluorophore. The amount of enzyme assayed was 2.5 ng/well.

Pin1 Assay

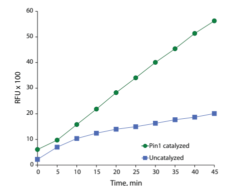

Figure 5. Pin1 assay kinetics. The amount of Pin1 enzyme was 1 µg/well.

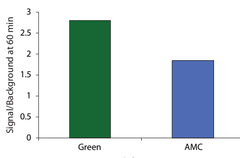

Figure 6. Pin1 assay: Green vs AMC substrate. Same peptide sequence substrate was labeled with Green or 7-amino-4-methylcoumarin (AMC) fluorophore. The amount of enzyme assayed was 500 ng/well.

Inhibition of Glutaminyl Cyclase *in vitro* and In Biological Samples

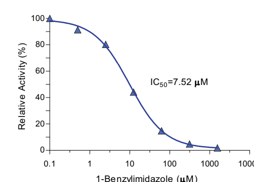

Figure 7. Inhibition of Glutaminyl cyclase with 1-Benzylimidazole *in vitro*. 1-Benzylimidazole was titrated 5-fold. The amount of Glutaminyl cyclase used was 10ng/well.

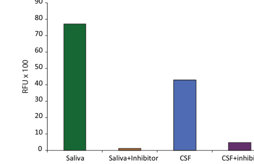

Figure 8. Glutaminyl Cyclase activity in human body fluids. Human saliva and CSF were diluted 2.5 times in the assay buffer and tested for the Glutaminyl cyclase activity with and without 100µM 1-Benzylimidazole.

Inhibitor Optimization for Pin1 Assay

Table 1. An example of Pin1 inhibitor screening. Three compounds were tested as potential Pin1 inhibitors using SensoLyte® Green Pin1 assay: Tannic acid, 3-nitrophenol, and 1,10-phenanthroline. Developer/inhibitor control was run to ensure that none of these compounds inhibits assay developer. Tannic acid was found to act as a Pin1 inhibitor. The amount of Pin1 enzyme used was 500 ng/well.

Sample	RFU at 60 min	S/B
Pin1, no inhibitor	2326	2.44
Pin1+10µM Tannic acid	1442	1.48
Pin1+20µM 3-nitrophenol	2165	2.42
Pin1+20µM 1,10-phenanthroline	2331	2.67
Developer+10µM Tannic acid	972	1.02
Developer+20µM 3-nitrophenol	893	0.94
Developer+20µM 1,10-Phenanthroline	872	0.91
Developer, no inhibitor	954	1.00

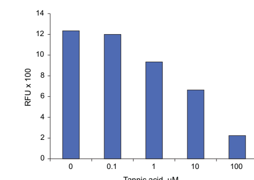

Figure 9. Tannic acid inhibits Pin1 activity. Tannic acid was titrated 10-fold. The amount of Pin1 used was 500 ng/well. Assay was run for 45 minutes.

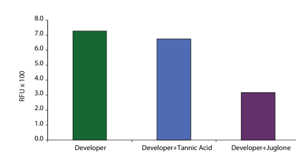

Figure 10. Inhibitors' effect on the Pin1 assay developer. Assay developer was mixed with the Pin1 green substrate in the presence of 10µM Tannic acid or 10µM Juglone. It was found that Juglone can inhibit assay developer.

Conclusions

- We have developed robust assays optimized to measure activity of human Glutaminyl cyclase and Pin1 enzymes.
- Optimized green substrate for both assay provides higher Signal/Background ratio compared to the conventional AMC substrate.
- We have demonstrated that SensoLyte® Green Glutaminyl cyclase Activity Assay can be used to measure activity of Glutaminyl cyclase in human saliva and CSF.
- We have identified Tannic acid as an inhibitor of human Pin1 enzyme.