Ribosome-inactivating proteins (RIPs) do as their name suggests: they inactivate the ribosomes crucial to protein synthesis at the cellular level, ultimately killing cells. While some are extremely toxic, ricin being the consummate example, most RIPs are not, and have been found useful in a number of medical and research applications such as in cancer and neurological research.

Ribosome-inactivating Proteins: Ricin and Related Proteins provides up-to-date information on all aspects of this broad-ranging family of proteins. The text includes comprehensive coverage of RIPs, providing detail on their distribution in nature, chemical structure, genetics, and chemical and immunological properties. The book also describes mechanistic aspects, including the enzymatic activity on various polynucleotide substrates; the interaction with and entry into cells; the toxicity to animals, pathology of poisoning; and the immunomodulatory and allergenic activity.

Ribosome-inactivating Proteins: Ricin and Related Proteins covers biological activities with potential applications in research in biology, medicine, and applied sciences; antiviral and insecticidal properties; and the conjugates of RIPs as immunotoxins and with other carriers capable of directing them toward specific target cells. Emphasis is given to the use of immunotoxins and other conjugates in clinical trials for the therapy of cancer and intractable pain. Finally, the possible uses of toxic RIPs for criminal uses and as biological weapons for warfare and terrorist attacks are summarized.

- Covers the use of RIPs in human therapies, such as targeted tumor treatment and pain management.
- Chapters on RIP activities as they relate to applications in research, agriculture, and medicine.
- Includes chapters on RIP antiviral properties, insecticidal properties, and use in neuroscience research.
- Describes the unique characteristics of RIPs in various plant families.
- Concluding section with a timely discussion of RIPs as bioweapons.

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Ribosome-inactivating Proteins
Ribosome-inactivating Proteins

Ricin and Related Proteins

Edited by
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Preface

Ribosome-inactivating proteins (RIPs) are a class of proteins that range from a few proteins known for more than a century, to a large number identified in the last few years. Some of them are potent toxins.

An abundant literature has appeared on the subject, with thousands of articles, reviews, and books. Research on RIPs has been stimulated not only for the sake of knowledge, but also for their potential applications, at first in medicine and subsequently in agriculture, some of which now seem to be close to use. In spite of this significant amount of research, a lot remains to be learned. Many questions remain unanswered, and new ones are posed by results obtained. To mention just one example, the role of RIPs in nature is still unclear.

These, in part unexpected, developments led us to plan a book on these proteins. We were fortunate enough to obtain the collaboration of some of the best experts on the various aspects of RIPs, who have well described the research on these proteins. They had absolute freedom, not only in reviewing the literature, but also in expressing their views and making new proposals, even when these were different from the opinions of other authors and, at times, even the editors. This, we hope, makes the book not only informative, but also a stimulus for further research.

The book is organized in 19 chapters, each assigned to relevant experts. It starts with an introduction summarizing the research that led from ricin to a new class of proteins and their possible practical applications, followed by a description of the occurrence and distribution of RIPs in nature, based on a modern original search on the genome of these proteins and their presence in the available genomes of plants and animals.

Almost all the type 1 RIPs are described, divided by plant families of origin—namely Phytolaccaceae, Caryophyllaceae, Cucurbitaceae, and Euphorbiaceae—followed by the non-toxic type 2 RIPs and the Shiga and Shiga-like toxins.

The properties of RIPs are extensively described, beginning with their structures, which are compared and related to their enzymatic activity and to the action of inhibitors. This is followed by the evolution of RIP genes. The true toxins enter cells by the clever and insidious use of a cell-binding protein; their traverse into the cell and to their target is a tale of incredible evolutionary prowess. Even RIPs with no cell-binding chain apparently enter cells for antiviral activity. The enzymatic action, the entry into cells, and the intracellular destination of RIPs are fully described. The pathological damage caused by toxic RIPs is also well discussed.

The antiviral, antifungal, embryotoxic, and abortifacient properties of RIPs lead to possible applications of these proteins in agriculture and in medicine, as they are or linked to antibodies or other carriers, with limitations due to their immunological properties which are well described and discussed.
RIPs conjugated to targeting proteins, such as antibodies that recognize cancer cell surface markers, have held promise over the years as miraculous anticancer agents if they could just be targeted specifically to the cancer cells and nothing else. The adventures and difficulties in developing a proper drug in which the marvels of modern science can be used is the subject of Chapter 16. This topic has been worked on for 40 years now and can, and should, be the subject of a completely separate book. The use of this same idea has been transferred to neuroscience research, described in Chapter 17, as scientists have begun the work on the Brain Activity Map project.

Finally, fears over the possible uses of toxic RIPs for criminal purposes and as biological weapons for warfare and terrorist attacks are summarized in Chapter 19 from a realistic viewpoint.

We are grateful to all our authors for having accepted our proposal and our comments on their work. These authors are leaders in their fields and have contributed their results, in several cases, for many years in the best peer-reviewed scientific publications. We, the editors, are proud that they have joined us in describing the many fascinating facets of ribosome-inactivating proteins. We also thank Wiley for their help in all aspects of this effort, including a most important aspect: publishing. We especially thank Denise Higgins for organizing and formatting all the chapters so that they all made sense. This was no small task and, without her help, this book would never have made it to the publishers. All of us, authors and editors, are tremendously grateful for her dedication.
1 Introduction and History

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Introduction

The history of RIPS, especially the toxic ones, has been well reviewed recently.\(^1\) This present chapter will summarize the research steps that in the last 40 years have led to significant advancements in the knowledge of these proteins, of their mechanism of action, and of their possible practical applications in medicine and in agriculture.

Ribosome-inactivating proteins (RIPS), initially discovered in higher plants, have been the subject of numerous studies (reviews by Van Damme,\(^2\) Nielsen,\(^3\) Hartley,\(^4\) Ćirbés,\(^5\) Stirpe,\(^6,7\) Ng,\(^8\) and Puri\(^9\)). More than 50 RIPS have been identified and purified, but it has become clear that they can, in some circumstances, be expressed in many plants and other organisms in which they have not been detected because of assay sensitivity or other reasons. Thus, they must have an important function to justify their persistence throughout the evolution of proteins, which are an expensive material to make. Furthermore, it is becoming more and more apparent that important uses of RIPS can be envisaged.

Identification and Distribution in Nature

The studies on the proteins that eventually were denominated ribosome-inactivating proteins (RIPS) began in Dorpat at the end of the nineteenth century when ricin, a potent toxin from the seeds of *Ricinus communis* (castor bean plant), was identified and isolated by Stillmark who described it in his thesis as a “ferment” (remarkably for the time!).\(^10\) Shortly afterwards, abrin, a toxin similar to ricin, was isolated from the seeds of *Abrus precatorius*.\(^11\)

Research on these toxins was then rather scarce for a long period, until a revival of the studies on ricin and abrin was prompted by the report that these proteins were more toxic to malignant, than to normal, cells.\(^12\) The same authors found that the toxins inhibited protein synthesis by cells, a first step toward the discovery of their mechanism of action.\(^13\) Unfortunately, subsequent interest in the toxins, especially ricin, was stimulated also by the fear of a possible use for warfare or terrorist actions, as in the case of a Bulgarian journalist murdered with a micro-bullet probably containing ricin.\(^14\)
Significant, pioneering progress in knowledge of the toxins was achieved by S. Olsnes, A. Pihl, and collaborators in the Institute of Biochemistry of the Norwegian Radium Hospital. They elucidated the structure of both ricin and abrin, establishing that they constituted two unequal polypeptide chains linked by a disulfide bond, an A chain which inhibited protein synthesis, and a B chain with the properties of a lectin specific for sugars with the structure of galactose. They found also that the latter chain binds to galactose residues on the surface of most cells, allowing the entry of the A chain, which exerts its toxic action. Furthermore, they confirmed that the toxins inhibit protein synthesis, not only in cells, but also in a cell-free system.16

The knowledge that ricin and abrin were produced by taxonomically unrelated plants, and still had a very similar structure and function, led our group to research whether other, similar, toxins were present in nature. In our laboratory this research was conducted by examining seed extracts from plants known to be toxic. On the basis of Olsnes’ observations, the extracts were tested for inhibition of protein synthesis in a cell-free system, a rabbit reticulocyte lysate, a much simpler and more rapid test to perform than to evaluate the effects on inhibition of protein synthesis by, or on viability of, cells. A number of seed extracts were screened in this way, starting from those from plants that in the old literature were reported as containing toxins similar to ricin, such as crotin and curcin. Much to our surprise (and for a while, disappointment!), we found that indeed in the seed extracts from many plants there were proteins that inhibited cell-free protein synthesis, but these were hardly toxic to cells. The meaning of these observations was elucidated when an antiviral protein (pokeweed antiviral protein, PAP) was isolated from the leaves of Phytolacca americana, that inhibited protein synthesis resembling the A chain of ricin. Thus, it soon became clear that the proteins present in our and other plant extracts also had antiviral activity against both plant and animal viruses (review by Kaur et al.). A new classification of RIs is proposed in Chapter 2. Another form of PAP was identified in pokeweed summer leaves, and another in pokeweed seeds. On the whole, these results indicated (i) that RIs could be present in the same plant as isoforms, which subsequent research showed could be several, and (ii) that in some plants RIs could be in different organs, and not only in seeds as ricin and abrin. It was found also that trichosanthin, a protein from Trichosanthes kirilowii and other plant proteins used in China to induce abortion, were RIs (reviews by Ng et al. and Chapter 4).

The structure of ricin, and subsequently of other RIs of both types, was elucidated by crystallographic studies (review by Robertus and Monzingo and Chapter 8), which led to the identification of active and sugar-binding sites.

The search for other toxins similar to ricin continued, and modeccin, a highly toxic protein described as a “toxalbumin” and already at that time suspected to be similar to ricin, was purified and characterized almost at the same time in Oslo and in Bologna. A galactose-specific haemagglutinating lectin purified from mistletoe turned out to be a type 2 RIP. Other type 2 RIs were found in several other plants, including some belonging to the genus Adenia. From Adenia stenodactyla stenodactylin was isolated, probably the most potent toxin of plant origin, with an LD₃₀ < 1 µg/kg of body weight. Lists of RIs are given in several reviews.

From the number of RIs isolated, it seemed that type 1 RIs were very common in plants, whilst type 2 RIs were rather scarce. This belief was somewhat changed: two barely toxic tetrameric agglutinins consisting of two A and two B chains are present in the seeds of Ricinus communis and Abrus precatorius; from Sambucus nigra a lectin, nigrin b, was purified and had structure and
biochemical properties similar to those of type 2 RIPS, and still a very low toxicity (Chapter 5), and other non-toxic type 2 RIPS have been isolated (see lists in [2, 5, 6]), so it is possible that other, possibly many, similar proteins are present in nature.

An evolutionary relationship between single-chain and two-chain RIPS was elaborated by Ready et al. [38] and the precursors in the synthesis of ricin were identified. [39]

Ribosome-inactivating proteins seemed particularly frequent in plants belonging to some families, namely Cucurbitaceae, Euphorbiaceae, and Caryophyllaceae. However, most results came from a search aimed at finding proteins present at a level sufficient to allow easy purification of significant quantities, and consequently the search was concentrated on plants taxonomically close to those already known to contain RIPS. Furthermore, a number of plants were not considered to contain a RIP when their extracts had protein synthesis inhibitory activity below a pre-set arbitrary level: thus it is possible that in many other plants RIPS are present, although at a low level or in an inactive form. This notion was supported by the study of plant genomes (Chapter 9) and by the observation that the expression of RIPS is stimulated or even appears under several conditions of stress. [40] However, the hypothesis that RIPS could be ubiquitous in plants was dismissed after it was reported that in the complete genome of Arabidopsis thaliana there are no sequences encoding for RIPS. [2]

There are reports, some of them controversial, that RIPS or RIP-like proteins are produced by organisms other than higher plants, namely algae, fungi, and microorganisms. Among bacteria, Shigella shigae and strains of Escherichia coli produce, respectively, Shiga toxin and Shiga-like toxins which have RIP activity (reviews by Reyes et al. [41] and Chapter 7), and the Streptomyces coelicolor genome appears to encode a type 1 ribosome-inactivating protein [41] (see Chapter 9).

Mechanism of Action

Our laboratory also became involved in the study of the mechanism of action of ricin and related toxins. The fact that RIPS inhibited cell-free protein synthesis led to an investigation on their effect in a system of purified ribosomes with necessary enzymes and cofactors. The experiments by L. Montanaro and S. Sperti established that ricin damaged ribosomes [42] more precisely their larger subunit, [43] rendering ribosomes incapable of binding Elongation Factor 2. [44] This effect was irreversible and occurred at a less-than-equimolar toxin: ribosome ratio, indicating that the toxins acted catalytically, that is enzymatically. The effect of the enzymatic activity was discovered by Endo and collaborators, who found that ricin is an N-glycosidase which removes a single adenine residue from rRNA (A from rat liver rRNA). [45, 46] Subsequently these results were extended to all RIPS of both types [47] and RIPS were officially denominated rRNA N-glycosidases (EC 3.2.2.22).

Unexpectedly, it was found that several saporins – the RIPS from Saponaria officinalis, and, to a lesser extent, some but not all other RIPS examined – removed more than one adenine per ribosome. [48] Subsequently it was observed that isoforms of saporin removed adenine not only from rRNA, but also from various other types of RNA, from poly(A) and from DNA. [49] These results were further extended, and it was found that many RIPS acted, to different extents, on DNA and some also on RNA, on viral RNAs, and on other polynucleotides; [50] consequently the denomination of adenine polynucleotide glycosylase was suggested as more appropriate for RIPS. [51] Whether the activities on substrates other than rRNA have a role in the function of RIPS in nature remains to be ascertained.

Furthermore, the presence in mammalian cells of a hitherto unknown enzymatic activity that removes adenine from DNA has been reported. [51] This possibly RIP-like activity appeared enhanced...
in cells subjected to stress (serum deprivation) or viral infection, as it occurs with RIPS in plants. Unfortunately, the supposed cellular enzyme involved defied all efforts toward purification in our laboratory, because the activity was very low, especially in organs of large animals, and was also labile, soon disappearing during the purification attempts. These results need to be confirmed and extended.

Other enzymatic activities of RIPS were reported, namely nuclease, superoxide dismutase, chitinase, phosphatase, and lipase (reviews in4, 7), but it is still a matter of discussion whether these activities are due to contaminants.

Effects on Cells and Animals

Flexner52 first described the severe lesions caused by ricin in rabbits’ and guinea pigs’ livers, kidneys, spleen, lymph nodes, and intestines, and noticed the early damage to sinusoidal lining of the liver. Few further studies were performed on the damages brought about by ricin and abrin until the macroscopical, histological, and ultrastructural lesions caused by ricin in rats were described.53 Necrotic lesions were observed in liver and spleen with hemorrhagic inflammation in the intestine and lymph nodes. In the liver, the Kupffer cells appeared damaged at an early stage. This result led to the finding of higher uptake of ricin by these cells, which was prevented only in part by galactose or mannose, and completely by both sugars. This was because the uptake of ricin occurred not only through the binding to the galactose residues on the cell, but also through the binding of the mannose present in ricin to the mannose receptors on Kupffer-54 and other macrophagic cells55, 56 that have a high uptake of, and are very sensitive to, ricin and other RIPS. This higher toxicity to macrophagic cells may account, at least in part, for the immunosuppressive activity of RIPS.57 On the other hand, ricin and other RIPS are strongly immunogenic58 and are potent allergens, causing formation of IgE in animals59 and humans, with severe allergic manifestations.60

The entry into cells and the intracellular fate of RIPS have been studied extensively (reviews by61–63). Ricin and other type 2 RIPS bind to galactosyl-terminated receptors on the cell surface, are endocytosed and transported to the Golgi apparatus and then to the endoplasmic reticulum and the cytosol. Once inside a cell, RIPS are partially exocytosed and/or degraded by proteolysis, but a fraction remaining intact is sufficient to inactivate ribosomes, with consequent inhibition of protein synthesis and cell death. There are differences in the binding, uptake, and intracellular processing of the various RIPS, and this may account, at least in part, for their differences in, or lack of, toxicity.64, 65

As per the mechanism of cellular damage, the first observations that ricin and abrin cause apoptosis66 were confirmed and extended to other type 2 RIPS and RIP-containing immunotoxins67 (reviewed in Chapter 15).

It was observed that ricin, and subsequently other type 2 RIPS, are transported retrogradely along peripheral nerves68 but only modeccin and volkensin, and not ricin and abrin, are transported retrogradely when injected in the central nervous system.69

Role in Nature

An important remaining question concerns the presence and role of RIPS in nature. Notions have been put forward about their role, but none of them are completely convincing.

A rather popular hypothesis is that RIPS could be a defense against predators and/or parasites. It is possible that the antiviral activity of RIPS may prevent or at least limit viral infections in some plants.
Interestingly, it was found that in pokeweed leaves PAP is located in the cell wall matrix, that is outside the cytoplasm, and the idea was put forward that it could be moved to the cytoplasm of virus-infected cells, thus inactivating ribosomes and preventing viral replication.\(^{38}\) Also, it is likely that toxic type 2 RIPS may deter animals from eating the plants containing them, but this is less probable in the case of type 1 RIPS, which are present in edible plants. However, the hypothesis of defense as the main role of RIPS is not generally accepted, and in any case it does not apply to RIP-producing microorganisms.

The expression of RIPS is enhanced in plants or leaves in conditions of senescence or subjected to a variety of biotic and abiotic stress, such as viruses, microorganisms, insects, fungi, heat, osmotic stress, cold, and salinity.\(^{70}\) This led to the notion that the function of RIPS could be to kill cells when they are bound to die.\(^{71}\) However, further possibilities arose when it was found that increasing the expression of RIPS renders plants more resistant to drought and salinity.\(^{72}\) These authors suggest that RIPS could have a role in the resistance of plants to stress, possibly “due to the re-organization of protein metabolism through inhibiting protein synthesis.”

### Practical Applications

Practical uses of RIPS in agriculture and in medicine have been envisaged. They could be used as such for their properties, namely their antiviral,\(^{21}\) insecticidal,\(^{73}\) antifungal,\(^{74}\) and abortifacient\(^{26}\) activities. Furthermore, RIPS have been linked to antibodies or other carriers to form “immunotoxins” or other conjugates capable of selectively directing them to target cells (see Chapters 15 and 16).

#### In Agriculture

Plants with low or no expression of RIPS have been transfected with RIP genes in order to confer resistance to viruses on them. In several cases success has been reported, however plants may be damaged if the expression of RIP is above some level.\(^{75}\)

RIPS of both types are toxic to some insect species and are investigated as biological insecticides.\(^{72}\) Plants transfected with a RIP acquired resistance to various Insecta.\(^{76}\)

Resistance to fungi was obtained in tobacco plants transfected with a type 1 RIP b-32 from barley.\(^{77}\)

Recently, the overexpression of an endogenous RIP obtained by duplication of the autologous relevant gene rendered rice plants more resistant to drought and salinity.\(^{72}\) If these results, important \textit{per se}, are extended to other plants, the economical impact will be substantial, if only a fraction of water for irrigation can be saved. Furthermore, the fact that the increased expression of an autologous RIP gene in rice plants did not cause any damage, suggests that these, and probably other plants, may be rendered more resistant to viral and other infections too.

#### In Medicine

Trichosanthin is used in the Chinese official medicine to induce abortion (see Chapter 18).

Several possibilities have been envisaged for the use of RIPS in therapy. Trichosanthin was explored as an antiviral agent for the therapy of AIDS. Unfortunately, attempts to treat HIV-infected patients resulted in no cure, and actually some aggravation of mental\(^{78}\) or neurological symptoms seemed to occur.\(^{79, 80}\)
More encouraging results were obtained by linking RIPS to antibodies or other carriers to form “immunotoxins” or other conjugates capable of selectively delivering them to cells to be eliminated. To this purpose, type 2 RIPS could not be used as such, because their B chain would link to all sorts of cells. Numerous conjugates have been prepared with separated A chains, mainly from ricin, linked to a variety of antibodies or other carriers to form “immunotoxins” or other conjugates (review by Fracasso et al.\textsuperscript{81}). When type 1 RIPS became available, they could be used to prepare conjugates, with the practical advantage of an easier and safer preparation. The feasibility of this approach was demonstrated when a first conjugate with a type 1 RIP was made with gelonin linked to concanavalin A,\textsuperscript{82} soon followed by an immunotoxin prepared with the same RIP linked to anti-Thy1.1 monoclonal antibody.\textsuperscript{58} Immunotoxins have also been prepared with non-toxic type 2 RIPS (see Chapter 15). With the advent of biotechnology, immunotoxins are prepared by the fusion technique, which gives the advantage of absolutely constant products.

Immunotoxins and other conjugates have been useful in research, particularly on the nervous system (review in Wiley and Lappi\textsuperscript{68, 83}), and have also been investigated for therapeutic purposes, especially for cancer. Actually, their administration often caused an improvement in cancer patients, with transient side-effects that usually could be controlled. Unfortunately, as foreign proteins, the immune response by the recipient posed a serious obstacle to prolonged treatments. However, this obstacle could be circumvented in several ways. Immunotoxins could be used for “external” applications, such as in the case of bladder cancer.\textsuperscript{84} Furthermore, (i) the immune reaction against the antibody component of the immunotoxins could be prevented with the use of humanized or, better, human antibodies and (ii) some RIP molecules can be modified to become less immunogenic by covalent conjugation with polyethylene glycol, (e.g., see Meng et al.\textsuperscript{85}) or by epitope mutation by genetic engineering.\textsuperscript{86} The latter authors obtained an immunotoxin consisting of bougainin, a RIP from Bougainvillea spectabilis, with mutated T-cell epitopes (debougainin) fused with an anti-EpCAM Fab fragment. This immunotoxin caused a significant antibody response in only two out of twelve patients receiving several doses over a four-week course.\textsuperscript{87} Furthermore, it has been reported that an immunotoxin constructed with recombinant gelonin caused only a limited immune reaction in a phase 1 clinical trial.\textsuperscript{88}

Immunotoxins could also be used if they were effective after a single administration, to eliminate small clumps of few malignant cells in the “minimal residual disease” after conventional therapy. Good results were obtained in experimental animals with saporin, a RIP from Saponaria officinalis, linked to the pain-processing peptide Substance P.\textsuperscript{89} A small number of cells that process pathological pain signals was removed, causing relief that appears to be permanent. Normal acute pain is unaffected. This approach seems very promising, because a single administration of the conjugate is sufficient to eliminate the target cells, thus without the adverse effects of immune reactions.

These are only the first encouraging results, but it took some 70 years for Ehrlich’s idea of a “magic bullet” to be tested, and the first immunotoxins were prepared less than 40 years ago.

**Bioweapons**

Regrettfully, it must be mentioned that ricin at least has been used for criminal purposes and that there are fears that it could be used as a weapon, especially by terrorists (reviews by Schep et al.,\textsuperscript{90} Griffiths,\textsuperscript{91} Anderson,\textsuperscript{92} and in Chapter 19). This led to the development of methods to detect ricin and abrin, to investigate the effects of ricin – especially if inhaled – and to develop vaccines. So, as often happens with research for military purposes, the result was an advancement of knowledge, which always is or will be useful.
Future Challenges

Following the identification of ribosome-inactivating proteins, a considerable amount of knowledge about them has been accumulated over the last 40 years. However, as is often the case in science, the unanswered questions probably outnumber the answered ones.

A challenging question is the role of RIPS in nature, which must be an important one, given the frequency, and sometimes abundant amounts of these proteins, which are energy-expensive materials to make.

Equally interesting would be a better definition of the distribution of RIPS among plants, as is advocated in Chapter 2. Obviously, a complete survey would be impossible, but at least the distribution among the main phyla could be ascertained. Also, the DNA glycosylase activity in animal cells, if confirmed, could be studied and its possible role in stress ascertained. One wonders whether the expression of this “RIP-like” activity could be artificially enhanced in mammalian cells and possibly in animals, and what the resulting effect would be.

Numerous developments can be foreseen for the practical applications of RIPS. In agriculture, an attractive possibility is to increase their expression without damage to the plants; at least plants that are not eaten raw could be safely modified, to confer on them protection against insects and/or viral and other infections. Also, plants could be rendered more resistant to stress, particularly drought and salinity.

In medicine, attempts to use RIPS as antiviral agents have not been successful. However, one wonders whether their oral administration could help to control intestinal viral infections.

The use of conjugates containing RIPS is becoming a realistic possibility. Obstacles to their use in human therapy are their side-effects, namely their unspecific toxicity, mainly the capillary leak syndrome, which seem to be controlled by drugs. Other problems come from the immune reaction against these foreign proteins. These, however, can be circumvented in various ways, as is outlined in the section “In Medicine” above.

It is noteworthy that research on RIPS was initiated for the sake of knowledge: its developments seem to confirm that, “First rate fundamental research, sooner or later, leads to important practical applications.”

References


2 Occurrence and Taxonomical Distribution of Ribosome-inactivating Proteins Belonging to the Ricin/Shiga Toxin Superfamily

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Introduction

Ribosome-inactivating proteins are by definition protein synthesis inhibitors that act at the level of the ribosome.\textsuperscript{1} Though the activity of ribosomes can be affected by different types of proteins/enzymes (e.g., proteases, RNases, ribosome-binding proteins) this contribution deals exclusively with proteins that by virtue of a well-defined RNA N-glycosidase activity are capable of depurinating a specific adenine in what is called the conserved α-sarcin/ricin loop of the large ribosomal RNA. The latter activity relies on the presence of a conserved structural domain (Pfam PF00161) of approximately 250 amino acid residues that occurs either as a single domain or in association with other proteins/domains. Proteins with such a domain are usually classified into the ricin/Shiga toxin superfamily because the latter two proteins are the best studied and most notorious examples of ribosome-inactivating proteins or RIPS. Though the name ricin/Shiga toxin superfamily is certainly informative, because it highlights the evolutionary relationship between two structurally different groups of toxins from two taxonomically distant phyla, it is too restrictive in the sense that there is no direct link with other types of RIPS. Therefore, it seems preferable to discuss the whole family of RIPS in terms of the presence of an RNA N-glycosidase domain structurally homologous to the A chain of both the Shiga toxin and ricin.

RIPS are classically subdivided in three main groups namely the (bacterial) Shiga and Shiga-like toxins (Stx) and the plant type 1 and type 2 RIPS. Shiga and Shiga-like toxins are built up of a catalytically active A subunit equivalent to a RIP domain and a B subunit, which itself is a pentamer – of five identical 89 amino acid residue polypeptides – that specifically binds to the glycolipid globotriaosylceramide. Both the A- and B-subunits are synthesized on a single dicistronic mRNA as two separate polypeptide chains.\textsuperscript{2} Type 2 RIPS from plants are also typically described in terms of an AB structure.\textsuperscript{3-5} It should be emphasized, however, that the overall structure of, for example, ricin is completely different from that of the Shiga toxin. First, the B subunit of ricin consists of a single polypeptide chain and, second, both the A and B chains are synthesized on the very same precursor polypeptide that is post-translationally processed through the excision of a linker polypeptide between the two domains. Plant type 1 RIPS are far simpler from a structural point of view since they
consist only of a RIP domain. However, this subgroup is less uniform than the type 2 RIPS and in some cases (e.g., a maize RIP b-32) enzymatic activity is acquired only after proteolytic processing of the A-domain into two smaller polypeptides. Though the mature maize RIP b-32 corresponds in fact to a proteolytically cleaved type 1 RIP, it was called a type 3 RIP to distinguish it from other type 1 RIPS known at that time. Unfortunately, the term type 3 RIP had already been introduced earlier for a 60 kDa jasmonate-induced barley leaf protein (JIP60) consisting of an N-terminal A domain fused to an unrelated domain with no known activity. The identification of JIP60 not only increases the complexity in terms of domain structure – of the RIP family, but also argues for a clear and unambiguous classification.

At present there is some confusion about the taxonomic distribution of RIPS. The isolation and characterization of numerous Shiga/Shiga-like toxins and both type 1 and type 2 RIPS leave no doubt for the occurrence of the RIP domain in both bacteria and plants. Though it is – especially in review papers – often stated that RIPS are also present in fungi, algae, and even in mammalian tissues no experimental evidence has been presented yet that in any of these cases the presumed RIP activity relies on the presence of a protein with a genuine N-glycosidase domain. On the contrary, it seems pretty evident that, for example, all so-called fungal RIPS belong to the family of fungal ribotoxins, which possess a ribonuclease rather than an N-glycosidase domain. However, it is questionable whether our current view on the taxonomic distribution of RIPS, which is merely based on studies of isolated proteins, reflects the actual occurrence of the N-glycosidase domain in living organisms. Most RIPS identified thus far are expressed at a level allowing purification by standard biochemical techniques. Moreover, even though (very) low levels of RIPS can be traced through their enzymatic activity, one cannot exclude that some escape detection or, alternatively, are for practical reasons not further investigated. As a result, the distribution of the RIP domain might well be underestimated, which is very unfortunate for two main reasons. First, some RIPS with unique properties/activities cannot be exploited. Second, no detailed study of the phylogeny and molecular evolution of proteins with an N-glycosidase domain can be elaborated in the absence of a comprehensive overview of the occurrence of the RIP domain throughout living organisms. Therefore the issue of the taxonomic distribution should be readdressed to extract an overview of all proteins/genes with an N-glycosidase domain.

How to Investigate the Distribution of Proteins with an N-glycosidase Domain?

The discussion of the taxonomical distribution is in most review papers primarily based on the list of RIPS that have been isolated and (partly) characterized. Though this approach is certainly helpful for scientists interested in the use – in the broadest sense – of RIPS, it is obviously too restrictive for in-depth studies of the phylogeny and molecular evolution of proteins with an N-glycosidase domain. As pointed out in a recent review, preliminary screenings of plant genome and transcriptome databases demonstrated that an extended list of expressed RIPS as well as putative RIP genes can already be added to the existing lists of documented RIPS. Moreover, analyses of the completed plant genomes revealed the occurrence of RIP gene families, some of which encode proteins with previously unknown domain architecture. For example, the Oryza (rice) genome contains a complex set of at least 30 different genes in which a RIP domain can be identified. Most of these genes encode (putative) proteins consisting of a single RIP domain, but some exhibit a chimeric overall structure in which an N-terminal RIP domain precedes an unrelated C-terminal domain.

Two important conclusions can be drawn from the results of the preliminary screening of the plant genomes/transcriptomes. First, the existing lists of RIPS have to be extended horizontally (more species) and vertically (more RIPS/RIP genes per species). Second, all evidence suggests that several types of
proteins with a novel domain architecture must be added to the present list of chimeric RIP genes (i.e., type 2 and type 3 RIPS). In addition, the identification of numerous “novel” plant RIPS by an *in silico* analysis raises the question of whether the same approach might reveal the possible occurrence of as yet unidentified RIPS/RIP genes outside the plant kingdom. Therefore, the issue of the taxonomic distribution of RIPS cannot be treated without a thorough exploration of the huge amount of data generated by genome and transcriptome sequencing programs of both prokaryotes and eukaryotes.

Since this contribution deals exclusively with proteins/genes possessing a canonical N-glycosidase domain (equivalent to the toxin part of Stx and ricin) the availability of a complete sequence or at least sufficient sequence information is a prerequisite for any entry to be included. Accordingly, the retrieval of RIPS and RIP genes is (apart from the Stx and plant type 1 and type 2 RIPS that have been studied in detail at the protein level) based in the first place on BLAST searches. These BLAST searches were not confined to protein databases (BLASTp) but covered also all publicly accessible genome and transcriptome databases (tBLASTn searches). In a first round of searches, sequences of well-studied RIPS (e.g., ricin, pokeweed antiviral protein, Shiga toxin) were used as queries. Subsequently, sequences of newly retrieved types of RIPS/RIP genes were used in a next round of BLAST searches. The latter process was repeated until no new sequences could be identified.

As will be discussed below, a more detail screening of the databases eventually resulted in the identification of (i) numerous novel rip genes and (ii) several previously unknown chimeric forms. Taking into consideration the existing ambiguity of the currently used classification (type 1, 2, and 3 RIPS) it seems rather inappropriate to increase the complexity of the system for the newly identified chimeric forms. Therefore, a novel system is introduced based on the domain architecture of the RIPS/RIP genes. Proteins/genes consisting of a single N-glycosidase domain will be referred to as the “[A] type” and the chimeric forms as the “[AN] type” whereby N stands for the different types of (unknown) C-terminal domains.

A final remark concerns the catalytic activity of the (putative) RIPS found in different organisms. Virtually all RIPS studied thus far exhibit N-glycosidase activity, but a few lectins closely related to type 2 RIPS (e.g., a lectin from *Bryonia dioica*) apparently possess an A chain homolog that lacks catalytic activity (unpublished data). Though the obvious occurrence of catalytically inactive RIP domains in some plants is rather anecdotal, it is highly relevant because it demonstrates that the presence of a domain that shares a reasonable sequence similarity with a genuine RIP does not necessarily imply that it possesses enzymatic activity. Therefore, it cannot be excluded that some of the RIPS/RIP genes discussed in the next section lack a catalytically active domain.

**Overview of the Occurrence of the N-glycosidase Domain in Living Organisms**

Hitherto, genuine RIPS have been isolated exclusively from bacteria and plants. It appears, however, that (expressed) RIP genes occur also in some fungi and in a few insects. Since plant RIPS are by far the largest and most heterogeneous group, they will be discussed first. Subsequently, bacterial, fungal, and insect RIPS will be discussed in separate sections.

**Plant RIPS**

As already suggested in an earlier review, neither the taxonomic distribution nor heterogeneity (in terms of domain structure) are accurately reflected by the (long) list of plant RIPS that have been purified and characterized in some detail. However, this does not imply that the N-glycosidase domain is
ubiquitous in plants. On the contrary, the absence of a RIP domain from the first completed plant genome of *Arabidopsis thaliana* provided evidence for the opposite more than a decade ago. In the meantime, numerous genomes of species covering all major plant taxa have been completed, which now allows a better overview of the occurrence and overall structure of genes with an N-glycosidase domain by an *in silico* approach that does not depend on the availability of “protein data.”

**What can be Learned from Analyses of Completed Plant Genomes?**

To identify RIP genes in the (nearly) completed plant genomes, extensive screenings based primarily on tBLASTn searches of genomic (nucleotide) sequences were set up. Searches were not confined to a BLASTp approach for the following reasons. First, several genomes are not yet annotated. Second, automated annotation of (putative) proteins does not identify all RIP domains. Third, the accuracy of the automated annotation is – especially for Poaceae species – insufficient (due to the presence of introns and the insertion of transposable elements).

The results of this extensive *in silico* screening covering a total number of 42 plant genomes are summarized in Figure 2.1, showing a phylogenetic tree of plant species with indication of the RIP gene complement. For most species, the RIP genes (if present) could readily be identified. However, for the Poaceae species the outcome of the screening is still preliminary because of the complexity of the RIP gene complement and the occurrence of (multiple) introns in some RIP genes. Apart from the identification of the genes, the *in silico* analysis revealed the occurrence of several as yet unknown chimeric RIPS (namely type [AD], [AC], [AX], [AP^M41], and [AP^C19]; for details see Figure 2.2) as well as at least two different lineages of the type [A] RIP. These latter two lineages are the type [A^AB] and type [A^AX] RIPS, which are derived by domain deletion events from the type [AB] and type [AX] chimeric RIPS, respectively (for details see Chapter 9). At present it is not clear how the multiple type [A] RIPS found in Poaceae species should be classified. Most probably they form a complex set of as yet unidentified type [A] RIPS and accordingly are, for practical reasons, classified here as type [A^a].

Several important conclusions can be drawn from Figure 2.1. First, RIP genes are apparently absent from 24 out of 42 completed genomes. Even within the group of flowering plants, more than half of all species investigated (20 out of the 38) lack RIP gene(s). Second, in some families (e.g., Euphorbiaceae and Poaceae) RIP genes are found in all sequenced genomes whereas in others (e.g., Rosaceae) RIP genes occur in some genomes (*Malus domestica* and *Prunus persica*) but are absent from others (*Fragaria vesca*). Third, there are striking differences between the RIP gene complement of the different species, ranging from a single gene to a complex set of genes encoding both type [A] and chimeric RIPS. Extended RIP gene families are apparently common in Poaceae species, but there are striking interspecific differences with respect to both the gene number and the domain architecture.

**Transcriptome Analyses Yield a More Complete Overview of the Occurrence of Expressed RIP Genes in Plants**

Though indicative, the summary presented in Figure 2.1 might not be very representative because it covers only three species from which one or more RIPS have been purified and characterized (namely maize (*Zea mays*), water melon (*Cucumis sativus*), and castor bean (*Ricinus communis*). Most RIPS have been isolated, indeed, from species that are not particularly interesting for whole genome sequencing. Therefore *in silico* searches for RIP genes were also extended to all other plant genome and transcriptome sequences.

A general overview of the documented occurrence of (expressed) RIP genes within the major taxa of green plants is presented in Figures 2.3 and 2.4. Apart from a single representative of the Gnetophyta (*Gnetum gnemon*), RIP sequences were exclusively found in flowering plants (angiosperms or Magnoliophyta). To illustrate the overall occurrence and phylogenetic distribution within
Figure 2.1 Schematic overview of the presence/absence of RIP genes in the currently completed plant genomes. The dendrogram reflects only the overall phylogeny of the species listed. The presence or absence of RIP genes is indicated. *denotes preliminary results.
**Figure 2.2**  Schematic overview of the domain architectures identified in plant RIP genes.