

Review of recently reported Ricin detection techniques focusing on combined immunoassay detection with abrin and saxitoxin in human plasma

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ABSTRACT

Increasing non-traditional threats from biological or chemical weapons, the Organisation for the Prohibition of Chemical Weapons (OPCW) have tried to perform the preliminary analysis of biotoxin sample to standardize analysis methods and strengthen analytical capabilities among OPCW member countries. With the changes of new analysis, ROK CBRN Defense Research Institute (CDRI) established enzyme-linked immunosorbent assay (ELISA) and cytotoxicity analysis methods for ricin, abrin, and saxitoxin through the OPCW exercise on Biotoxin sample analysis. Thus, this study aims to established analytical methods (ELISA and cytotoxicity analysis) for the biological toxins called ricin, abrin and saxitoxin according to recent OPCW Biotoxin detection exercise. In particular, to refine practical and effective methods of biological analysis, we reviewed recent research on scientific analysis of ricin as a potential bioterror weapon, letter with ricin sent in White House, and suggested future agendas for preparedness testing.

Keywords : biotoxin, ricin, abrin, prohibition of chemical weapons, immunoassay detection platforms

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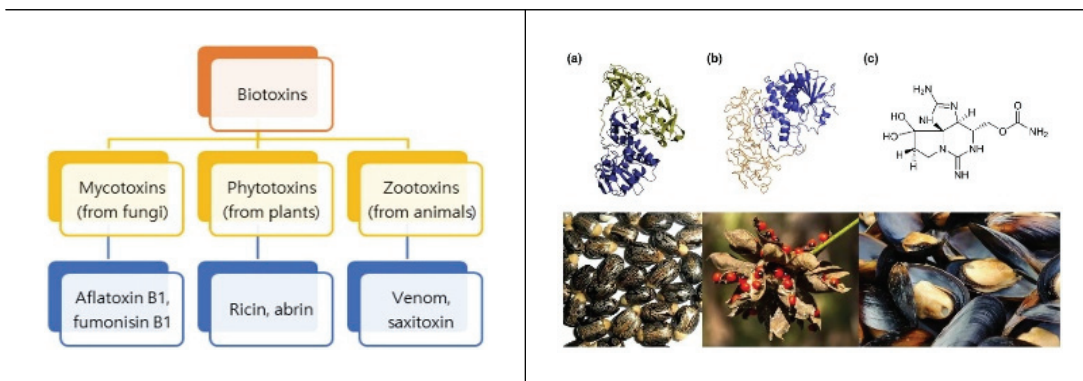
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I. INTRODUCTION

Toxins are naturally present in minor amounts in a wide variety of organisms worldwide. These chemicals provide an important security line for the natural defense of organisms in their native environment. To survive in nature, nearly every living organism produces some form of bio-toxin to protect itself. As far as humans are concerned, certain bio - toxins are very harmful, and they have been used to incapacitate or kill other living organisms or other human beings. Among such bio - toxins are substances that are readily highly toxic. Therefore, there is a natural concern regarding substances that are accessible, and thus easily obtained and exploited by humans for terrorism intent. As a result, such substances are listed as significantly more threatening and require state-of-the-art detection capabilities (Pancrazio, 2007).

The biotoxins discussed in this article are part of a standardized testing protocol, and the molecules that possess toxicity and accessibility selected for this study were ricin (castor), abrin (rosary pea), and saxitoxin. Ricin and abrin are prepared from seeds of castor oil plants, are highly toxic, and can be found worldwide. In Figure 1, Ricin is isolated from castor seeds *Ricinus communis* and consists of two polypeptide chains (A and B - chain subunits) linked with disulfide bonds (Audi et al., 2005; Franz & Jaax, 1997; Lord et al., 2003).



<Figure 1> Overview of Biotoxin

Note. (Left) Biotoxin classification by living organism type. (Right) Chemical structures of bio - toxins and materials: (a) ricin and castor beans (b) abrin and jequirity seeds and (c) saxitoxin and mussels. (protein structure graphics obtained from Wikipedia).

Although complex, the molecular structures of these toxins allow for a well-understood mechanism of action. The B-chain of the ricin protein can attach to the end of the galactose or mannose residues present in the glycoprotein of the cell membrane, and this conjugate enters

through endocytosis. Most ricin proteins that enter living cells are thought to either return to the cell surface or be degraded by lysosomes. Significantly small amounts of ricin reach the endoplasmic reticulum (ER) via the trans - Golgi network. After reaching the ER, ricin is separated into its A and B - chains, which requires the scission of the disulfide bond, and the separated toxin is then inserted into the ER membrane and is no longer degraded by proteolytic enzymes. The A - chain binds to the ribosomal rRNA and interferes with protein elongation. One molecule of the A - chain inactivates 1500 - 2000 ribosomes per minute and causes cytotoxicity through this biological mechanism (Doan, 2004; Endo & Tsurugi, 1987; Olsnes, Refsnes, & Pihl, 1974).

Abrin is a natural protein toxin isolated from rosary pea seeds of the plant *Abrus precatorius*. The structure and toxicity mechanisms of abrin are similar to those of ricin. For this reason, abrin and ricin is historically been among the most frequently used toxic substances, and continue to be used in terrorism (Melati et al., 2022). In 2020, ricin was sent to the White House to threaten the political executive leadership of the United States though land mail (Bradberry, 2016; Olsnes et al., 1975; Olsnes, 2004). Because these cases occur occasionally, it is important for technology and science to focus on the detection of ricin and related molecules to produce improved sensing.

Saxitoxin is also known as the paralytic shellfish toxin (PST), and is a neurotoxin produced in shellfish such as mussels and oysters as well as by dinoflagellates (Schantz et al., 1975). Because saxitoxin chemically consists of carbamate groups, it is also referred to as “carbamate toxin.” The mechanism of toxicity involves saxitoxin bindings to voltage - gated sodium channels in the synapse clefts. It blocks sodium ion entry into nerves and muscles by occluding voltage - gated sodium channels, which causes muscle paralysis (Andrinolo, Michea, & Lagos, 1999). Most cases of human saxitoxin contamination are caused by the ingestion of naturally tainted marine shellfish such as mussels, oysters, scallops, and geoducks, or the inhalation of vapors that contain saxitoxin (Johnson et al., 2009). Unlike ricin and abrin, saxitoxin does not appear to be frequently used in terrorism. Saxitoxin is generated from spoiled mussels or oysters in foods, and is claimed to be responsible for accidental poisoning of numerous individuals annually (Silva et al., 2010). In Table 1, toxicities of biotoxins were compared with chemical warfare agents.

<Table 1> Comparative toxicities of biotoxins and related molecules

Toxic material	Toxicity (i.p. ^[a] LD50, $\mu\text{g/kg}$)	Origin	Chemical structure
Abrin	0.7	Plant	Proteotoxin
Ricin	3.0	Plant	Proteotoxin
Saxitoxin	10.0	Marine dinoflagellate	LMW ^[b]
Tetrodotoxin	8.0	Pufferfish	LMW
VX nerve agent	15.0	Synthetic small molecule	LMW
Sarin nerve agent	100.0	Synthetic small molecule	LMW
Aflatoxin B1	500.0	Aspergillus flavus	LMW

Note. It recited from Pöhlmann & Elßner(2020). [a] indicates intraperitoneal injection and [b] states low molecular weight compounds.

Scientists and bureaucracies have attempted to (i) discover new science, and (ii) implement proper, efficient, and workable protocols against bio-toxins. There should be synergy between policy and science. Once the detection limits are established scientists can develop more sensitive devices. Largely, these efforts are led by the analytical chemistry community, whose work merges with the biochemistry and synthetic organic and instrumental/physical chemistry communities. Several analytical methods such as Enzyme-linked immunosorbent assays (ELISA), liquid chromatography (LC), LC-mass spectrometry (MS) have been studied to effectively analyze bio-toxins for decades (Medlin, 2013). Among these methods, ELISA, which uses antigens or antibodies, has the advantages of selectively and sensitively detecting bio-toxins (Dubois et al., 2010). ELISA is a qualitative and quantitative immunological analysis method that uses antibody-antigen determined using enzyme measurements, instrumental methods, and the human eye. ELISA has the following advantages: (i) rapid analysis time, (ii) long shelf life, (iii) high selectivity, (iv) sensitivity, (v) less radiation exposure, (vi) rapid performance, and (vii) versatile analysis of infectious agents. For these reasons, ELISA is used in various fields such as infectious agent detection, drug and tumor marking, clinical research, hormone quantitative checking, and vaccine quality controlling (Syaifudin, Jayasundera, & Mukhopadhyay, 2009). ELISA are divided into two types: competitive and non-competitive methods. The non-competitive method version includes, direct, indirect, and sandwich ELISA modes depending on the intended antigen under detection. Cytotoxicity assays indirectly measure the loss of cellular or intercellular structure and function, including lethal cytotoxicity, by counting healthy cell populations (Gatto-Menking et al., 1995).

The on-site selective detection of analytes and protocols is an important issue. Regarding

toxicity and easy accessibility, bio - toxins such as ricin, abrin, and saxitoxin are significantly dangerous to humans. For this, the Organization for the Prohibition of Chemical Weapons (OPCW) considers them potential biological and chemical warfare agents. Thus, OPCW has been regularly conducting bio-toxin exercises to help evaluate the analytical capabilities for the detection of ricin, abrin, and saxitoxin at different institutes worldwide since 2016 (Long-Hui et al., 2021). The OPCW recommends that participants in bio - toxin exercises analyze bio - toxins using at least two different methods: ELISA and other methods. Therefore, the focus of our study will be on the detection and remediation of these analytes as unwanted toxins. In this study, analysis of ricin, abrin, and saxitoxin in human serum using ELISA and cell cytotoxicity tests was performed and recent analyte research is reviewed (*vide infra*).

II. EXPERIMENTS SECTION

2.1 Sample preparation: ELISA testing procedures of ricin and abrin

A capture antibody sample (55 mL) was added to 11 mL of phosphate-buffered saline (PBS) and then added to 100 mL of the capture antibody solution to all wells. The wells were then covered and incubated overnight at 4 °C. Next, the contents of each well were aspirated, and the wells were washed using 100 mL of PBST (phosphate-buffered saline with Tween™) in each well (more than four times). This washing procedure bound the targeted protein and antibody by weakening the binding force of the impurities and the capture antibody (weakening the binding forces between unnecessary proteins and the capture antibody and washing away extra proteins for binding the capture antibody). Then, a sample of 150 mL blocking solution was added to all wells and then incubated at 37 °C for 1 h. A blocking solution was then used to block non - specific binding of the antibody. The solutions in each well on the plate were then aspirated, before washing with 100 mL PBST (more than four times). Standard sample (100 mL) was added to the wells and the plate was covered and incubated at 37 °C for 1 h. The solution was aspirated into each well and washed with 100 mL of PBST in each well (more than four times). The detection antibody (55 mL) was diluted to 11 mL, the blocking buffer was added, and 100 mL of the diluted detection antibody was added to each well. The plate was covered and incubated at 37 °C, before being washed with 100 mL PBST (more than four times).

A detection antibody was used to increase the sensitivity of ELISA. The conjugated antibody

(4.4 mL) was added to 11 mL of dilution/blocking buffer, 100 mL of diluted conjugated antibody was added to each well, and the plate was covered and incubated at 37°C with the plate covered. Then, the plate was washed using 100 mL PBST (at least four times). Next, 100 mL of the ABTS peroxidase substrate was added to each well and incubated at 37 °C for 0.5 h. Absorbance was read at 405~410 nm.

2.2 ELISA of saxitoxin

Fifty microliters of each saxitoxin standard sample were added in duplicate in the wells. Fifty microliters (50 μ L) of each sample were added in duplicate into different sample wells. 50 μ L of saxitoxin-horseadish peroxidase (HRP) conjugate were added to each well. 50 μ L of anti - saxitoxin antibody was then added to each well immediately following by pipetting up and down once. The plate was then incubated for 30 min at room temperature (20 - 25 °C) in the dark. The solution from each wells was thoroughly decanted and aspirated, and the liquid was discarded. The plate was washed thrice with 250 μ L of a 1 x wash solution. Thereafter, the plate was inverted and gently tapped to allow drying, followed by blotting on paper towels. The TMB substrate (100 μ L) was then added. The solution was mixed by gentle manual rocking of the plate for 1 min during incubation. Incubation was maintained for 15 min at room temperature (20 - 25 °C) in the dark. After incubation, 100 μ L of stop buffer was added to terminate the reaction. The absorbance at 460 nm was measured directly following the addition of a stop buffer.

2.3 Cytotoxicity sample preparation

The entire procedure for determining the cytotoxicity of ricin, abrin, and saxitoxin was conducted at Biosafety Level 3 to prevent external contamination. The frozen Vero cells (1 mL) were thawed in a static bath for approximately 1 min. The cells were transferred from cryogenic storage to Falcon tubes and 1.0 mL of cell culture media was then added. The mixture was then centrifuged again. After centrifugation, the supernatants were discarded. One milliliter of the cell culture was added to a Falcon tube, and the cells that were attached to the tube wall were detached. Then, 1 mL of Vero cell and 4.0 mL of cell culture media were transferred in 5.0 mL cell culture flasks and then incubated under 37 °C and 5% CO₂ for 24 h. PBS (1.0 mL) was added to wash the incubated solution, then the PBS was removed.

Next, 1 mL of trypsin-ethylenediaminetetraacetic acid (EDTA) was added and the cell culture media was treated carefully to detach the cells from the flask walls. Centrifugation was repeated to remove the cell culture medium. Cells (1 mL) and 14 mL of cell culture were transferred to a 25 mL sample of cell culture flask and then incubated under 37 °C and 5% CO₂ for 24 h. Then, the cells were sub - cultured to three cell culture flasks. For cell counting, 2 mL of cells and 18 mL of trypan blue were added to a new 1.5 mL tube. Cells and trypan blue solution were placed in hemocytometers, and the cells were counted (quantified) using a scientific microscope. An estimated 10,000 cells were transferred to each of the 96 wells, and 10 µL of the OPCW samples were transferred to 96 well plate and incubated in the dark (37 °C and 5% CO₂) for 24 h. Deionized water was then added to the blank wells. WST-8 test compound solution (10 µL) was added to each well and incubated under 37°C for 4 h. After incubation, absorbance was recorded at 460 nm.

III. RESULTS AND DISCUSSION

3.1 OPCW unknown sample testing

Two different ELISA principles were used in our study to identify bio-toxins in the OPCW samples. One method was the Sandwich method, used for ricin and abrin, and the other was a competitive method to make determinations for saxitoxin. Three types of ELISA kits were used to identify the exact bio - toxins in the unknown samples. Human plasma samples contain numerous proteins, nutrients, and waste molecules. Parts of proteins and others in the plasma are likely to strongly interfere with the ELISA results in some cases.

The OPCW did not provide information on the concentrations of bio - toxins for the unknown samples in any form; therefore, selecting the optimal dilution ratio for the ELISA trials was the main focus of the research team. The samples were diluted 1, 20, 200, and 2,000 times with ricin and 1, 10, and 20 times with abrin, and the absorbance within the calibration curve was determined. The standard solution was diluted to eight different concentrations to determined the concentration at which the antigen did not specifically bind to the antibody. No bio-toxins were detected in the **BT20/PL03/27** and **BT20/PL07/27** samples. Ricin was detected in the **BT20/PL01/27** and **BT20/PL04/27**, whereas, abrin was detected in the **BT20/PL04/27** and **BT20/PL08/27** samples. Saxitoxin was detected in three samples, **BT20/PL05/27**, **BT20/PL06/27**,

and **BT20/PL08/27**. This study elucidated that the concentrations of three different biotoxins could be analyzed using ELISA. The concentration of bio-toxin in OPCW samples at the conclusion of our investigation were determined to be 15.1 mg mL⁻¹ ricin in **sample 1**, 0.65 mg mL⁻¹ ricin in **sample 2**, 1.85 mg mL⁻¹ ricin and 59.8 mg mL⁻¹ abrin in **sample 4**, 1.78 ng mL⁻¹ saxitoxin in **sample 5**, 0.9 ng mL⁻¹ saxitoxin in **sample 6**, 28 ng mL⁻¹ abrin and 2.4 ng mL⁻¹ saxitoxin in sample 8.

The cytotoxicity of bio - toxins in the OPCW samples to Vero cells was then studied. Lactate dehydrogenase (LDH) is produced when Vero cells die or are damaged. the tetrazolium salts in the cell counting kit were reduced to formazan using LDH. The number of apoptotic and necrotic cells and, formazan production increased, resulting in ultraviolet-visible (UV-vis) optical absorption at 460 nm. The absorption values at 460 nm for Vero cells and bio - toxins using WST - 8 are provided in Table 2.

<Table 2> ELISA and cytotoxicity assay results for bio - toxin detection from samples provided by OPCW for blind testing

Sample number ^[a]	Types of bio - toxins	Absorbance wavelength (nm) used in determinations	Quantitative analysis (conc. of bio - toxins)	ELISA types	Cytotoxicity (Vero cell) ^[b]
BT20/PL01/27	Ricin	405-410	15.13 mg/mL	Sandwich	100/38
BT20/PL02/27	Ricin	405-410	0.65 mg /mL	Sandwich	100/45
BT20/PL03/27	None	-	-	-	-
BT20/PL04/27	Ricin Abrin	405-410 405-410	1.85 mg/mL 59.8 mg/mL	Sandwich Sandwich	100/63
BT20/PL05/27	Saxitoxin	450	1.78 ng/mL	Competitive	100/46
BT20/PL06/27	Saxitoxin	450	0.9 ng/mL	Competitive	100/50
BT20/PL07/27	None	-	-	-	-
BT20/PL08/27	Abrin Saxitoxin	405-410 450	28 ng/mL 2.4 ng/mL	Sandwich Competitive	100/50 100/55

Note. Measurements were made in human blood plasma matrix. [a] from OP and, [b] Relative OD (%), 460 nm dying cells/living cells.

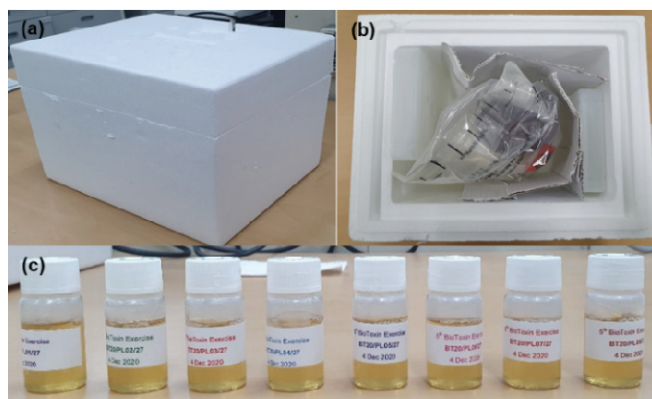
The relative optical density (OD) is the absorbance density of apoptosis, necrosis results from the presence of biotoxins, and OP is the absorbance density of the remaining living Vero cells.

This study indicated immunoassay detection using ELISA is a good analytical tool for the detection of ricin, abrin, and saxitoxin in human plasma. Unknown samples provided by the OPCW using ELISA to determine the types and amounts of bio - toxins. Because there are

numerous additive proteins and chemicals that can be detected through ELISA in human plasma, finding the proper concentration for non - specific binding to antibodies is very important. ELISA exhibited good sensitivity and selectivity for the identification of unknown bio - toxin samples. The cytotoxicity of Vero cells and bio - toxins indicated that ricin, abrin, and saxitoxin caused apoptosis and necrosis through direct contact with cells. This result indicated that the vitro toxicity of the actual cells and bio - toxins supported the ELISA results.

3.2 Materials and methods

Bio - toxin samples of ricin, abrin, and saxitoxin in human plasma were obtained from the OPCW through the Chemical Defense Research Institute (Seoul, Republic of Korea) and were subjected to the 5th bio - toxin sample analysis exercise. The OPCW provided eight vials that may have contained bio - toxins of unknown concentrations and identities. Therefore, the OPCW did not provide information on which samples contained the types of bio - toxins (Figure 2).



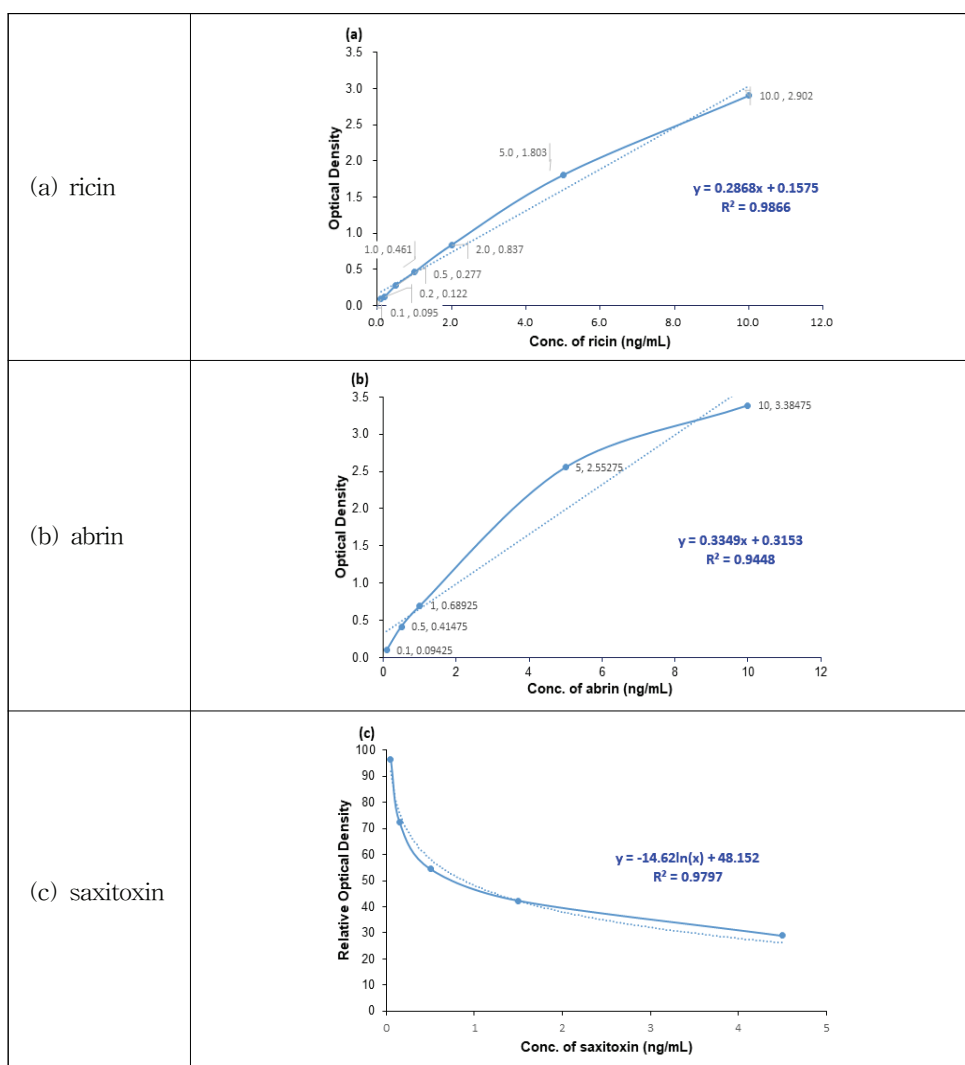
<Figure 2> The bio - toxin samples in their containers as provided/shipped by OPCW

Note. (a) triple package box; (b) interior of packed box; (c) eight samples in vials provided for testing

The bio - toxin samples were wrapped in aluminum foil to guard against exposure to light (ambient light and sunlight) and were stored at 4 °C to avoid thermal degradation of the chemical samples. High-performance liquid chromatography (HPLC) grade acetonitrile, methanol, formic acid and water were purchased from Sigma - Aldrich and used without further purification. ELISA kits for ricin and abrin were purchased from Tetracore Inc. (Rockville, MD 20850). The Saxitoxin ELISA kit was purchased from BIOO Laboratories (Hercules, CA, USA). The PBST (with TweenTM) was purchased from Sigma - Aldrich. The PBST was prepared and

used on the same day to minimize errors in the ELISA results. Dulbecco's modified Eagle's medium (DMEM), 1% fetal bovine serum (FBS), penicillin, and trypsin-EDTA was purchased from Sigma - Aldrich. Vero cells were purchased from the Korean Cell Line Bank. The cell counting kit (CCK)-8 (WST - 8, ab228554) was purchased from Abcam.

According to experiments with methods and kits above mentioned, standard ELISA curves could be present in Figure 3. With this standard curves, concentration of sample can be predicted based on measured optical density.



<Figure 3> Standard ELISA curves for (a) ricin, (b) abrin, and (c) saxitoxin in OPCW bio - toxin exercise samples

3.3 Contemporary analytical chemistry research on ricin detection

First reported in 1971, ELISA has become the mainstay for the determination of analytes of interest, in which the analyte is often a full-length globular protein. In these assays, the absorbance values can be plotted as a function of the protein concentration, in which the linearity of the curve is related to the effectiveness of the concentration (Pöhlmann & Elßner, 2020). The analytical chemistry of ricin has been the focus of researchers since at least 2010 (Baldoni et al., 2010; Sousa et al., 2017). In addition to studies using ELISA, other efforts have been made. Recently, optical probes for ricin have been developed and related studies is also actively conducted (Table 3).

<Table 3> Recent colorimetric and related studies on ricin

Mode and description	Limit of detection	Optical signal	Reference
Liquid crystal detection	10 mg/mL	-	Zhao et al. (2011)
Gold nanoparticles, surface functionalized, and lactosylated cysteine substrate.	13 ng/mL	Colorimetric; red → blue λ_{abs} , max = 519 → 670 nm	Kandasamy et al. (2019)
Digital holographic Microscopy	-	-	Makdasi et al. (2019)
Biolayer interferometry-based assay	10 pg/mL	-	Mechaly et al. (2016)
Surface plasmon resonance, cell surface oligosaccharides on glyco chips	30 ng/mL	Response signal for detection; λ_{abs} = 550 nm	Nagatsuka et al. (2013)
Galactose - terminated bead extraction coupled with spectrometric detection	8 ng/mL	-	Hoyt et al. (2021)
Depurination activity detection using RNA substrate	1 ng/mL	-	Liang et al. (2021)

IV. Conclusions

We have described the ELISA and discussed the recent literature on these interesting protein - based analytes. Blind testing assists with preparedness against bio-toxin poisoning and is a

realistic test, and ELISA has been shown to be efficient. With this background and preparedness testing, we continued the discussion to put these compounds, testing, preparedness, and rationales pertinent to the context through a brief mention of recent related reports and their limits of detection. The concentration of bio - toxin in OPCW samples were found to be 15.13 mg/mL ricin in sample 1, 0.65 mg/mL ricin in sample 2, 1.85 mg/mL ricin and 59.8 mg/mL abrin in sample 4, 1.78 ng/mL saxitoxin in sample 5, 0.9 ng/mL saxitoxin in sample 6, 28 ng/mL abrin and 2.4 ng/mL saxitoxin in sample 8.

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Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author contributions

Conceptualization: Kang, S., Kim, S., and Jang, Y.; Literature review: Kim, S., Churchill, D. G., Jang, Y.; Resources and Data curation: Kang, S., Kim, S., and Jang, Y.; Investigation and Methodology: Kang, S., Kim, S., Kang, K., and Jang, Y; Writing: Kang, S. and Jang, Y.; Project administration and Supervision: Kang, S. and Jang, Y.

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생물독소의 면역효소흡착법과 세포독성분석법 연구 리뷰

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국문초록

본 연구는 최근 비전통 위협이 증가함에 따라 OPCW에서 신설한 생물독소 분석 예비평가에 따른 리신, 아브린, 색시톡신이라는 생물독소 분석법(면역효소흡착법, 세포독성분석법)을 정립하고자 한다. 우리 주변에서 흔하게 얻을 수 있는 독성이 강한 생물독소(리신, 아브린, 색시톡신)에 의한 생물테러의 경우 빠른 시간 내에 정확히 분석하는 것이 매우 중요하다. 이런 생물독소의 분석법을 표준화하고 분석능력을 향상하기 위해서 화학무기금지기구(OPCW)는 가입국의 생물독소 정밀분석 예비평가를 신설하였다. 이에 따라 국군화생방방어연구소는 OPCW 생물독소 분석능력 예비평가에 참여하여 리신, 아브린, 색시톡신의 생물학적 분석방법인 효소결합면역흡착법(ELISA)과 세포독성분석법을 정립하였다. 본 연구는 최근 백악관에 편지 테러로 사용되고 있는 리신 분석법에 관한 최근 연구동향을 탐색을 통해 현실적이고 효과적인 생물학적 분석방법의 준비성 테스트 방안을 제시하고자 하였다.

주제어 : 생물독소, 리신, 아브린, 화학무기금지기구, 면역효소흡착법

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