Inactivation of Toxins

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Biological Toxins

Biological Toxins are at the interface of classical B- and C-agents:

- **produced by living organisms, but not ‚living' or able to replicate**
- **share many characteristics with classical chemical agents**
- **many high molecular weight toxins exert an enzymatic activity within the body**

  >> amplification of potency, higher specific toxicity in humans
- **detection & identification has been somewhat neglected in the past**

Biological Toxins

Detection of biological toxins: a challenge

- **active in the absence of the producing organism and its genetic information**
- **detection of nucleic acid insufficient**
  → detection of protein / toxin necessary
- **very high toxicity poses a challenge to detection technology**
- **biological toxins are often produced in numerous variants or isoforms which differ in their characteristics**

Lethal dose of selected toxins in mice

Toxin	LD_{50} [µg/kg; *i.p.*]	MW [Da]	source
Botulinus Neurotoxin A	0,0003	150 000	bacteria *C. botulinum*
Tetanus Neurotoxin	0,001	150 000	bacteria *C.tetani*
Abrin	0,04	65 000	plant *Abrus precatorius*
Diphtheria toxin	0,10	52 000	bacteria *C. diphtheriae*
Iotatoxin	0,2	47 500	bacteria *C. perfringens E*
Ricin	3,0	64 000	plant *Ricinus communis*
Tetrodotoxin	8,0	320	bacteria
Saxitoxin	10,0	300	Dinoflagellat
T-2 Trichothecene	1210,0	466	Fungi *Tr. lignorum*
Plutonium-239	1000,0 (*i.v.*)	239	element
KCN	3000,0 (oral)	65	chemical

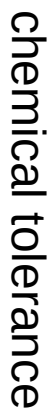
How to proof inactivation

Residual biological toxin after inactivation might still comprise lethal dose:

→ Validation of in-house method using toxin specific bioassays

Functional method for detection:

- **in vivo (mouse bio assay)**
- **ex vivo (e.g. mice phrenic nerve hemidiaphragm for BoNT)**
- **in vitro:**
  - **cell culture systems**
  - **enrichment method & highly sensitive functional MS**

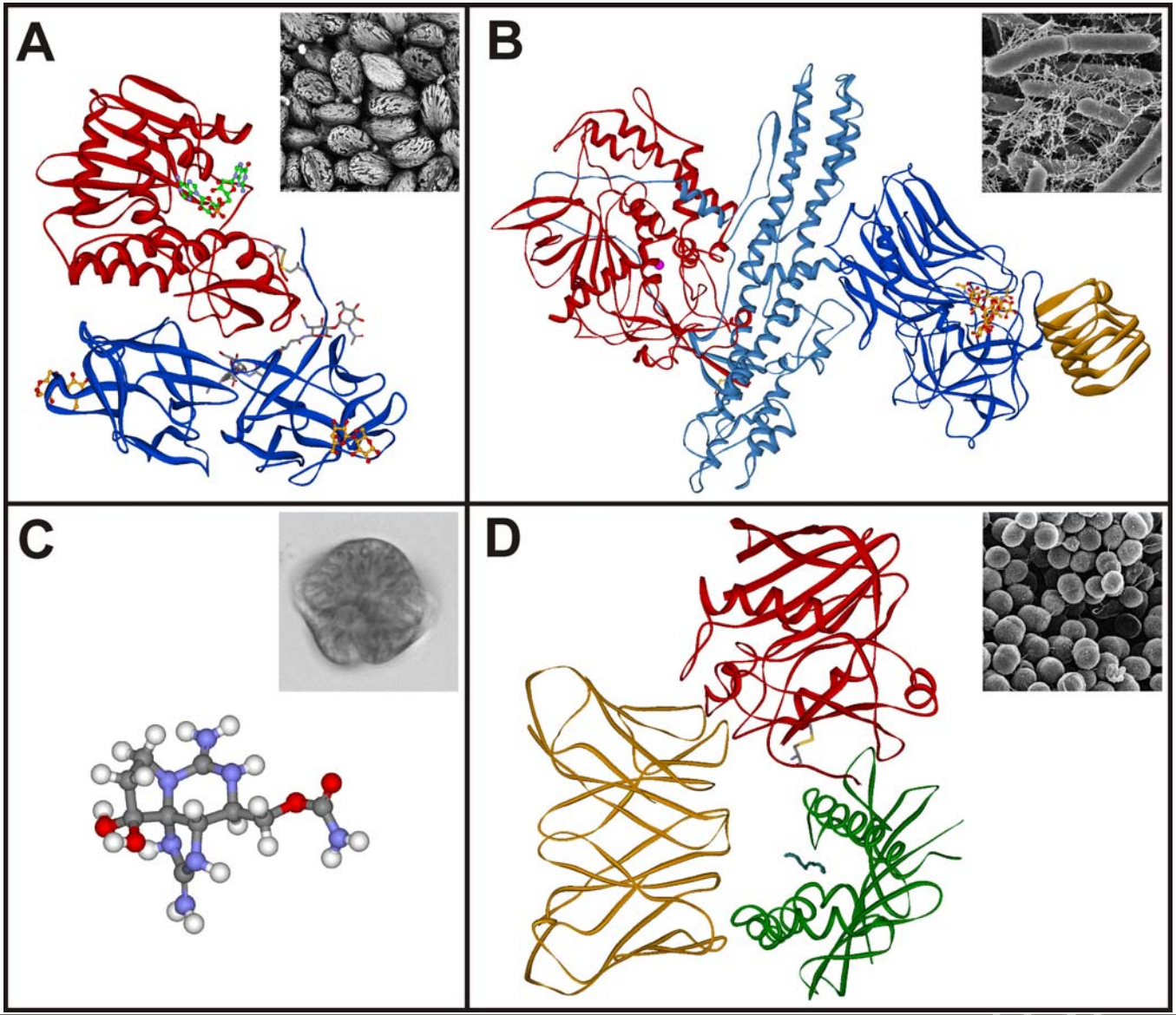


chemical tolerance

Biological Toxins

Ricin

BoNT



Saxitoxin

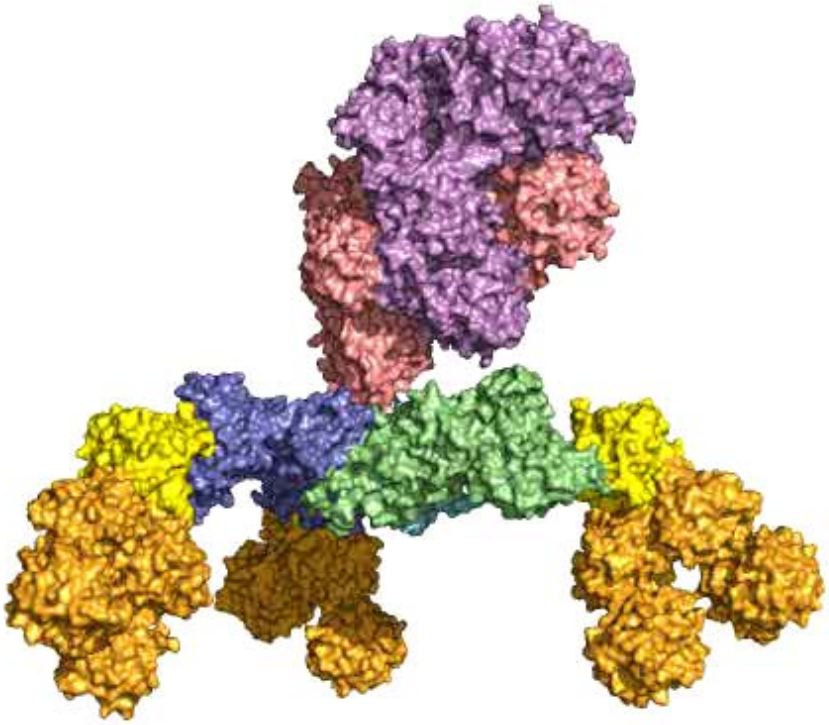
SEB

Biological Toxins

	Ricin	BoNT	SEB	STX
Producing organism	Plant *Ricinus communis*	Gram positive bacteria *Clostridium botulinum* group I-IV, *C. baratii*, *C. butyricum*	Gram positive bacteria *Staphylococcus aureus*	Dinoflagellates Cyanobacteria
Molecular weight	~63 kDa (576 AA)	~150 kDa (1251-1296 AAs)	28.4 kDa (SEB, 239 AAs)	299.29 g/mol
Toxin type & mechanism of action	A-B type: RNA *N*-glycosidase: depurination of adenine 4324 within sarcin-ricin loop of 28S-rRNA → halt of protein biosynthesis inducing cell death[1]	A-B type: Targets exclusively neurons; hydrolyses specifically members of SNARE protein family → blocking neurotransmitter release leading to flaccid paralysis[2]	Cross-links MHC II molecules on antigen presenting cells with T-cell antigen receptor → inducing massive release of chemokines and proinflammatory cytokines → lethal shock syndrome[3]	Neurotoxin voltage-gated sodium channel (Na v) blocker
Molecular variants/ closely related molecules	Ricin D and ricin E RCA120: highly related lectin co-expressed in the plant[4]; Diff. glycoforms due to potential N-glycan sites in A-chain (N10, N236) and B-chain (N95, N135)	Seven serologically different types BoNT/A-G; Mosaic toxins of different serotypes; Subtypes: variants of BoNT/A, B, E & F with up to 36% difference in AA sequence → >40 different BoNT molecules identified [2]	>23 SE: SEA-SEE, SEG-SET, SE/U, SE/U2, SE/V, TSS toxin-1 [5]	57 PSP analogues e.g. NeoSTX GTX1-6 C2, C4 [6]

[1]Spooner, R. A. & Lord, J. Ricin Trafficking in Cells. *Toxins* 7, 49-65, doi:10.3390/toxins7010049 (**2015**).
[2]Rummel, A. The long journey of botulinum neurotoxins into the synapse. *Toxicon* 107, Part A, 9-24 (**2015**).
[3]Marrack, P. & Kappler, J. The staphylococcal enterotoxins and their relatives. *Science* 248, 705-711 (**1990**).
[4]Chan et al. Draft genome sequence of the oilseed species Ricinus communis. Nat. Biotechnol. 28, 951-956 (**2010**).
[5]Hennekinne et al., S. aureus and its food poisoning toxins: characterization and outbreak investigation. FEMS Microbiol. Rev. 36, 815-836 (**2012**).
[6]Wiese et al. Neurotoxic alkaloids: saxitoxin and its analogs. Mar. Drugs 8, 2185-2211 (**2010**).

EM/X-ray structure of 14-mer 760 kDa L-PTC/A



Lee et al., PLOS Path. 2013

Table 1. Physical Inactivation of Selected Toxins

Toxin	Steam Autoclave	Dry Heat (10 min)	Freeze-thaw	Gamma Irradiation
Botulinum neurotoxin	Yes [a]	> 100° C [b]	No [c]	Incomplete [d]
Staphylococcal Enterotoxin	Yes [e]	> 100° C; refolds [f]	No [g]	Incomplete [h]
Ricin	Yes [i]	> 100° C [i]	No [j]	Incomplete [k]
Microcystin	No [l]	> 260° C [m]	No [n]	ND
Saxitoxin	No [l]	> 260° C [m]	No [n]	ND
Palytoxin	No [l]	> 260° C [m]	No [n]	ND
Tetrodotoxin	No [l]	> 260° C [m]	No [n]	ND
T-2 mycotoxin	No [l]	> 815° C [m]	No [n]	ND
Brevetoxin (PbTx-2)	No [l]	> 815° C [m]	No [n]	ND

U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention & National Institutes of Health. Biosafety in Microbiological and Biomedical Laboratories. 5th edn. (2009).

Thermal Inactivation of Toxins[1]

Ricin	BoNT	SEB	STX
60 min dry heat of >100ºC 1 h steam at >121ºC Heat-denatured ricin can undergo limited refolding (<1%) to yield active toxin.	10 min dry heat >100°C 1 h steam at >121ºC	10 min dry heat >100°C 2 h steam at >121ºC partial refolding occurs	10 min dry heat of >260°C

[1]U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention & National Institutes of Health. Biosafety in Microbiological and Biomedical Laboratories. 5th edn. (2009).

Problems:

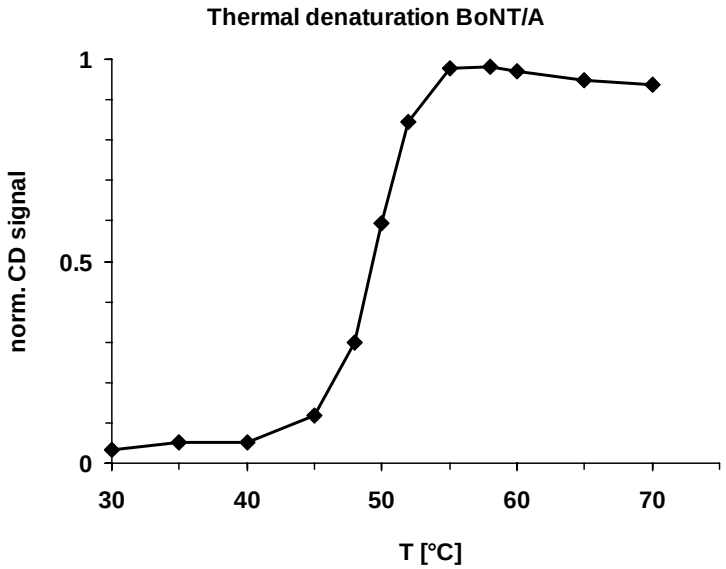
- LMW toxins >>> stable than HMW toxins

- HMW/protein toxins display different stability

- refolding of protein toxins

→ functional methods for detection

→ CD spectroscopy



Thermal Inactivation of BoNT

Analyzing a bioterror attack on the food supply: The case of botulinum toxin in milk

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assumption: 77°C for 15 min
→ 68.4% inactivation

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2010, p. 3293–3300 Vol. 76, No. 10
0099-2240/10/$12.00 doi:10.1128/AEM.02937-09

The Case of Botulinum Toxin in Milk: Experimental Data▽†

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evidence: 72°C for **15 sec**
→ 99.99% inactivation of BoNT
→ 99.5% inactivation of BoNT complex

Thermal Inactivation of BoNT

Toxin	Time sample held at 72°C (s)	Dilution of BoNT-spiked milk	No. of mice surviving/ no. of mice tested	MLD before heating/ MLD after heating	Reduction in toxic activity (%)
BoNT/A					
	Not heated	—[a]	0/2	10,000/10,000	0
	1	—	2/2	10,000/<1	>99.99
	5	—	2/2	10,000/<1	>99.99
	15	—	2/2	10,000/<1	>99.99
	30	—	2/2	10,000/<1	>99.99
BoNT/A complex					
	Not heated	—	0/2	10,000/10,000	0
	1	—	0/2		
		1:10	0/5		
		1:50	5/5	200/<1	>99.5
		1:100	2/2		
	15	—	0/2		
		1:10	0/5		
		1:50	5/5	200/<1	>99.5
	180	—	0/5		
		1:10	5/5	1,000/<1	>99.9
BoNT/B					
	Not heated	—	0/2	10,000/10,000	0
	1	—	2/2	10,000/<1	>99.99
	5	—	2/2	10,000/<1	>99.99
	15	—	2/2	10,000/<1	>99.99
	30	—	2/2	10,000/<1	>99.99
BoNT/B complex					
	Not heated	—	0/2	10,000/10,000	0
	1	—	0/2		
		1:10	0/2		
		1:50	0/5		
		1:100	5/5	100/<1	>99.0
	15	—	0/2		
		1:10	0/5		
		1:50	5/5	200/<1	>99.5
	180	—	0/5		
		1:10	5/5	1,000/<1	>99.9

[a] —, no dilution.

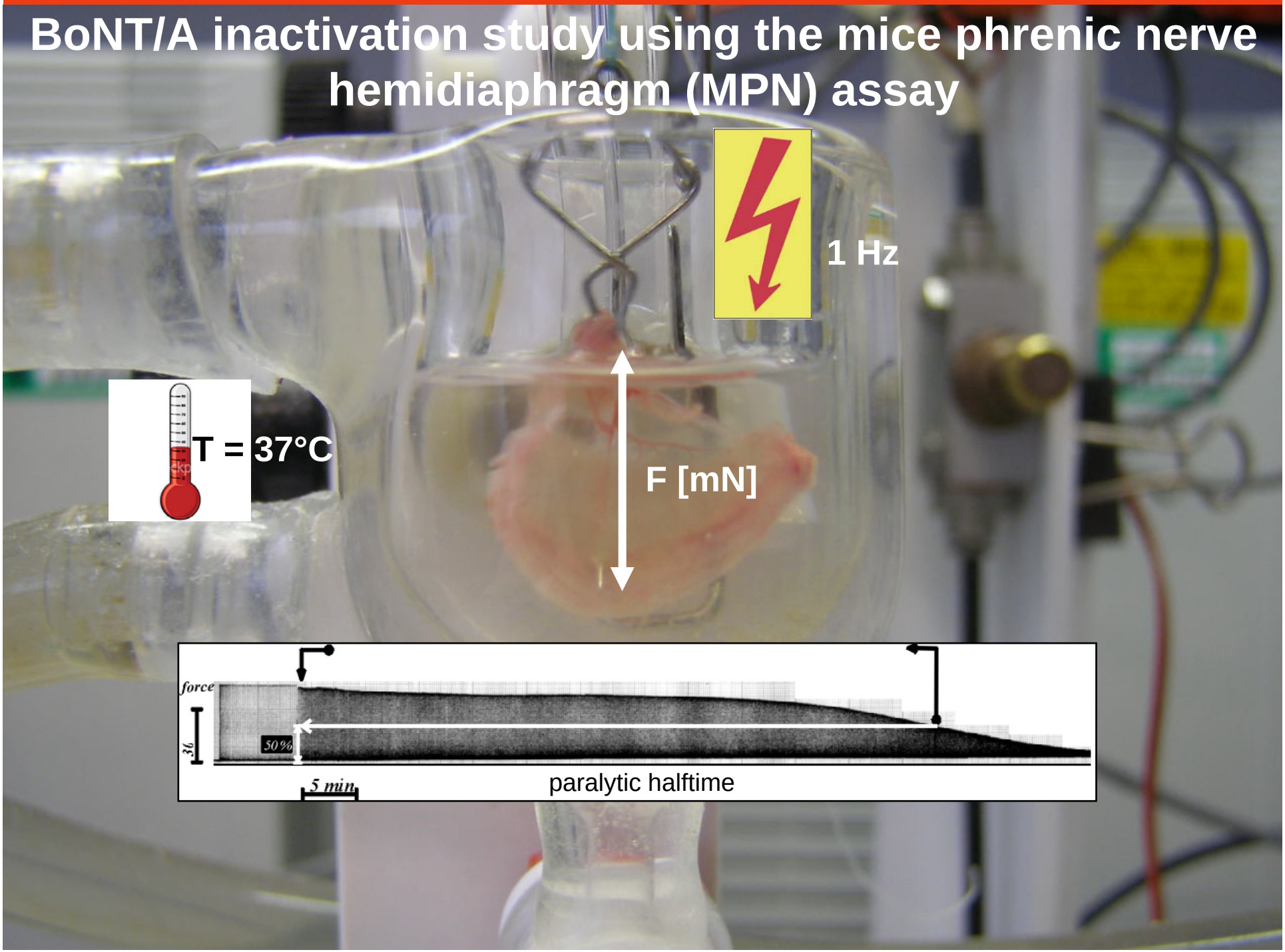
Weingart et al. 2010

Table 2. Chemical Inactivation of Selected Toxins

Toxin	NaOCl (30 min)	NaOH (30 min)	NaCOl + NaOH (30 min)	Ozone Treatment
Botulinum neurotoxin	> 0.1% [a]	> 0/25 N	ND	Yes [b]
Staphylococcal Enterotoxin	> 0.5% [c]	> 0.25 N	ND	ND
Ricin	> 1.0% [d]	ND	> 0.1% + 0.25N [e]	ND
Saxitoxin	≥ 0.1% [e]	ND	0.25% + 0.25N [e]	ND
Palytoxin	≥ 0.1% [e]	ND	0.25% + 0.25N [e]	ND
Microcystin	≥ 0.5% [e]	ND	0.25% + 0.25N [e]	ND
Tetrodotoxin	≥ 0.5% [e]	ND	0.25% + 0.25N [e]	ND
T-2 mycotoxin	≥ 2.5% [e, f]	ND	0.25% + 0.25N [e]	ND
Brevetoxin (PbTx-2)	≥ 2.5% [e, f]	ND	0.25% + 0.25N [e]	ND

U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention & National Institutes of Health. Biosafety in Microbiological and Biomedical Laboratories. 5th edn. (2009).

BoNT/A inactivation study using the mice phrenic nerve hemidiaphragm (MPN) assay



In-house BoNT/A inactivation study

Method:

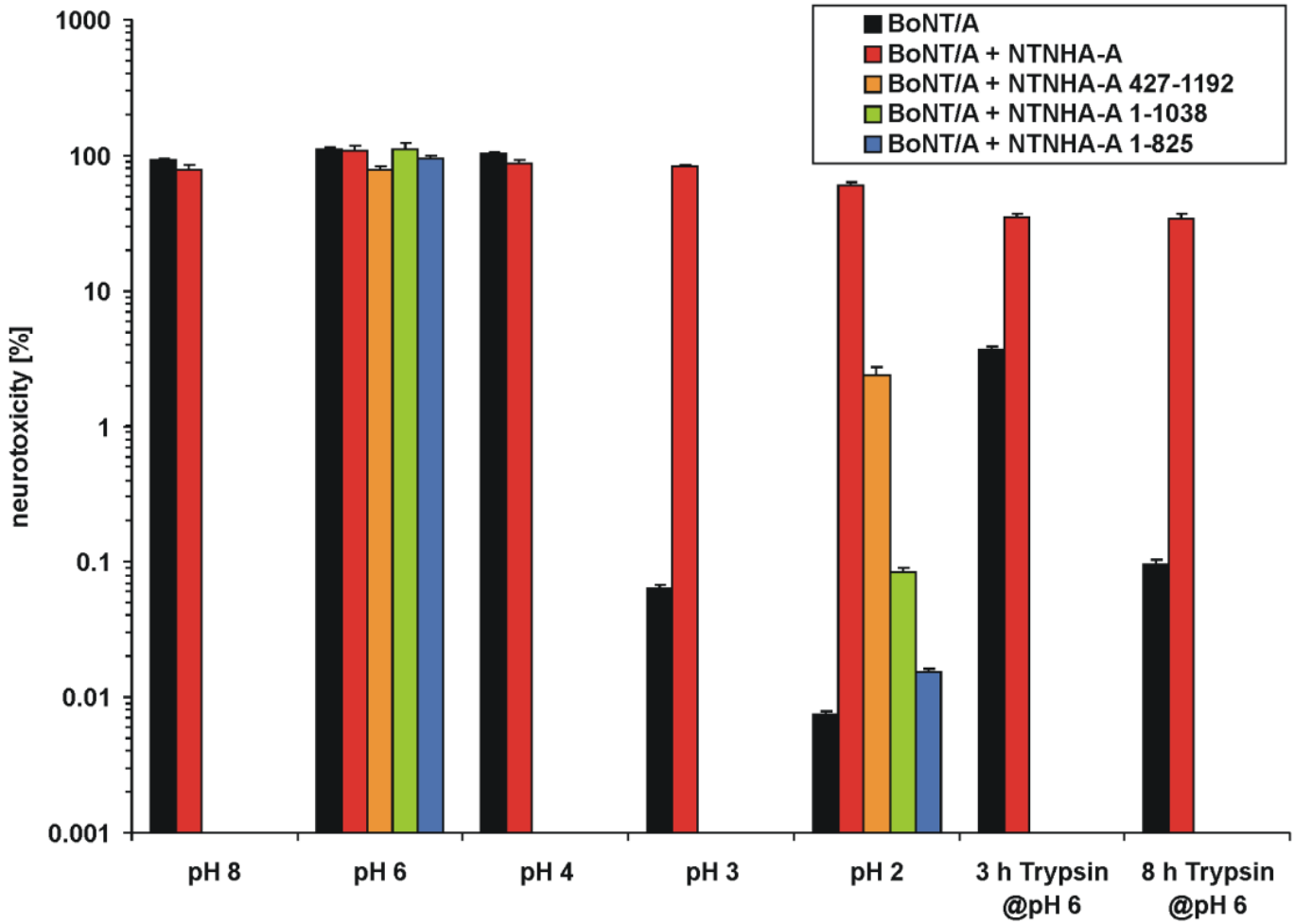
- Incubation of 10 µg BoNT/A for 5 min with 1% Dismozon, 70% Ethanol, 50% Isopropanol, 0.1% SDS or 0.1 M NaOH or water as control
- Dilution by 5.000 to 100.000-fold → MPN assay
- Dialysis 24-48 h → dilution by 5.000 to 100.000-fold → MPN assay

Results:

- BoNT/ A inactivated (residual activity <0.001%) within 5 min by
  - Dismozon ad 1%
  - Ethanol ad 70%
  - Isopropanol ad 50%
  - SDS ad 0.1%
  - Natronlauge ad 0.1 M
- Inactivation is irreversible

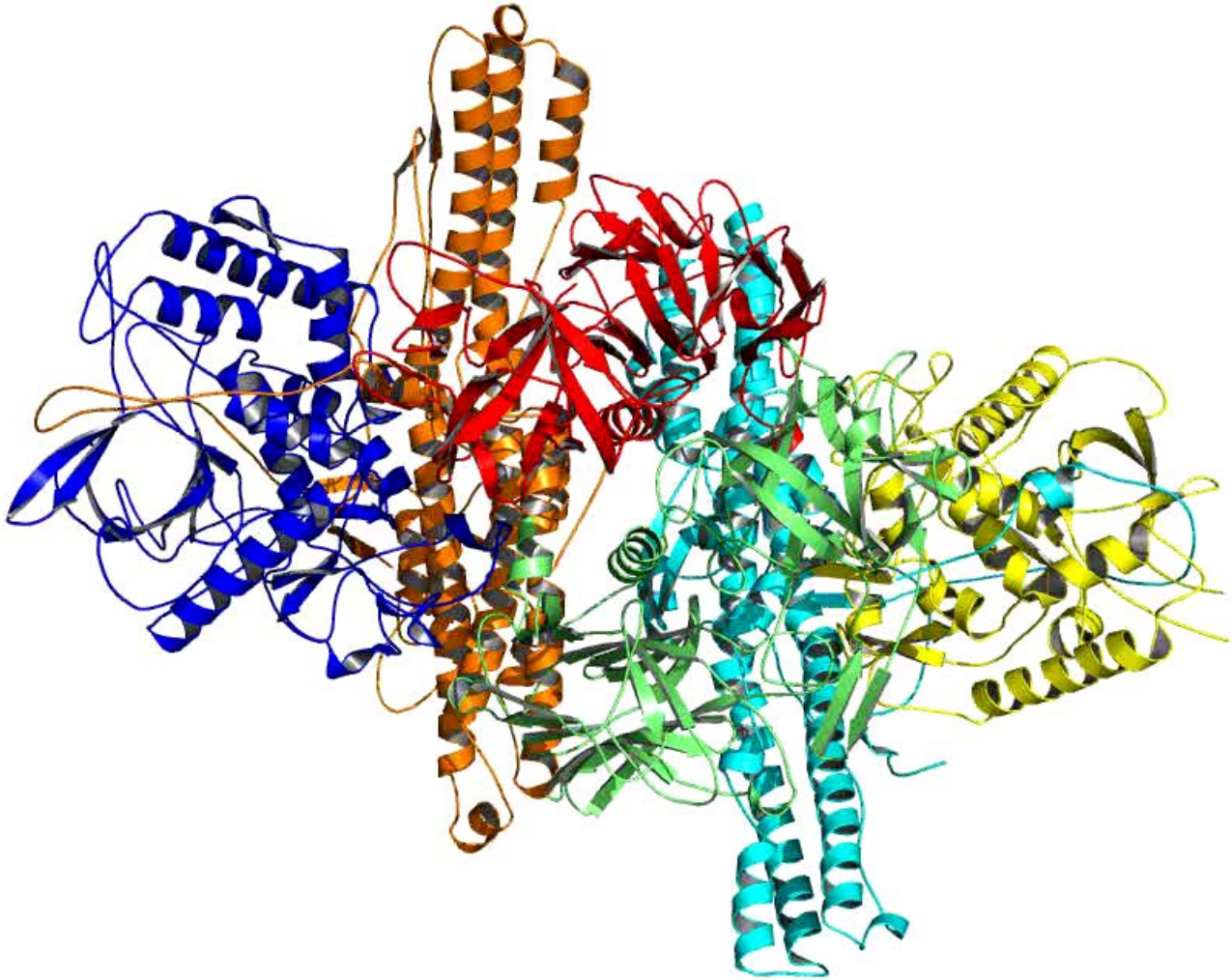
NTNHA-A protects BoNT/A at low pH

biological activity at MPN assay



Gu et al., Science, 2012

Crystal structure M-PTC, 3.9 Å; +VHH: 2.7 Å



Gu et al., Science, 2012

Conclusions

- **Biologicals toxins are chemicals from biological sources with catalytic properties amplifying their potency**
- **No ‘one size fits all’ inactivation**
  → consider toxin type (stability, refolding, pH sensitivity…)
  → matrix/surface
- **Validate your method using toxin specific bioassays**

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