New high affinity antibodies against Botulinum Neurotoxin Type A have been developed in chickens. Production of polyclonal IgY is both easy and cost effective. A single egg contains as much antibody as a single bleed from a rabbit and is a simple and less invasive production method. Eggs from immunized chickens represent a continual source of polyclonal antibody.

The antigen used to challenge the chicken was a recombinant Heavy Chain Binding domain fragment (HoA) from Type A toxin. IgY derived from eggs was affinity purified using an anti-Heavy chain-coated column. To test the affinity purified antibodies we determined the titer of the anti-HoCα antibodies against Botulinum Neurotoxin Type A (BTA) holotoxin, List Product #130. The ability of the anti-HoCα antibodies to capture BTA was also tested using a “Sandwich” ELISA.

Fluorimetric assays: Continuous assays were performed on a SPECTRamax GEMINI XS fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) using Greiner TRU-TRAC black flat-bottomed plates (E&K Scientific, Campbell, CA). Assays to determine specificity constants, $k_{cat}/K_m$, were performed with plates containing 10 nM LcA. For the sensitivity assays, plates containing anti-HoCα-HRP antibody were used at 1:1000 and 1:2000 respectively, to detect the binding of BTA to IgY. 1-Step Turbo TMB from Pierce was used as peroxidase substrate to develop. The midpoint of the detection curve is 2.9 ng/ml and as low as 0.244 ng/ml can be detected above background.

Fluorimetric assays: Continuous assays were performed on a SPECTRamax GEMINI XS fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) using Greiner TRU-TRAC black flat-bottomed plates (E&K Scientific, Campbell, CA). Assays to determine specificity constants, $k_{cat}/K_m$, were performed with plates containing 10 nM FRET peptide, pre-incubated for 15 mins to ensure equilibrium prior to addition of 10 nM LcA. For the specificity assays, plates containing serial dilutions of LcA were equilibrated for 15 min at 37°C prior to addition of 10 μM substrate. For both assays, the time-dependent increase in fluorescence intensity was monitored at 37°C. The excitation and emission wavelengths were set to 325 nm and 418 nm, respectively, for the o-Abz-substrate, 325 nm and 398 nm for the Mca-substrate, and 490 nm and 535 nm using a cutoff filter of 495 nm for the FITC-substrate.

Determination of specificity constants, $k_{cat}/K_m$: Measurements of kinetic parameters using FRET peptides may be limited by peptide solubility and the inner filter-quenching effects observed at concentrations approaching $K_m$. Specificity constants were determined using the progress curve method at substrate concentrations much lower than the $K_m$. The apparent first order rate constant was calculated by fitting the progress curves to:

$$RFU(t) = [RFU]_0 - [RFU]_1\exp(-k_{obs}t) + RFU_0;$$

where $[RFU]_0 = [RFU]_{dig} - [RFU]_1$.

The second order rate constant, $k_{cat}/K_m$, was calculated using:

$$k_{cat}/K_m = k_{obs}[E_0]$$

All non-linear regressions were performed using KaleidaGraph software.

Comparison of FRET Substrates for Botulinum Neurotoxin Type A

A new FRET substrate for the zinc endopeptidase activity of the 50 kDa light chain (LoA) of the type A Botulinum neurotoxin has been designed and evaluated. This new substrate, which contains the FRET pair Mca/Dnp was compared to the SNAPside® substrate (Product #520 and #521) FRET peptides, which contain the oAbz/Dnp and FITC/DABCYL FRET pairs, respectively (FIGURE 1). The substrates were evaluated on the basis of specificity constants, $k_{cat}/K_m$, quenching efficiency, and the increase in relative fluorescence units (RFU) observed after complete hydrolysis (Table 1). Tests to estimate sensitivity with LoA were also performed (FIGURE 2).

$$RFU = RFU_{max} - RFU_0$$

$\Delta RFU = RFU_{1} - RFU_0$$

$\%q.e. = (1 - RFU_0/RFU_{1}) \times 100$

$$RFU_{sec} = RFU_{1}/RFU_0$$

Table 1: Hydrolysis data for FRET peptides as substrates for LoA.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$k_{cat}/K_m$ (mM⁻¹s⁻¹)</th>
<th>$RFU_{sec}$</th>
<th>$RFU_{max}/RFU_0$ (%)</th>
<th>$%q.e.$</th>
<th>$RFU_{sec}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNAPside® (Mca/Dnp)</td>
<td>250,330</td>
<td>4,970</td>
<td>10</td>
<td>94</td>
<td>0.62</td>
</tr>
<tr>
<td>SNAPside® (Mca/Dnp)</td>
<td>63,879</td>
<td>17,546</td>
<td>12</td>
<td>91</td>
<td>14.87</td>
</tr>
<tr>
<td>SNAPside® (FITC/DABCYL)</td>
<td>22,319</td>
<td>38,168</td>
<td>11</td>
<td>90</td>
<td>7.72</td>
</tr>
</tbody>
</table>

Sensitivity: The hydrolysis of the three peptides after 2 hrs at 37°C was evaluated as a function of LoA concentration in order to determine the minimum amount of toxin detectable using these FRET substrates. The Mca/Dnp FRET substrate is the most sensitive with a detection limit around 0.01 nM LoA or 0.5 ng/ml. The FITC/DABCYL peptide is not useful for detection of the toxin but is valuable for inhibitor screening where UV absorbing compounds could be evaluated without interference.

Conclusions: The o-Abz/Dnp SNAPside® is the best FRET peptide based on the increase in fluorescence for the fully digested peptide (RFU/RFU_0) and the quenching efficiency as given in Table 1. The best FRET substrate for the BTA enzyme, as measured by the $k_{cat}/K_m$, is also the oAbz/Dnp SNAPside®. The Mca/Dnp SNAPside® produces the strongest signal measured as RFU/sec and as such is the preferred peptide for detection of low levels of botulinum toxin (FIGURE 2).

FIGURE 1: The three FRET pairs used in peptides designed as substrates for the BTA enzyme.

FIGURE 2: The fluorescence intensity as a function of LoA concentration for the FRET substrates after 2 hrs digestion at 37°C. A linear relationship between the RFU and the concentration of LoA is observed.

FIGURE 3: The fluorescence intensity as a function of LoA concentration for the FRET substrates after 2 hrs digestion at 37°C. A linear relationship between the RFU and the concentration of LoA is observed.