FRET peptide substrates for the Botulinum toxins type A, B, and E and for anthrax lethal factor.

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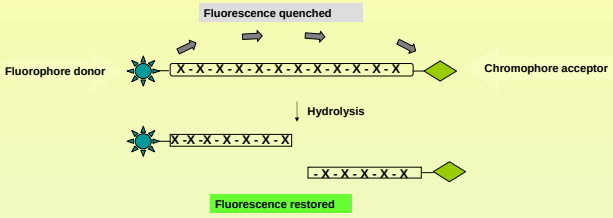
FRET substrates

The potent toxicity of both the botulinum neurotoxins and anthrax lethal toxin is due to a zinc-dependent proteolytic activity associated with the toxins. Measurement of this enzymatic activity provides for:

1) potentially sensitive and direct means for **detection** of the toxin, and

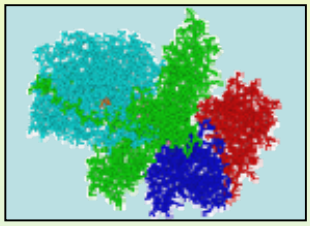
2) a method for identifying potential toxin **inhibitors** using high throughput screening.

A highly efficient approach for monitoring enzymatic activity is based on the use of fluorescence resonance energy transfer (FRET) substrates. These fluorogenic peptides (**-X-X-X**) contain a fluorescent group ( ) at one end and a suitable chromogenic acceptor group ( ) at the other. As shown below, the fluorescence is quenched initially by intramolecular energy transfer between the donor fluorophore and the acceptor chromophore. Cleavage of the FRET substrate by the appropriate enzyme releases the fluorophore and full fluorescence is restored. The increase in fluorescence intensity is directly proportional to the amount of enzyme present. Enzymatic activity can be monitored continuously by recording the increase in fluorescence intensity with time.



Botulinum neurotoxins

are among the most potent toxins in nature. They are synthesized as single 150 kDa polypeptide chains which are subsequently cleaved to produce a heavy chain and a light chain linked by a disulfide bond. The crystal structure of botulinum toxin type A (BTA) is shown here. There are three functionally distinct domains that mediate either cell binding (blue and red domains), translocation through the membrane (green domain), or cleavage of specific proteins in neuronal cells. The domain responsible for cleavage, a 50 kDa zinc-dependent endoprotease, is shown in aqua.



The potency of these toxins in humans is due to the cleavage and inactivation of one of the SNARE membrane fusion proteins. Each of the seven serotypes of the neurotoxin, A-G, selectively cleaves a single neuronal protein at a specific site. Types A, B , E, and F cause human intoxication. Neurotoxin types A and E cleave the 25 kDa synaptosomal protein, SNAP-25, and types B and F hydrolyze the vesicle-associated membrane protein, VAMP-2 (synaptobrevin-II).

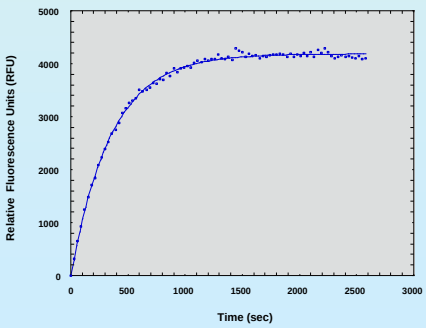
The following FRET peptides have been designed at List Biological Laboratories as substrates for the botulinum toxin enzymes.

SNAPtide®, Product #520 and #521 (U.S. Patent, No. 6,504,006) is readily recognized and cleaved by the Botulinum toxin type A (BTA), Product #130, and the BTA light chain (LcA), Product #610A. One of the substrates, Product #520, contains an oAbz/DNP FRET pair and the other, Product #521, a FITC/DABCYL FRET pair. Representative data are given below.

VAMPtide®, Product #540 is readily recognized and cleaved by the Botulinum toxin type B light chain (LcB), Product #620A. This FRET substrate contains an oAbz/DNP FRET pair.

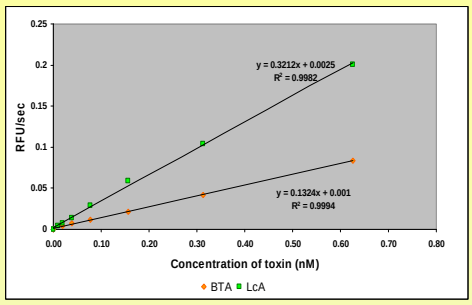
SNAP Etide™, Product # 550 is readily recognized and cleaved by the Botulinum toxin type E light chain (LcE), Product #635A. This FRET substrate contains an oAbz/DNP FRET pair.

Cleavage of SNAPtide®, Product #520, by LcA



Representative plot of fluorescence intensity (RFU) versus time observed for cleavage of 10 μM SNAPtide®, Prod #520 with 10 nM LcA at 37º C in 50 mM HEPES, pH 7.4 with 0.05% Tween-20. The k_{cat}/K_m obtained from this progress curve is 285,470 $M^{-1}sec^{-1}$. An 18-fold increase in fluorescence intensity is observed when the oAbz/DNP substrate is fully hydrolyzed.

Response Curves for BTA and LcA



The rate of cleavage (RFU/sec) of 8 μM SNAPtide®, Prod #520 as a function of LcA (green) and BTA (orange) concentration. The excitation and emission wavelengths were set to 321 nm and 418 nm, respectively. Endopeptidase reactions were conducted in 20 mM HEPES, pH 8.0, 0.3 mM $ZnCl_2$, 1.25 mM DTT, and 0.2% TWEEN 20 for 1.5 hrs at 37ºC. The buffer used was optimized for hydrolysis with BTA. A linear relationship between the RFU/sec and the concentration of toxin is observed.

Limit of detection (LOD) for BTA and LcA

	LOD for BTA	LOD for LcA
SNAPtide® Prod #520	48 pM (7.2 ng/ml)	24 pM (1.20 ng/ml)
SNAPtide® Prod #521	31 pM (4.65 ng/ml)	25 pM (1.25 ng/ml)

LOD studies were carried out using 8 μM of each FRET substrate and 1.5 hrs digestion at 37ºC. Conditions for SNAPtide®, #520 are given in the legend above. SNAPtide® #521 digestions were conducted in 20 mM HEPES, pH 8.0, 0.3 mM $ZnCl_2$, 1.25 mM DTT, and 0.1% TWEEN 20 using excitation and emission wavelengths of 490 nm and 523 nm, respectively, and a cutoff filter of 495 nm.

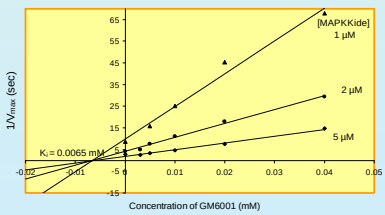
Lethal factor

(LF) is the enzymatic component of anthrax lethal toxin which specifically cleaves the MAPK-kinase proteins. LF is also an ideal target for therapeutic inhibitors. FRET substrates for LF, MAPKKide®, Product #530 and 531, have been designed at List Biological Laboratories. One of the substrates, Product #530, contains an oAbz/DNP FRET pair and the other, Product #531, a FITC/DABCYL FRET pair. Product #531 is especially well suited for high throughput screening for the IC_{50} and inhibitor modality of potential inhibitors. The results obtained for several compounds using Product # 531 are shown in the table below.

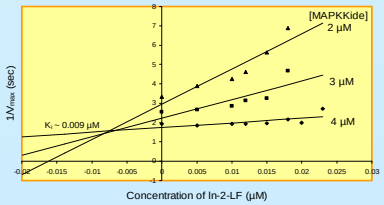
IC_{50} values for a series of potential LF inhibitors

Compound	IC_{50} (μM) with LF
tetracycline	> 1000
EGCG	2
Actinonin	29
MMP-3 Inhibitor II	145
MMP-8 Inhibitor I	44
GM6001	9
GM6001, negative control	>500
CL82198	156
In-2-LF	0.021

Dixon plots for LF inhibitors, GM6001 and In-2-LF



Dixon Plot obtained for the matrix metalloproteinase inhibitor, **GM6001**, using 10 nM LF, Product #172, and 1, 2, or 5 μM MAPKKide®, Product #531, at 37ºC. The plot indicates that GM6001 behaves in a non-competitive manner.



Dixon Plot obtained for the Anthrax Lethal Factor Protease Inhibitor, **In-2-LF** using 10 nM LF, Product # 172, and 2, 3, or 4 μM MAPKKide®, Product #531, at 37ºC. For **In-2-LF**, the plot indicates competitive inhibition. The value obtained for K_i is 9 nM which is also in the range previously reported by [Tonello et al., Nature, 418:386, 2002].