Ribosome-Inactivating Proteins from *Abrus pulchellus*

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Abstract Pulchellins are highly toxic type 2 ribosome-inactivating proteins (RIPs) expressed in the seeds of *Abrus pulchellus tenuiflorus*. Four pulchellin isoforms have been characterized, allowing their classification into two subgroups based on the toxicity levels and sugar-binding specificity. The residues involved in the catalytic mechanism are all conserved amongst the pulchellins, suggesting that the differential toxicity is related to variations in their B-chain behavior. In this chapter, insights into the successful production of active A- and B-chains via heterologous production in *Escherichia coli* and their assembly in vitro to produce an active heterodimer are discussed in some detail. Additionally, some features of subcellular sorting of native pulchellins are presented. Pulchellin comparative studies have contributed to the knowledge of the molecular bases of biochemical processes, such as sugar binding and toxicity, observed among RIPs.

1 Introduction

The ribosome-inactivating proteins (RIPs) belong to a class of enzymes (EC 3.2.2.22) widely distributed among plants, fungi, algae, and bacteria (Girbés et al. 2004). Members of this group play a role in the history of clinical medicine and biomedical research (reviewed in Olsnes (2004)). These proteins exhibit rRNA *N*-glycosidase activity, which leads to the excision of a specific adenine residue from a conserved loop of the large rRNA (Endo and Tsurugi 1987). The latter effect is irreversible and prevents the association of the elongation factors with the 60S ribosomal subunit, resulting in the inhibition of protein synthesis and subsequent cell death (Hartley et al. 1991). Moreover, it has been reported that some RIPs

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possess additional enzymatic activities. These include phosphatase activity on lipids, as well as chitinase, DNase, and superoxide dismutase activities (Li et al. 1996; Shih et al. 1997; Helmy et al. 1999; Wang and Tumer 2000).

The vast majority of RIPs are divided into two groups, which can be distinguished according to the absence (type 1 RIPs) or presence (type 2 RIPs) of a lectin chain (the B-chain), which is linked to the toxic chain (A-chain) by a disulfide bond (VanDamme et al. 1998; Peumans et al. 2001). The A-chain exhibits the toxic rRNA *N*-glycosidase activity (Olsnes and Pihl 1973a; Endo and Tsurugi 1987). The lectin activity of the B-chain is targeted toward specific carbohydrate moieties on the mammalian cell surface (Peumans and van Damme 1995; VanDamme et al. 1998) and mediates the entry of the A-chain (Olsnes and Pihl 1973b; Endo and Tsurugi 1987). The absence of the lectin chain significantly limits the access of type 1 RIPs into cells, resulting in lower cytotoxicity levels. However, the presence of the B-chain alone is not enough to guarantee high levels of cytotoxicity to type 2 RIPs (Stirpe and Battelli 2006).

Most plant RIPs have been found in a small number of families, namely Caryophyllaceae, Sambucaceae, Cucurbitaceae, Euphorbiaceae, Phytolaccaceae, and Poaceae (reviewed in Girbés et al. (2004)). Within the Leguminosae-Papilionoideae family, in particular, type 2 RIPs have been found and characterized in seeds of only two species, both of the genus *Abrus*: *Abrus precatorius* and *Abrus pulchellus tenuiflorus*. The *Abrus* genus is composed of about 15 species, being mostly found in tropical and subtropical areas in Asia, Africa, and South America (Parrotta 2001). Contrasting with the well-known seeds of *A. precatorius*, which exhibit a glossy red coloration with a black spot, *A. pulchellus tenuiflorus* seeds are thinner and brownish (Fig. 1).

Pulchellins are extracted from the seeds of *A. pulchellus tenuiflorus* and exhibit specificity for galactose and galactose-containing structures. This lectin property causes the agglutination of human and rabbit erythrocytes. Additionally, their toxic property kills mice when injected intraperitoneally (Ramos et al. 1998; Silva et al. 2005). In keeping with other type 2 RIPs, pulchellins are heterodimeric proteins composed of a toxic A-chain (29 kDa) in conjunction with a lectin B-chain (31.5 kDa), and are also highly toxic proteins (Ramos et al. 1998; Silva et al. 2005; Castilho et al. 2008).

Seeds of *Abrus pulchellus* present several pulchellin isoforms and four of them have already been isolated and few aspects of their sugar-binding properties have been investigated in detail (Castilho et al. 2008). All pulchellins are synthesized as precursor forms including an N-terminal presequence and a short intervening linker peptide joining the A- and B-chains, both of which are removed during protein maturation. The presequence, as observed in other type 2 RIPs, contains an endoplasmic reticulum targeting signal peptide that directs the proteins to the secretory pathway (Castilho et al. 2008). As observed by Jolliffe et al. (2003) for proricin, propulchellins possess similar intervening linker peptides (Castilho et al. 2008) which possibly contain a vacuolar targeting signal (Nielsen and Boston 2001).

Regarding cell entry during the intoxication process, subcellular sorting in mammalian cells reveals that the endosomal internalization pathway, as well as

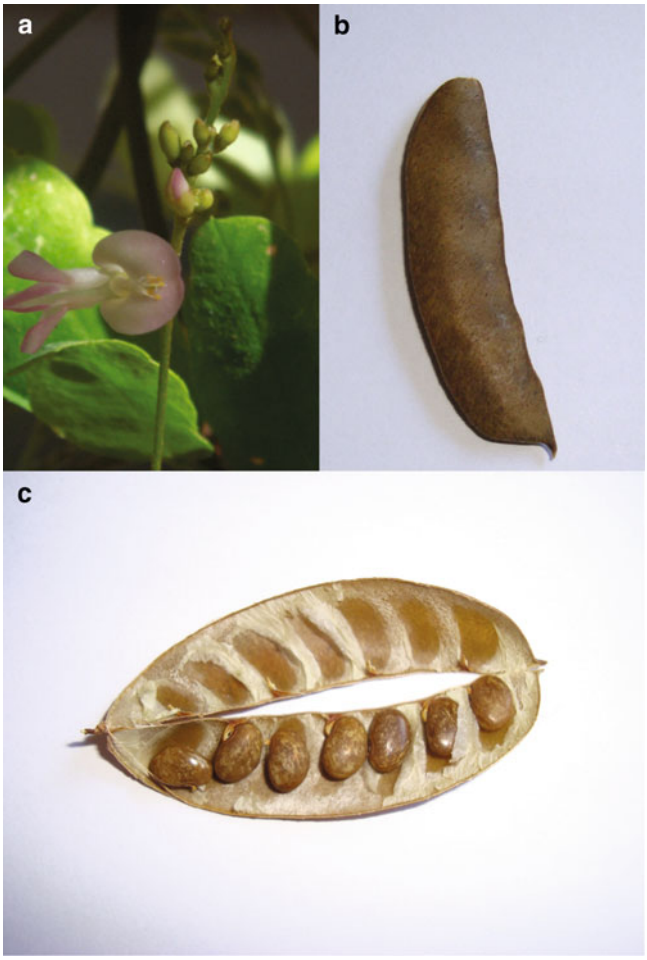

Fig. 1 *Abrus pulchellus tenuiflorus*. Plant details showing: (**a**) a close-up view of the flower; (**b**) a mature fruit; and (**c**) an open fruit showing the seeds. (Photos **a**, **b**, and **c** are not to scale)

retrograde transport through the Golgi apparatus, might be used by both native protein and single pulchellin B-chains.

In order to obtain an alternative source of these holotoxins, other than their direct extraction from seeds, pulchellin synthesis from callus culture has been investigated (Silva et al. 2003) which represents a convenient system of production for biotechnological applications. Callus culture from immature seeds of *A. pulchellus* is able to express pulchellins and their levels of expression are similar to those described for other RIPs (D'Silva et al. 1993; Silva et al. 2003). Although the isolation of pulchellins from callus culture is possible, extraction from seed cotyledons is more efficient, probably because of the higher percentages of protein present in the latter.

The heterologous expression of type 2 RIPs in *Escherichia coli* is an attractive strategy. However, pulchellin is synthesized as a single polypeptide precursor that must be cleaved in order to yield the independent, mature protein chains. Thus, the use of a prokaryotic expression system, such as bacteria, suffers from the shortcoming of having to produce the A- and B-chains separately (although in studies which focus on either one of the chains in isolation, this could be advantageous). Another major difficulty is the empirical refolding process typically required for B-chains.

2 Pulchellin Isoforms

Plants usually produce several RIP isoforms that show variation in their physical and therefore biological properties. RIP isoforms may be present in one or more plant tissues (Stirpe and Battelli 2006) and/or in a season-dependent fashion (Stirpe et al. 1992); however, the reason for such wide heterogeneity remains unclear. Several isoforms were found in the seeds of *A. pulchellus* and four of them, named P I, P II, P III, and P IV, have been isolated and studied in some detail (Castilho et al. 2008).

The pulchellins exhibit isoelectric point between 5.2 and 5.8 and possess subtle differences in their migration pattern on SDS-PAGE. Recently, biochemical characterization has been performed to investigate both their toxic and lectin-like properties. The addition of pulchellin isoforms to cultures of HeLa cells resulted in a high inhibition of protein synthesis. The half-maximal inhibitory concentration (IC_{50}) values showed that P I and P II have similar cytotoxicity (21.7 ng/ml (0.375 nM), and 22.7 ng/ml (0.391 nM), respectively) and are approximately fivefold more potent then P III and P IV (101.9 ng/ml (1.76 nM) and 98.4 ng/ml (1.7 nM), respectively). In agreement, median lethal doses (LD_{50}) in mice pointed at P II as the most toxic isoform (15 μg/kg), followed by P I (25 μg/kg), P IV (60 μg/kg), and P III (70 μg/kg). According to the toxicity values, one can conclude that although the pulchellin isoforms are less toxic than the two well-known RIPs, ricin (IC_{50} = 0.001 nM and LD_{50} = 2.6 μg/kg) (Olsnes and Pihl 1973a) and abrin (IC_{50} 0.0037 = nM and LD_{50} 0.56 = μg/kg) (Olsnes and Pihl 1973b), they are still highly toxic proteins.

The difference in the cytotoxicity values (IC_{50}) shown by pulchellin isoforms and those by abrin and ricin is still controversial because the protein samples used in the pulchellin assays were lyophilized. When the analyses were carried out with fresh samples, higher cytotoxicity values were obtained (unpublished data). This suggests that lyophilization of pulchellins should be avoided as the resuspension of dry protein probably leads to heterogeneity in the structure and activity of the protein samples.

Regarding the lectin properties of pulchellins, even though all isoforms are capable of binding to galactosides, the mechanisms of sugar interaction may vary. Haemagglutination inhibition assays on proteins, preincubated with several serially diluted sugars, showed that whereas agglutination was inhibited by

galactose and its derivatives (such as *N*-acetylgalactosamine (GalNAc), methyl-α-D-galactopyranoside), it was evident that at doses up to 100 mM, glucose, mannose, α-methylmannoside, fucose, maltose, xylose, and saccharose did not inhibit agglutination at all. The failure to bind to these sugars implies that an axial hydroxyl group at C4 is crucial and that a reversed configuration at this position might prevent sugar recognition. Besides, the haemagglutination inhibition caused by methyl-α-D-galactopyranoside suggests that the OH group on C2, C3, and C4, which displays the same configuration as those in galactose and lactose, is responsible for the strong interaction between this sugar and the four isoforms. Interestingly, P II is the only isoform with affinity for rhamnose, lacking the galactose and/or *N*-acetylgalactosamine specificity that is a characteristic feature of the archetypal type 2 RIP, for which there are few exceptions.

The most relevant difference regarding pulchellin–sugar interaction is related to the ability to bind GalNAc. For example, cytotoxicity assays, in which the four pulchellins were preincubated or not with free GalNAc, revealed that for P I and P II increased levels of cellular protein synthesis were seen as the concentration of premixed GalNAc was increased (Fig. 6). However, in contrast with the rescue of protein synthesis observed for P I and P II, GalNAc protection against P III and P IV was only marginal, even when toxin was pretreated with 100 mM GalNAc. That is, pulchellin isoforms P I and P II exhibit a remarkable difference in lectin-like properties compared with P III and P IV because in the presence of this sugar, both haemagglutination and cytotoxicity are differently inhibited. Moreover, these data suggest that the binding and uptake of P III and P IV into cells might not be dependent on receptors containing GalNAc.

On the basis of their toxicity and sugar-binding specificity, therefore, the four pulchellins can be roughly divided into two subgroups: P I and P II, which are more toxic and capable of binding to GalNAc; and P III and P IV, which are less toxic and incapable (or much less capable) of binding to GalNAc. The amino acid sequence alignment of the four isoforms (deduced from cDNA cloning and confirmed by mass spectrometry analysis) shows that the residues of the catalytic site are all conserved. In addition, the highest similarity within each subgroup is in the second domain of the B-chain (see alignment in Castilho et al. (2008)). These findings suggested that what dictates the different levels of toxicity for the two subgroups is the variation seen in the B-chain. That is, the two subgroups might interact with different binding sites on the cell surface, which might at least partially explain the differences in toxicity, given that the active site on the A-chain is conserved in both subgroups.

3 The Heterologous Expression of Pulchellins

Expression in *E. coli* of type 2 RIPs is an attractive strategy, since it may represent an unlimited source of protein (some RIPs are present in a seasonal dependent manner). Additionally, problems regarding differences on the expression patterns – which often occurs and may influence the reproducibility/reliability of the studies – may be overcome.

3.1 The Pulchellin A-Chain

In the 1990s, cloning of several type 2 RIPs, such as ricin and abrin, had already been obtained in several research laboratories (Wood et al. 1991; Roberts et al. 1992; Hung et al. 1994). Analysis of these genes showed that these RIPs belong to multigene families and had no introns. The latter information, in conjunction to the high sequence identity between abrin and ricin allowed the genomic cloning of prepulchellin A-chain (Silva et al. 2005).

In contrast to the recombinant production of the pulchellin B-chain (discussed latter), the A-chain is soluble when overexpressed as a GST-fusion protein in *E. coli* (Silva et al. 2005). The recombinant protein is able to depurinate rRNA in vitro releasing the Endo's fragment (defined below) after acid aniline treatment, as does native, reduced pulchellin, confirming that it was enzymatically active (Silva et al. 2005). It should be recalled that the evaluation of RIPs' enzymatic activity is based on RIP-mediated depurination of the large ribosomal subunit, which results in a susceptibility of the RNA to hydrolysis at the depurination site (Endo et al. 1987). This action leads to the release of a small fragment of nucleotides from the 3′-end of the rRNA (Endo's fragment).

It is known that the recombinant A-chain lacks the lectin-like activity. An effective method to check its toxic activity in cells or animals is via a reassembly the holotoxin by association of the single chains in vitro. Obviously, the protocols for the heterologous production of the B-chain or for its isolation from natural sources must already have been determined. The reconstruction of recombinant heterodimer of pulchellin has been shown to be feasible, and its resultant toxicity is comparable to that of the native molecule when injected into mice (Fig. 2, Silva et al. 2005). Without doubt, the toxic activity itself is accounted for solely by the A-chain. However, the full pulchellin intoxication pathway is clearly dependent on B-chain shuttling into mammalian cells, since neither isolated recombinant A- nor B-chains showed lethality in mice after intraperitoneal injection (Silva et al. 2005). Analogous reassociation of the A- and B-chains was also performed by Eck et al. (1999) for mistletoe lectin. The recombinant A-chain produced by this heterologous system is active and available for the preparation of toxic moieties of conjugates and immunotoxins with great potential as therapeutic agents.

3.2 The Pulchellin B-Chain

Only few studies involving isolated type 2 RIPs B-chains from recombinant expression sources have been reported (Tonevitsky et al. 1994; Pevzner et al. 2005; Chambery et al. 2007). This might be due to the difficulty in overexpressing RIP B-chain genes in *E. coli*. Pulchellin B-chain, which shares 81% sequential identity within the abrin-a isoform and up to 58% identity to ricin (including all key residues for proper folding and function), comes from a refolding process from

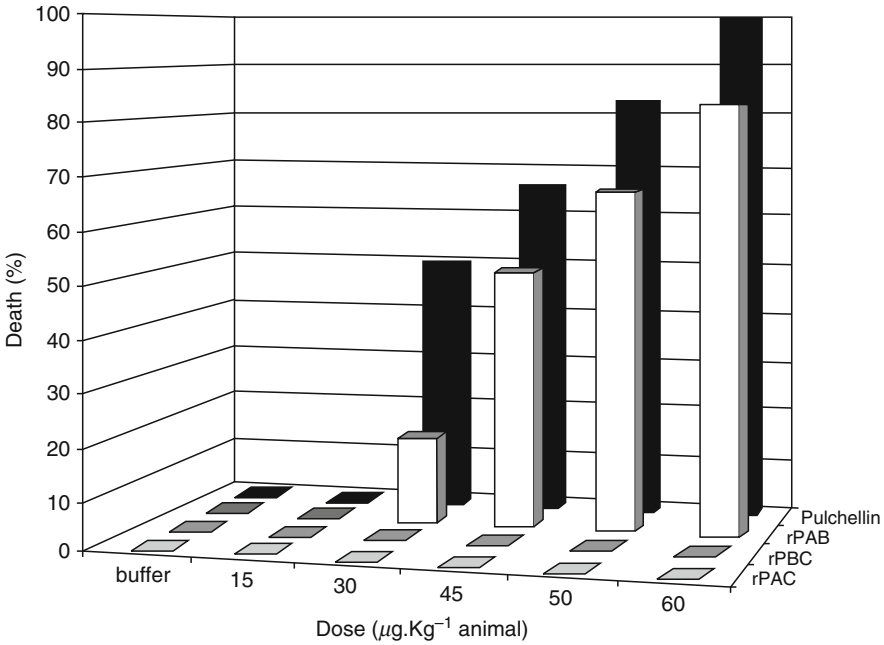

Fig. 2 Lethal activity determined by intraperitoneal injection in mice. Recombinant pulchellin A-chain (rPAC), recombinant pulchellin B-chain (rPBC), recombinant holotoxin pulchellin (rPAB), and native pulchellin were injected at the doses indicated. The buffers of each protein were used as negative control. Groups of six animals were used per dose of each protein preparation. The toxic effects were determined after 48 h. (Figure from Silva et al. 2005)

insoluble inclusion bodies (Goto et al. 2003) which may be considered a relatively difficult process. Although one might imagine that the refolding of the pulchellin B-chain may serve as a guideline for how to refold any similar type 2 RIP B-chain, the process of refolding may be affected by several factors (Maachupalli-Reddy et al. 1997; Tsumoto et al. 2003), many of which are empirical. Consequently, the pulchellin refolding protocol might not be generally applicable to every type 2 RIP B-chain. For the best results, it should be adjusted for each particular case. The problem of protein refolding is beyond the scope of the work discussed here, and there are plenty of refolding protocols available (Zardeneta and Horowitz 1994; Hashimoto et al. 1998; St John et al. 1999; Rariy and Klibanov 1999; Gu et al. 2001; Clark 2001; Tsumoto et al. 2003). What is expected to be true for most (if not all) type 2 RIP B-chains is that they are produced as insoluble inclusion bodies if overexpressed in a cytosolic *E. coli* system. This is due to the intrinsic nature of type 2 RIP B-chain structure which contains multiple disulfide bonds (Lilie et al. 1998; Kadokura et al. 2003). Attempts to obtain active recombinant type 2 RIP B-chains should focus on refolding protocols or changing the host expression system (Wales et al. 1991; Frankel et al. 1994; Sehnke et al. 1994; Sphyris et al. 1995; Frigerio et al. 1998; Chamberlain et al. 2008).

The refolding of recombinant pulchellin B-chain has been shown to be feasible and some characterization has been performed. The recombinant lectin chain was isolated as a monomeric polypeptide (free of a carrier peptide). Circular dichroism measurements were in agreement with the expected β-sheet-rich structure. Haemagglutination activity and inhibition of haemagglutination by the addition of D-galactose corroborated the expected lectin properties (Goto et al. 2003). The recombinant pulchellin heterodimer, reconstituted from individual recombinant expression of both chains, showed a very close median lethal dose (LD_{50}) in mice (45 μg/kg) to that found for a native toxin extract (30 μg/kg) (Silva et al. 2005).

One interesting point coming from the in vitro assembly of the holotoxin was the verification that the heterodimer retained characteristics very similar to those of the native proteins. Reconstituted pulchellin showed very similar toxicity, molecular masses, and CD spectra to that of the native protein (Figs. 2 and 3) (Silva et al. 2005). It is noted that the pulchellin B-chain, responsible for the holotoxin cell uptake, is harmless (at least for the test systems used so far) when administrated to living subjects. One potential advantage of the recombinant pulchellin B-chain over the native B-chain is that the former was free of glycans which are known to affect the applicability of other native RIPs for therapeutic purposes (Blakey and Thorpe 1988;

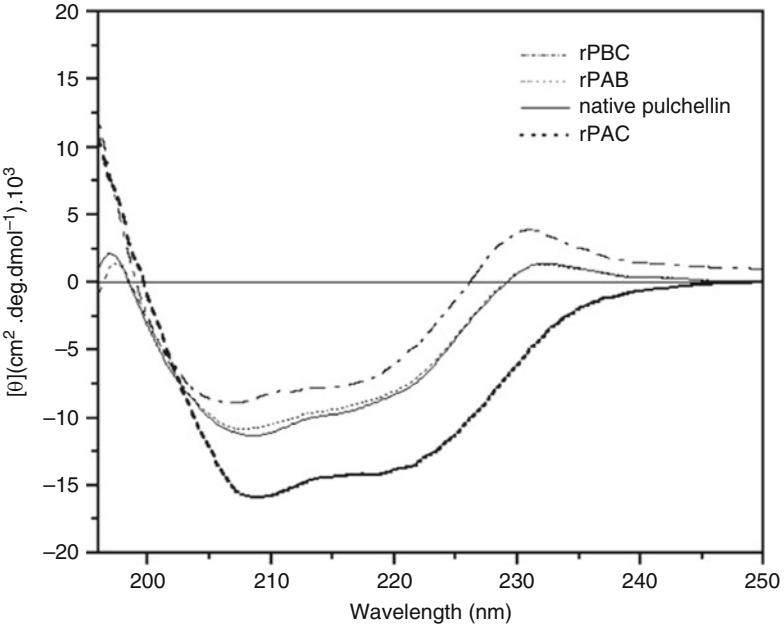

Fig. 3 CD spectra of recombinant pulchellin A-chain (rPAC), recombinant pulchellin B-chain (rPBC), recombinant pulchellin (rPAB), and native pulchellin. The spectra were obtained from each protein at a concentration of 0.3 mg/ml in 20 mM Tris–HCl, pH 8.0. The measurements were performed using quartz cuvettes of 1 mm path length and recorded from 195 to 250 nm as the average of 16 scans at 25°C. (Figure from Silva et al. 2005)

Jansen et al. 1992). The method used for coupling the recombinant chains results from the recombinant B-chain retaining a useful free cysteine residue (most likely the one nearest to the N-terminus) which may be available for coupling to other type of molecules.

4 Pulchellin Endocytosis in Mammalian Cells

The pulchellin B-chain could be used as a targeting molecule mediating cell uptake. This is based on the cell specificity displayed by recombinant pulchellin B-chain, a result of its lectin-like properties which guide it preferentially to given cell types via specific recognition of their carbohydrate composition. Indeed, recombinant pulchellin B-chain showed preferential cell type discrimination, leading to adhesion of MDA-MB-231 (human breast cancer) and K-562 (human bone marrow leukemia cancer) cell lines. This effect was not observed for mice fibroblasts (ATCC CCL-1.3) and human cervix adenocarcinoma (ATCC CCL-2) cells (Goto et al. 2007). On the other hand, native pulchellin, which includes a mixture of isoforms with different properties (Castilho et al. 2008), is able to interact with HeLa cells (ATCC CCL-2), in contrast to the recombinant refolded pulchellin B-chain, as the latter represent only one isoform that is unable to recognize external carbohydrate structure of HeLa cells. A series of chromatographic and surface plasmon resonance experiments has shown that native pulchellins have, besides β-D-galactose, remarkable affinity also for lactose, *N*-acetylgalactosamine and lacto-*N*-biose structures (Ramos et al. 2001).

Endocytosis of pulchellin B-chain should follow a very similar route to that described for other RIPs (Hartley and Lord 2004) as, in fact, this has been shown by confocal laser scanning microscopy subcellular localization experiments (Goto et al. 2007). Recombinant pulchellin B-chain (and also native pulchellin) were both found to accumulate in the region of the Golgi apparatus (Fig. 4), as would be expected if the endocytosis-ERAD retrograde transport mechanism is being used as has been claimed for all type 2 RIPs endocytosis/intoxication pathways (Hartley and Lord 2004; Roberts and Smith 2004).

Despite their similarity, a point of interest comes from the great variation in toxicity found within RIPs, which cannot be accounted for by structural variations or enzymatic activities of the A-chains (Barbieri et al. 2004). This is probably due to differences in B-chains, which may be more or less effective in the intoxication process (Svinth et al. 1998; Barbieri et al. 2004). In this respect, it should be pointed out that the LD_{50} in mice of around 45 μg/kg indicates that the recombinant pulchellin B-chain is a very effective RIP lectin chain, even without its natively attached carbohydrate which could account for an additional form of interaction important for cell entry (Simmons et al. 1986, Magnusson et al. 1993). The reasons for this come from structural features particular to this RIP B-chain and are subjects of current investigation.

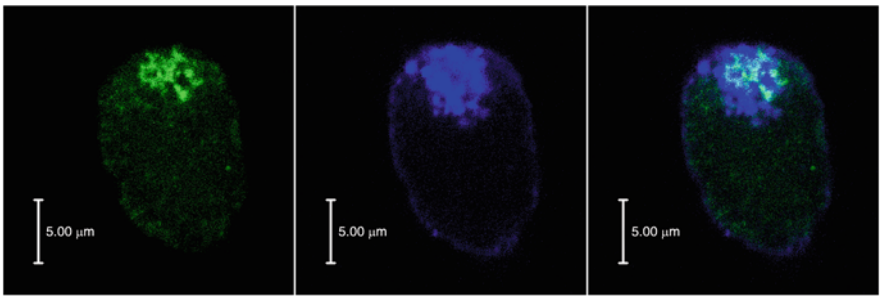

Fig. 4 Subcellular localization of pulchellin by confocal laser scanning microscopy. (**a**) K-562 cell fluorescently immunostained in *green* for golgin, a *trans*-Golgi protein. (**b**) Endocytosed fluorescently labeled pulchellin localization (*in blue*). (**c**) Overlaid images showing that pulchellin accumulated in the Golgi apparatus region (modified from Goto et al. (2007))

5 Structure of Pulchellin

The structure of pulchellin isoform P II (see below) was solved by X-ray crystallography (Fig. 5 (Navarro et al. unpublished)). Overall, the pulchellin fold resembles the structures of the well-known type 2 RIPs abrin, ricin, and mistletoe RIP, as indicated by the root-mean-square deviation calculated for the structurally equivalent Cα atoms (0.718, 1.131, and 1.031 Å for abrin, ricin, and mistletoe I, respectively). As expected, the structure is divided into two chains (the catalytic A-chain and the lectin B-chain) connected by a covalent disulfide bond between the Cys residue at the C-terminus of the A-chain and the Cys residue at the N-terminus of the B-chain (Cys240 and Cys263 respectively; numbering as Castilho et al. (2008)).

The catalytic A-chain (composed of 248 residues) possesses a globular fold and the *N*-glycosidase active site is located at the interface of three defined domains: the A1 domain, formed by the first 107 residues and mainly consisting of a six-stranded β-sheet and two α-helices; the A2 domain, formed by 86 residues organized into five α-helices, being the most conserved region of the A-chain (Bagaria et al. 2006); and the A3 domain, formed by 55 residues at the C-terminus organized as two α-helices, two antiparallel β-sheet strands and an unstructured coil (Fig. 5). All residues (Asn72, Tyr74, Tyr113, Arg124, Gln160, Glu164, Arg167, Glu195, Asn196, Trp198) involved in the catalytic mechanism of type 2 RIPs (Tahirov et al. 1995) are conserved in pulchellin.

The B-chains of type 2 RIPs fold into two globular domains, each one with at least one carbohydrate-binding site, which result in the agglutinating properties of the lectin chain (Rutenber et al. 1987; Sphyris et al. 1995). The B-chain of all the type 2 RIPs is thought to have emerged from a series of gene duplications, as each of the two globular domains seem to be built up from three similar ancestral peptides, named α, β, and γ (Rutenber et al. 1987; Rutenber and Robertus 1991).

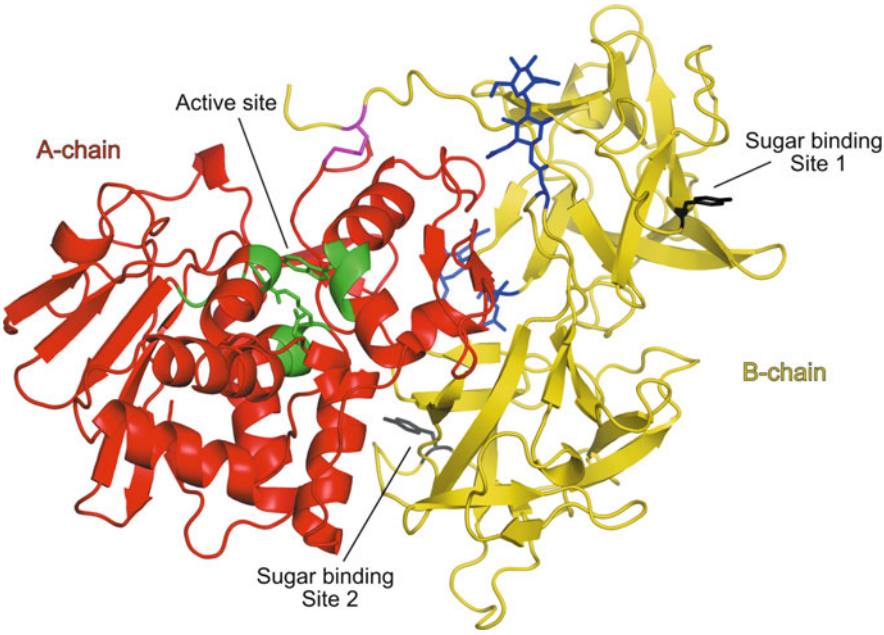

Fig. 5 Crystal structure of Pulchellin (isoform P II). Cartoon representation of the A-chain (*red*) and B-chain (*yellow*). The active site region is highlighted in *green* and the two conserved aromatic residues, which provide the binding platform for the sugar recognition, in *black*. The two glycosylations found in the B-chain are in *blue* and the disulfide bond connecting the catalytic and lectin chains is in *magenta*

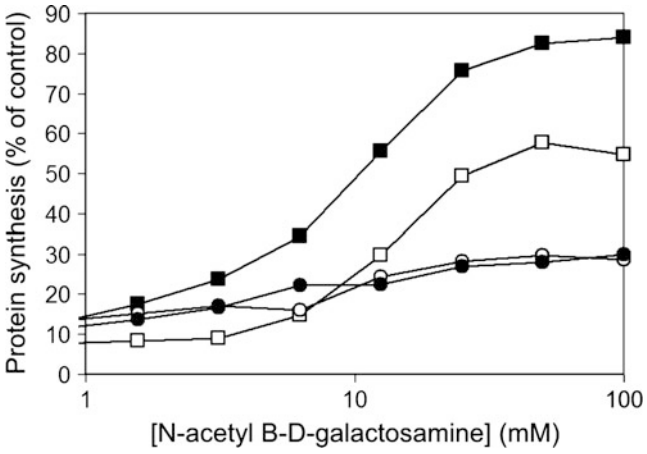

Fig. 6 Competition of pulchellin entry by *N*-acetyl-D-galactosamine. A single dose of toxin (200 ng/ml P I and P II or 800 ng/ml P III and P IV), previously shown capable of inhibiting 90% protein synthesis within 4 h, was used in all preincubations. Each toxin was mixed with increasing concentrations of GalNAc in DMEM/FCS for 30 min at 37°C. The mixtures were added to cells for 4 h and remaining protein synthesis determined as detailed in the Castilho et al. 2008. (Figure extracted from Castilho et al. 2008) (*open square*) P I; (*filled square*) P II; (*open circle*) P III; (*filled circle*) P IV

The pulchellin lectin chain, like other B subunits of type 2 RIPs, contains 256 amino acids arranged into two subdomains well conserved amongst this protein family, exclusively constituted of β-sheet regions interconnected by coiled structural elements. These subdomains contain the two galactose-binding sites. All the residues constituting the abrin B-chain carbohydrate-binding site 1 are conserved in the pulchellin B-chain: Asp274, Ile287, Tyr289, Asn298, and Gln299. Similarly, the residues of the abrin B-chain galactose-binding site 2 are conserved in pulchellin: Asp486, Ile498, Tyr500, Asn508, and Gln509. In both sites, the only one exception is the replacement of the aromatic residue that serves as the platform for sugar-binding (a Trp in abrin is replaced by Tyr in pulchellin). As in all type 2 RIPs, pulchellin also possesses *N*-glycans, and the crystal structure revealed that two (B-chain Asn347 and Asn387) out of the three predicted sites (Castilho et al. 2008) are indeed glycosylated.

6 Conclusion

Pulchellins have been described only recently. They are members of the highly toxic type 2 RIP family. Seeds of *A. pulchellus* present several different isoforms and four of them (PI, PII, PIII, and PIV) have already been isolated and studied in some detail. The toxicity values exhibited by the isoforms showed that they are less toxic than ricin and abrin, but nevertheless they are still highly toxic proteins. P I and P II are the most toxic isoforms and they are able to bind GalNAc; P III and P IV are about five times less toxic and not capable of binding to GalNAc. Taken together, an analysis of the sequence alignments and experimental results suggests that minor changes to the sugar-binding site domains could account for the differences in the isoform toxicities, since their catalytic sites of the A-chain and their ability to depurinate rRNA is conserved in both subgroups.

Additionally, the PII A-chain has been expressed in a heterologous system allowing for the production of a soluble and active toxic chain. This protein is available for the preparation of toxic moieties of conjugates and immunotoxins with potential for use as a therapeutic agent. The B-chain has also been overexpressed in a prokaryotic system and its refolding is feasible. Thus, it is possible to reconstruct the holotoxin from the recombinant A- and B-chains resembling the native protein in terms of toxicity and structure. The recombinant pulchellin B-chain has also the potential to be used as a cell targeting molecule and mediator of cellular uptake.

The crystal structure of at least one isoform from each subgroup would provide a deeper and more detailed understanding of the sugar-binding site. Moreover, the knowledge of the structure–function relationship of the residues interacting with galactosides would help outline the distinct structural features that are relevant for galactose binding and thereby the modulation of toxicity, which could help make them more appropriate for therapeutic use. Further studies comparing the different pulchellin isoforms may contribute to the understanding of how sugar binding is related to cell toxicity in type 2 RIPs.

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