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Detection of ricin and other ribosome-inactivating proteins by an immuno-polymerase chain reaction assay

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Abstract

Ribosome-inactivating proteins (RIPs) are plant proteins with enzymatic activity, classified as type 1 (single chain) or type 2 (two chains). They are identified as rRNA *N*-glycosidases (EC 3.2.2.22) and cause an irreversible inhibition of protein synthesis. Among type 2 RIPs, there are potent toxins (ricin is the best known) that are considered as potential biological weapons. The development of a fast and sensitive method for the detection of biological agents is an important tool to prevent or deal with the consequences of intoxication. In this article, we describe a very sensitive immuno-polymerase chain reaction (IPCR) assay for the detection of RIPs—a type 1 RIP (dianthin) and a type 2 RIP (ricin)—that combines the specificity of immunological analysis with the exponential amplification of PCR. The limit of detection (LOD) of the technique was compared with the LODs of the conventional immunological methods enzyme-linked immunosorbent assay (ELISA) and fluorescent immunosorbent assay (FIA). The LOD of IPCR was more than 1 million times lower than that of ELISA, allowing the detection of 10 fg/ml of dianthin and ricin. The possibility to detect ricin in human serum was also investigated, and a similar sensitivity was observed (10 fg/ml). IPCR appears to be the most sensitive method for the detection of ricin and other RIPs.

Keywords: Ribosome-inactivating proteins; Immunoassay; Detection; Quantification; Ricin; Dianthin

Ribosome-inactivating proteins (RIPs)[1] are a group of proteins widely distributed among higher plant species that damage ribosomes in an irreversible manner [1,2]. RIPs can be classified as type 1 or type 2. Type 1 RIPs are basic proteins of approximately 30 kDa consisting of a catalytic chain (A-chain). They are very potent inhibitors of protein synthesis in a cell-free system, but they are relatively nontoxic to intact cells, in which they enter with difficulty due to the lack of a carbohydrate-binding B-chain. Type 2 RIPs consist of a similar catalytically active A-chain linked to a lectinic B-chain by a disulfide linkage. Their toxicity is the result of the combined action of both chains [3]. The B-chain facilitates the transport of RIPs into cells by binding to specific sugar residues of glycoproteins or glycolipids on the cell membrane, and then internalization occurs by endocytosis. Then the A-chain exerts its RNA *N*-glycosidase activity, by which it brings about depurination of RNA, removing a single highly conserved adenine residue (A_{4324} in the rat liver 28S rRNA). This activity prevents the formation of a critical stem–loop configuration, to which

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[1] *Abbreviations used:* RIP, ribosome-inactivating protein; ELISA, enzyme-linked immunosorbent assay; FIA, fluorescent immunosorbent assay; IPCR, immuno-polymerase chain reaction; LOD, limit of detection; BoNT/A, *Clostridium botulinum* neurotoxin A; HIV, human immunodeficiency virus; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); FITC, fluorescein isothiocyanate; ANOVA, analysis of variance; ANCOVA, analysis of covariance.

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the elongation factor 2 is known to bind during the translocation step of translation. The end result is a complete inhibition of cellular translation with consequent cell death.

Subsequently, it was found that RIPs remove adenine residues from DNA and other polynucleotide substrates [4,5]; therefore, the denomination of adenine polynucleotide glycosylase was proposed for these proteins [6]. Some type 2 RIPs are potent toxins (ricin is the best known), whereas others have a structure and an enzymatic activity similar to toxins but have a much lower toxicity. This difference presumably is due, at least in part, to diversities in their intracellular localization and processing [7,8].

Because of the extreme potency of toxic type 2 RIPs, such as ricin, growing concerns have arisen about the possibility of abusing these agents as weapons for warfare. In the past, ricin has been used for suicidal and homicidal purposes. In 1979, Georgi Markov, a Bulgarian exiled in London, was killed by a small bullet, and the lesions found at postmortem were attributed to ricin poisoning [9,10]. More recently, in January 2003, suspected terrorists were arrested because they were producing ricin in a north London apartment. Never before has there been such a strong possibility that biological agents might be used indiscriminately on civilian populations.

This alarming situation prompted the search for, and the development of, methods for the detection and dosage of biological agents considered as possible weapons [11,12]. Ideally, these methods should be both fast and sensitive to allow a rapid detection of minute amounts of the suspected toxin and should represent an important tool to prevent, or deal with the consequences of, contamination and intoxication.

Currently, qualitative and quantitative detection of RIPs is carried out using traditional immunological methods such as Western blot analysis, enzyme-linked immunosorbent assay (ELISA), and fluorescent immunosorbent assay (FIA). During recent years, numerous techniques have been developed as valid alternatives to traditional immunological methods; for example, a colloidal gold-based assay allows the detection of ricin in less than 10 min [13], and hydrogel-based protein microchips [14] and array biosensors [15] provide the possibility of analyzing several toxins in the same sample. A complete list of detection methods used to reveal and detect ricin is reported in Table 1. The maximum sensitivity of conventional and newly developed methods, reported in the table, is in the order of magnitude of nanograms or picograms of toxin.

This article describes the use of the immuno-polymerase chain reaction (IPCR) for the detection of a type 1 RIP (dianthin) and of a type 2 RIP (ricin). IPCR is a very sensitive antigen detection method, first described in Ref. [33], that combines the specificity of immunological analysis with the exponential amplification of PCR. As a result, the limit of detection (LOD) of an ELISA is generally enhanced 100- to 10,000-fold by the use of PCR as a signal amplification system. Since its initial description, IPCR technology has evolved and today enables a broad range of applications, including the detection of toxins such as *Clostridium botulinum* neurotoxin A (BoNT/A) [34] and rViscumin [35], of allergens such as gliadin [36], and of pathogens such as hepatitis B virus [37,38] and human immunodeficiency virus (HIV) [39].

In the current study, the method was first set up with dianthin, a type I RIP from the seeds of *Dianthus*

Table 1
Overview of ricin detection techniques

Antigen	Detection method	Limit of detection (ng/ml)	Reference
Ricin	Quartz crystal microbalance sensors	5000	[26]
Ricin	Fluoroimmunoassay	1000	[21]
Ricin	Biosensor assay	320	[25]
Ricin	Lateral flow devices	250	[19]
Ricin	ELISA	80	[17]
Ricin	Inhibition of lysozyme	80	[16]
Ricin	Immunochromatographic assay	50	[13]
Ricin	Microarray biosensor assay	10	[27]
Ricin	Immunoassay	5	[20]
Ricin	ELISA	5	[19]
RCA	Fluoroimmunoassay	1	[22]
Ricin	Biosensor assay	0.5	[15]
Ricin	Microelectromechanical sensors assay	0.4	[29]
RCA	Microarray biosensor assay	0.18	[28]
Ricin	Biosensor assay	0.1	[24]
Ricin	Immunoassay on gel-based microchips	0.1	[14]
Ricin	Chemiluminescence ELISA	0.1	[18]
Ricin	Protein array	0.1	[32]
Ricin	Luciferase-based assay	0.001	[31]
Ricin	MALDI–MS HPLC–electrospray	<5% of crude extract	[30]
Ricin	Piezoelectric detection	10 μg/crystal	[23]
Ricin	IPCR	0.00001	Current work

Note. RCA, *Ricinus communis agglutinin.*