Bacterial expression of biologically active recombinant musarmin 1 from bulbs of *Muscari armeniacum* L. and Miller

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This paper is dedicated to Manuel Ruiz Amil, Professor of Biochemistry and Molecular Biology at the Faculty of Pharmacy of the Complutense University in Madrid, Spain, on the occasion of his retirement.

Abstract

Musarmins are type 1 ribosome-inactivating proteins with *N*-glycosidase activity on the 28 S rRNA that are present in bulbs of *Muscari armeniacum* L. and Miller at rather low concentrations. In the present work, a cDNA fragment coding for musarmin 1 was sub-cloned and expressed in *Escherichia coli*. The recombinant protein (rMU1) was synthesised as a polypeptide of 295 amino acids that was delivered to the periplasm and processed. Recombinant musarmin 1 present in the periplam has two forms: insoluble with a molecular mass of 29,423 and soluble with a molecular mass of 29,117 because of a small proteolytic shortening with respect to the insoluble one, presumably in the C-terminal. The yield of protein homogeneous by polyacrylamide gel electrophoresis was 23 mg l^{-1} of bacterial culture. The recombinant musarmin 1 forms isolated from both the soluble and the insoluble (upon refolding) fractions retained full translational inhibitory and 28 S rRNA *N*-glycosidase activities as compared with the native protein. The recombinant protein displayed great stability towards trypsin, collagenase, rat plasma and rat liver protein extract, but was sensitive to the action of papain and proteinase K. The easy availability and full activity of the recombinant musarmin 1 makes it a good candidate for the preparation of immunotoxins for targeted therapy and for the construction of transgenic plants expressing it as antipathogenic agent.
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1. Introduction

Ribosome-inactivating proteins (RIPs) are translational inhibitors that catalytically and irreversibly inactivate sensitive ribosomes, thus arresting protein synthesis (Barbieri et al., 1993; Hartley et al., 1996; Girbés and Ferreras, 1998). The number of RIPs that have been characterized is increasing and these

Abbreviations: CTAB, cetyltrimethylammonium bromide; IPTG, isopropyl β-D-thiogalactoside; RIP, ribosome-inactivating protein; MU, musarmin

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compounds have been found in plants (Barbieri et al., 1993; Girbés and Ferreras, 1998), bacteria (Suh et al., 1998) and fungi (Wang and Ng, 2001), and recently a RIP-like activity has been found in mammals (Barbieri et al., 2001). Initially RIPs were found to display *N*-glycosidase activity on the large rRNA, which results in the release of the A_{4324} present in the highly conserved loop of mammalian ribosomes (Barbieri et al., 1993; Girbés and Ferreras, 1998). Nonetheless, some RIPs trigger rRNA multidepurination (Girbés and Ferreras, 1998). The current view is that RIPs are polynucleotide adenine glycosidases acting on both synthetic and natural RNA (Girbés et al., 1996b; Barbieri et al., 1996).

RIPs can be classified in two main categories according to their ability to bind sugars (Barbieri et al., 1993). Type 1 RIPs are composed of a single catalytic polypeptide chain with an apparent Mr range of 25,000–32,000. Type 2 RIPs are composed of two polypeptide chains; a catalytic A chain with *N*-glycosidase activity, which is equivalent to a type 1 RIP, linked by disulfide bonds to a B chain with sugar-binding ability that confers the whole RIP the nature of lectin. Type 2 RIPs may be classified as highly toxic like ricin and related toxins (Lord et al., 1994) and non-toxic as compared with ricin-like a nigrin b and ebulin l found for the first time in *Sambucus* spp. and related proteins (Ferreras et al., 2000; Girbés et al., 2003).

Despite the enormous efforts dedicated to researching RIPs, the biological role of these substances in plants remains to be elucidated. Notwithstanding, roles for them as anti-viral or anti-feedant agents have been proposed. The anti-viral hypothesis considers that homologous RIPs would trigger ribosome inactivation in infected cells and hence cell death (Bonness et al., 1994), and some evidence seems to support such a proposal. First, exogenous application of RIPs prevents infection by plant viruses containing either genomic DNA or RNA (Bolognesi et al., 1997; Girbés and Ferreras, 1998). Second, transgenic plants expressing RIP genes become more resistant to virus and fungi infection (Lodge et al., 1993; Jach et al., 1995; Krishnan et al., 2002). Third, two type 1 RIPs named beetins are induced in sugar beet upon infection by plant RNA viruses or following treatment of virus-free plants with mediators of the systemic acquired resistance of plants: either H_2O_2 or salicylic acid (Girbés et al., 1996a). Another interesting hypothesis suggests that some type 1 RIPs could act as a death signal in senescence (Stirpe et al., 1996; de Benito et al., 1998).

Interest in RIPs is increasing because of their use as a toxic moiety in the construction of immunotoxins (Barbieri et al., 1993; Girbés et al., 1996b; Kreitman, 2000; von Mehren et al., 2003) and conjugates for targeting therapy (Muñoz et al., 2001; Citores et al., 2002). In addition, many type 1 RIPs have found to be active against the HIV-1 (reviewed in Girbés et al., 1996b).

Success in the production of active recombinant RIPs has varied. In some cases, the recombinant protein was inactive or much less active than the native protein (Wu et al., 1998; Arias and Girbés, unpublished results). In contrast, others are so active that they strongly promote bacterial ribosome inactivation, thus inhibiting cell growth (Cho et al., 2000; Iglesias and Girbés, unpublished results). Accordingly, the availability of recombinant RIPs retaining full activity as compared with the native form is a desirable goal, especially in cases where the protein is scarce or where no plant material is available. It has recently been described that bulbs of *M. armeniacum* L. and Miller have a family of at least three RIPs: namely, musarmins 1–3 (Arias et al., 2003). Since all three proteins (MUs) are present in rather low amounts in the bulbs of *M. armeniacum* L. and Miller, it was decided to approach RIP production by recombinant expression of MU1. As reported here, rMU1 is produced in a form that is as active as the native MU1 in a homogeneous state and in high amounts. This could therefore be a suitable source for large-scale production and also for the construction of immunotoxins and conjugates for targeting therapy: cancer and AIDS, for example.

2. Materials and methods

2.1. Materials

Chemicals and biochemicals were obtained as described previously (Girbés et al., 1993; Citores et al., 1997; Citores et al., 1998). Pfu DNA Polymerase and *Escherichia coli* strain XL1-Blue were purchased from Stratagene. Luciferase T7 control DNA, T7 RNA Polymerase and the Luciferase assay reagent were obtained from Promega.

RNAguard Ribonuclease Inhibitor, SureClone Ligation kit, Superdex 75 Hiload column and SP-Sepharose gel were purchased from Amersham Pharmacia Biotech. The thermal cycler used in PCR amplifications was the Geneamp PCR system 2400 from Perkin Elmer. Oligonucleotides were synthesised by Amersham Pharmacia Biotech. Restriction endonucleases, Nitro Blue Tetrazolium (NBT), 5-Bromo-4-chloro-3-indolyl-D-galactopyranoside (X-phosphate) and proteases were obtained from Roche (Boehringer). Alkaline phosphatase-conjugated goat anti-rabbit IgG was purchased from Chemicon. BL21(DE3) and BL21(DE3)pLysS strains and pET-25b(+) plasmid were obtained from Novagen.

2.2. Construction of the expression plasmid encoding rMU1

The scheme for the construction of the expression plasmid is shown in Fig. 1. The plasmid containing the *MU1* cDNA (GenBank accession no. AF289118) was amplified by PCR using the primers 5′-GTC GCC ATG GCC GGT CAA GGC TTT-3′, which introduces a *Nco* I site at the N-terminus, and 5′AGT GAA AGG TGG CCT AAC GCA TCA AGC TTA AG3′, corresponding to the C-terminal sequence and which includes the *Hin*d III site present in the *MU1* gene overlapping the stop codon. The reaction mixture for amplification contained 100 ng of MU1 plasmid, 20 mM Tris–HCl, pH 8.75, 10 mM KCl, 2 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 100 μg ml^{-1} BSA, 0.2 mM dNTPs, 5 U of Native Pfu DNA Polymerase, and 0.5 μM of specific primers in a 100 μl reaction volume. After denaturation of DNA for 5 min at 95 °C, amplification was performed for 25 cycles through a regime of 45 s at 95 °C; 30 s at 60 °C, and 2 min at 72 °C. Analysis of PCR products was carried out by electrophoresis in a 1.5% (w/v) agarose ethidium bromide gel. The product was purified from the gel, sub-cloned into

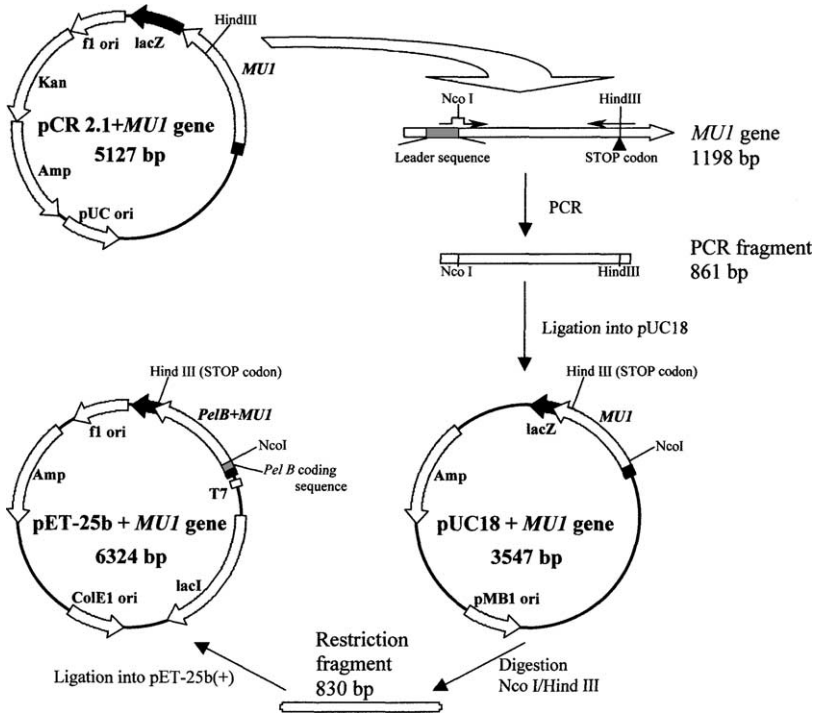

Fig. 1. Scheme for the construction of the expression plasmid. The cloning vector pCR 2.1 containing the *MU1* gene (1198 bp) was amplified by PCR to insert a *Nco* I restriction site in the 5′ end of the mature *MU1* coding sequence. The resulting fragment (861 bp) was subcloned into the pUC18 vector and sequenced. That controlled sequence was digested with *Nco* I and *Hin*d III and ligated to the expression vector pET-25b after the Pel B leader coding sequence. The resultant plasmid pET-25b + MU1 was then introduced into the expression *E. coli* strains BL21(DE3) and BL21(DE3)pLysS.

the pUC 18 vector using the SureClone Ligation kit, and fully sequenced to confirm that no changes had been introduced during the amplification step. The resulting plasmid was digested with *Nco* I and *Hin*d III and the MU1 fragment purified from the gel was sub-cloned between the *Nco* I and *Hin*d III sites of the expression vector pET-25b(+).

2.3. Expression of rMU1

Expression of rMU1 was induced essentially following the manufacturer's instructions (Novagen). The expression plasmid was introduced into the BL21(DE3) and BL21(DE3) pLysS strains and the transformant was grown at 25 °C in Luria–Bertani medium containing ampicillin (0.2 mg ml^{-1}) and chloramphenicol (34 μg ml^{-1}) to an optical absorbance of 0.8 at 600 nm. After induction with 1 mM isopropyl β-D-thiogalactoside at 25 °C for 6 h, the cells were harvested and the pellet was suspended in 0.05 vols. of 10 mM Tris–HCl, pH 8, and 150 mM NaCl. The cells were lysed by freeze/thawing and incubation for 5 min at 37 °C. The DNA was broken down by passing the extract several times through a 21-gauge needle. The supernatant collected after centrifugation represented the soluble protein fraction. The insoluble protein fraction was obtained as described elsewhere (Rajamohan et al., 1999). Briefly, the inclusion bodies were washed several times and suspended in buffer containing 1.5% (w/v) cetyltrimethylammonium bromide (CTAB). After removing insoluble materials by centrifugation, 20% (v/v) glycerol (final concentration) was added to the supernatant and this was slowly dialysed in 5 mM K_2HPO_4 (pH 8) to obtain biologically active protein.

2.4. Purification of rMU1

The soluble protein fraction was acidified to pH 4 with acetic acid and the precipitate was removed by centrifugation at 27,000 × *g* for 25 min at 4 °C. The supernatant was applied to a SP-Sepharose Fast Flow column previously equilibrated with 10 mM sodium acetate buffer (pH 4.5). The column was next washed first with the same buffer and then with 5 mM sodium phosphate buffer (pH 7.0). Protein was eluted at a rate of 2 ml min^{-1} with a 0–60 mM sodium chloride gradient in the same buffer. Protein fractions were tested by ELISA, using polyclonal anti-MU1 at a dilution of 1:2500 as primary antibody. The fractions selected were collected, dialysed against Milli-Q-purified water, and finally freeze-dried. The insoluble protein fraction, previously solubilised, was subjected to ion-exchange chromatography as described above. After ELISA the fractions selected were collected and concentrated with an Amicon YM10 membrane and applied to a Superdex 75 HiLoad column equilibrated with 0.4 M NaCl containing 5 mM Na-phosphate (pH 7.0). Protein was eluted with the same buffer at 2 ml min^{-1}. The protein contained in the major peak was dialysed against Milli-Q-purified water and finally freeze-dried.

2.5. Assay of cell-free protein synthesis

Rabbit reticulocyte lysate was obtained according to a standard procedure (Pelham and Jackson, 1976). Inhibition of protein synthesis was performed with a coupled transcription–translation in vitro assay using the rabbit reticulocyte lysate system essentially as described previously (Shih et al., 1998). The reaction mixture (8 μl) contained the following: 5 μl of rabbit reticulocyte lysate, 6.5 U ribonuclease inhibitor, 4 U T7 RNA polymerase, 0.25 μg Luciferase T7 plasmid, 0.4 mM rNTP's each, 2 μM protein amino acid each, 10 mM Tris–HCl (pH 7.8), 0.2 mM spermidine, 28 mM KCl, 1 mM $MgCl_2$, and nuclease-free water. The reaction mixtures were incubated at 30 °C for 10 min and then placed on ice. Following this, 2 μl of either water or different concentrations of the corresponding RIP were added and the samples mixture were incubated at 30 °C for 30 min. After this time, 25 μl water at room temperature was added and 25 μl of the resulting solution was removed and mixed with 25 μl of room temperature Luciferase Assay Reagent in a luminometer tube. The Luciferase activities of the samples were determined in a Luminova 1254 luminometer (BIO ORBIT) for 10 s with an initial delay of 2 s. To determine background luminescence, 2 μl of water was added to the reaction mixture and, performing a measurement immediately thereafter.

2.6. Western blot analysis

SDS-PAGE and electroblotting were carried out essentially as described elsewhere (Citores et al., 1998).

After blotting, the membrane (Immobilon P, Millipore) was blocked as indicated by the manufacturers and then treated for 1.5 h at 37 °C with TBS containing 0.5% BSA, 0.05% Tween 20 and polyclonal antibody anti-MU1 (dilution, 1:2500). After a wash in TBS–BSA–Tween solution, the membrane was incubated for 1 h at room temperature with the same solution but containing alkaline phosphatase-conjugated goat anti-rabbit IgG at a dilution of 1:7500. After a further wash, colour was developed by the addition of a freshly prepared solution containing NBT (0.385 mg ml^{-1}) and X-phosphate (0.188 mg ml^{-1}), after which the reaction was stopped by extensive washing with distilled water.

2.7. Protease treatment

Portions of 30 μg of rMU1 refolded from the insoluble fraction were subjected to different treatments in reaction mixtures containing: 3.75 μg of one of each protease (trypsin, papain, collagenase or proteinase K), 0.2 mg of rat liver protein extract prepared as described elsewhere (Girbés et al., 1993) or 0.7 mg of rat plasma, and buffer up to 50 μl. The control buffer was 5 mM sodium phosphate (pH 7.5) containing 100 mM NaCl. The buffers used for each protease treatment were those recommended by the protease manufacturers. The rat liver protein extract buffer was 20 mM Tris–HCl (pH 7.6) containing 150 mM KCl, 3 mM Mg-acetate and 5 mM dithiothreitol. Incubations were carried out for 13 h at 25 °C, except for proteinase K (30 min at 37 °C) and for papain (1 h at 25 °C). Finally, 250 ng of the treated rMU1 was analysed by Western blotting as indicated above. Variable amounts of treated rMU1 were analysed for inhibitory activity on translation as described above.

2.8. Analytical methods

Polyclonal primary antibodies were raised in rabbits using purified native musarmins as immunogens (Arias et al., 2003). ELISA analysis was carried out as described previously (Muñoz et al., 2001). 28 S rRNA *N*-glycosidase analysis was performed as indicated elsewhere (Citores et al., 1997). Protein concentrations were determined according to a standard procedure (Kalb and Bernlohr, 1977). Mass spectrometry analysis was performed on a matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometer as described previously (Arias et al., 2003).

3. Results

3.1. Expression of rMU1 in E. coli

We have recently cloned and analysed four genes from *M. armeniacum* coding for type 1 RIPs that we named musarmins (Arias et al., 2003). The gene encoding MU 1 was amplified by PCR using two specific primers designed to avoid the *MU* gene leader extension. The fragment was cloned into the expression vector adjacent to the vector-encoded 22 amino acid pelB leader sequence, which targets the recombinant protein (rMU1) to the periplasmic space, where an endogenous peptidase must cleave the signal peptide. The open reading frame encodes a sequence of 295 amino acids consistent with a molecular mass of 32,631. Accordingly, the mature rMU1 polypeptide should contain 273 amino acids and should have a molecular mass of 30,419.

For comparative purposes, the resulting recombinant plasmid was used to transform the two *E. coli* strains indicated in Section 2. Expression of the recombinant RIP was assessed by Western blotting. The extracts from the expression hosts showed similar levels of rMU1 expression in both the soluble and insoluble fractions (Fig. 2, inset). This indicated that either of both strains can be used and, therefore, we chose the *E. coli* BL21(DE3)pLysS strain (*E.coli* $rMU1^{+}$) as the most appropriate one since it expresses endogenous T7 lysozyme, which reduces the basal expression of toxic proteins and facilitates the preparation of cell extracts. As shown in Fig. 2, non-induced *E. coli* $rMU1^{+}$ cells grew like the *E. coli* harbouring the empty expression vector. The induction promoted a slight decrease in growth in both strains.

3.2. Purification and characterization of rMU1

Since MUs are strongly basic proteins, purification of rMU1 was performed by chromatography through SP-Sepharose Fast Flow. The insoluble fraction was refolded as indicated in Section 2 and was finally chromatographed through SP-Sepharose Fast Flow,

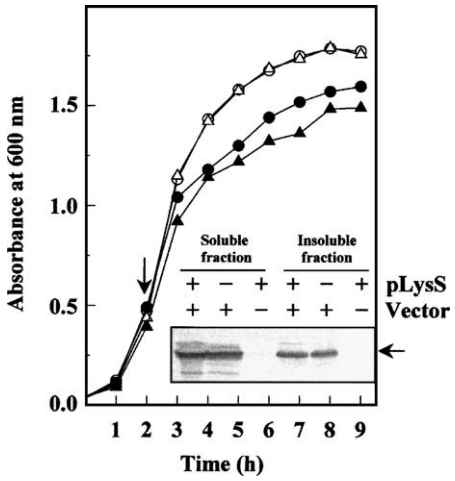

Fig. 2. Expression of rMU1 in *E. coli*. Time-course of bacterial growth of *E. coli* strain BL21(DE3)pLysS harbouring the expression vector with (triangle) or without (circle) the *MU1* gene. Open symbols indicate the bacterial growth of non induced-cells while closed symbols indicate the bacterial growth of *E. coli* after induction by IPTG. The vertical arrow indicates the addition of IPTG. Inset: Western blot analysis of bacterial extracts expressing the rMU1. pLysS indicates that the bacterial strain carries on the lysozyme gene. Vector indicates that the bacterial strain carries on the expression vector. The horizontal arrow indicates the position of the protein marker carbonic anhydrase (Mr 29,000).

yielding a peak with protein that was reactive with anti-MU1 antibodies (Fig. 3A). Further chromatography of the fractions from the highest part of the peak through Superdex 75 (Fig. 3B) yielded a SDS-PAGE homogeneous protein with an apparent Mr value of 28,700 (Fig. 3D). SP-Sepharose Fast Flow chromatography of the soluble fraction yielded several peaks. The fractions of peak 2 (Fig. 3C) reacted with anti-MU 1 antibodies and SDS-PAGE revealed a homogeneous protein with almost the same apparent Mr value as the insoluble form (Fig. 3A). To assess the molecular mass values of both forms of rMU1, mass spectrometry analysis was carried out. The values obtained were 29,423 for the insoluble form and 29,117 for the soluble one (data not shown). rMU1 displayed better cross-reactivity with rabbit anti-MU1 polyclonal antibodies than with those raised against MU2 and MU3 (data not shown). The yields of rMU1 were 3.5 and 20 mg l^{-1} of bacterial culture for the soluble and insoluble refolded forms, respectively.

3.3. Enzymatic activity

rMU1 strongly inhibited protein synthesis as assessed by the Luciferase biosynthesis assay. As shown in Fig. 4 (left), the soluble and the insoluble forms of rMU1 had IC_{50} (concentration of protein inhibitor that gives 50% inhibition of translation) values of 25 and 27 ng ml^{-1}, which match the value of 20 ng ml^{-1} obtained for the native MU1. Both forms of rMU1 displayed the *N*-glycosidase activity of the 28 S rRNA, which upon treatment with acid aniline splits the so-called diagnostic fragment characteristic of RIPs (Fig. 4, right).

3.4. Stability towards proteases

One feature of type 1 RIPs is their resistance to proteolytic degradation (Barbieri et al., 1993). Since type 1 RIPs are frequently glycosylated, it is possible that such resistance might be related to the presence of sugar chains in the native proteins. In fact, MU1 is glycosylated and is not attacked by either trypsin or chymotrypsin (Arias et al., 2003). Therefore, we investigated whether the absence of glycan chains in rMU1 might increase its sensitivity to proteolysis. As shown in Fig. 5, the rMUs remained stable after incubation with rat liver extract, rat plasma or collagenase. Incubation with trypsin shortened rMU1 very slightly, while incubation with either papain or proteinase K led to the disappearance of rMU1 from the gel. On the other hand, we found that the treatments of rMU1 with rat plasma, rat liver extract, collagenase or trypsin did not promote substantial changes in the translation inhibitory activity of the RIP (data not shown).

4. Discussion

In some cases, the expression of RIPs in bacterial systems affords proteins with a variable degree of activity (Wu et al., 1998; Cho et al., 2000). The cDNA of some type 1 RIPs has been expressed successfully and at a high level of expression (Rajamohan et al., 1999; Chi et al., 2001). In order to optimise the expression of recombinant anti-ribosomal protein, here we selected targeting to the periplasm, induction and growth at 25 °C to slow down cell growth, and used an *E. coli* strain to reduce the basal expression of

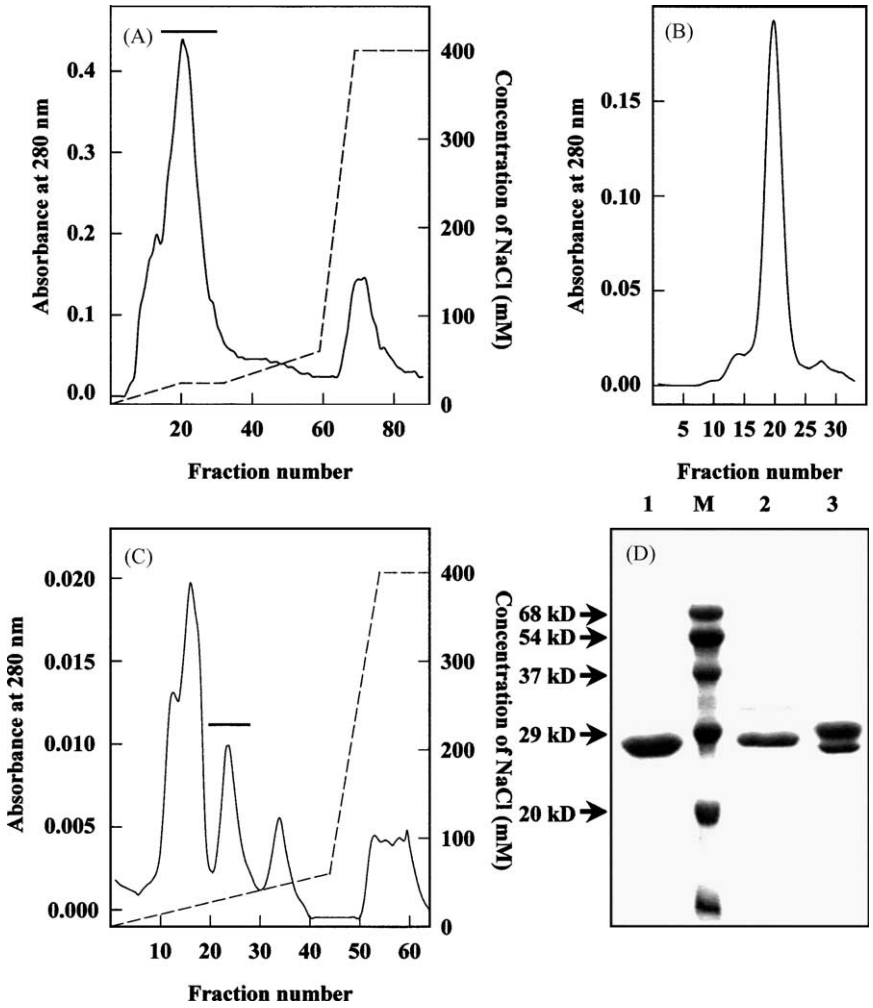

Fig. 3. Purification of rMU1. Panel A: Ion-exchange chromatography of insoluble and refolded rMU1. The insoluble fraction from *E. coli* cells expressing the rMU1 was solubilised and dialysed as described in Section 2. Folded proteins were then chromatographed on a SP-Sepharose column. The horizontal bar indicates the position of the rMU1 as detected by the ELISA test. Panel B: Gel filtration of rMU1. Fractions collected from the SP-Sepharose column (Panel A) were chromatographed on a Superdex 75 column as described in Section 2. Panel C: Ion-exchange chromatography of soluble rMU1. The soluble fraction from *E. coli* cells expressing the rMU1 was chromatographed on a SP-Sepharose column as described in Section 2. The horizontal bar indicates the fractions containing the rMU1 as determined by the ELISA test. Panel D: Analysis of rMU1 by SDS-Polyacrylamide (15% w/v) gel electrophoresis in the presence of 2-mercaptoethanol. Lane 1: rMU1 from the insoluble fraction. Lane 2: rMU1 isolated from the periplasmic soluble fraction. Lane 3: a mixture of native MU1 (Mr 28,700) and MU2 (Mr 30,000) isolated from bulbs. M: molecular-weight markers. Each lane contained 10 μg of protein.

recombinant proteins. A cDNA fragment coding for MU1 was expressed and the resulting protein (rMU1) was purified to homogeneity as judged by SDS-PAGE (Fig. 3D) with a very good yield and purity as compared with some other recombinant type 1 RIPs (Wu et al., 1998; Rajamohan et al., 1999; den Hartog et al., 2002). To improve the targeting of rMU1 to the periplasm, the native leader of MU1 was replaced by the very commonly used pectate lyase leader PelB. The construct was directed to the periplasm, where the leader was excised, as revealed by mass spectrometry. However, the true Mr of rMU1 was slightly lower

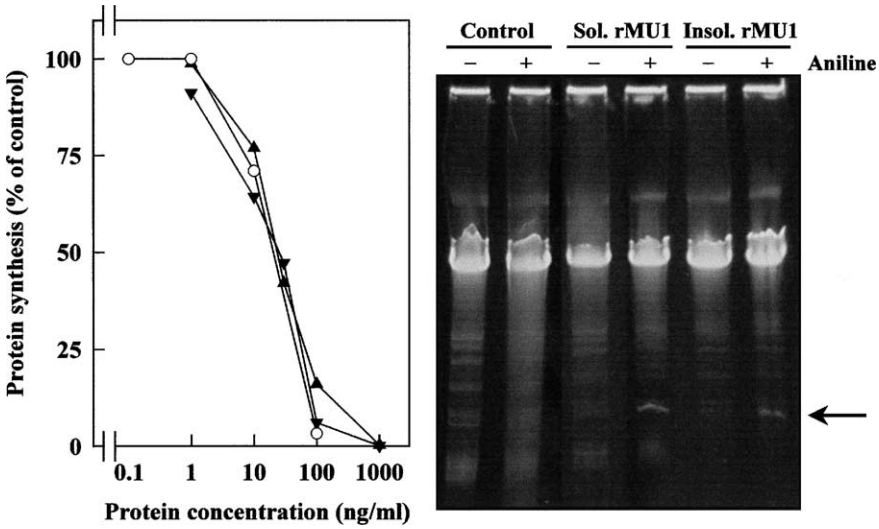

Fig. 4. Anti-ribosomal activities of rMU1. Left panel: Effects of MU1 and rMU1 on protein synthesis carried out by the rabbit reticulocyte lysate system. Symbols: (○) MU1; (▲) rMU1 purified from the soluble fraction; (▼) rMU1 purified from the insoluble fraction. A control was run in the absence of inhibitor. Right panel: 28 S rRNA *N*-glycosidase activity of type 1 RIP rMU1 purified from both the soluble and the insoluble fractions. Each lane contained 5 μg of total lysate RNA. The arrow indicates the diagnostic RNA fragment released upon treatment of RIP-treated lysates with acid aniline (Citores et al., 1997).

than expected, most probably because of further proteolytic processing, consisting of the excision of nine amino acids (data not shown). Although we cannot offer any explanation for this, a similar complementary processing affecting a fragment higher than the leader has recently been described for MU1 in *M. armeniacum* (Arias et al., 2003). Part of the rMU1 secreted to the periplasm was entrapped as protein bodies, thus hindering further proteolytic degradation, and a minor part of rMU1 was maintained as soluble protein, which underwent a slight degradation, accounting for three amino acids as assessed by mass spectrometry

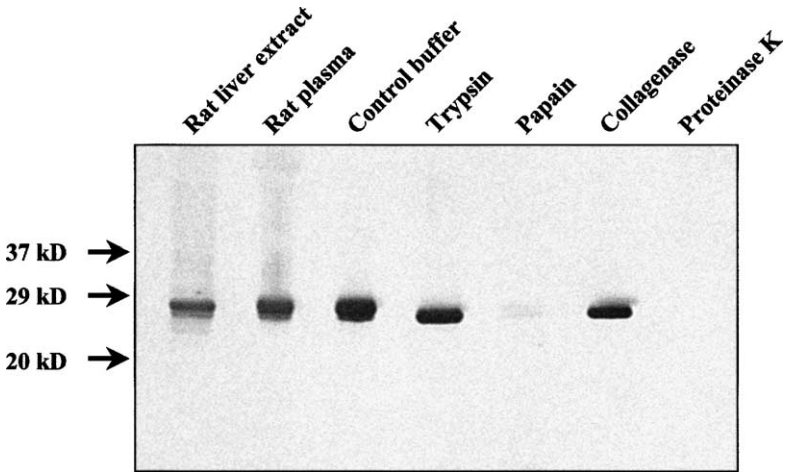

Fig. 5. Stability of rMU 1 towards proteases. rMU1 was treated with proteases and mammalian extracts and then analysed by Western blotting to ascertain potential degradation, as described in Section 2.

(data not shown). Another reason to obtain recombinant proteins from the periplasm is that this extracellular space accumulates only few of the cell proteins. Therefore, targeting rMU1 to the periplasm should allow easier purification as compared with MUs (Arias et al., 2003), the concentration of the recombinant protein, and the avoidance of intracellular degradative enzymes: examples of the latter would be proteases and nucleases that might affect the stability and action of the recombinant protein for its use in the construction of therapy conjugates and immunotoxins.

Analysis of the enzymatic activity essential to RIPs—namely, depurination of the 28 S rRNA, revealed that rMU1 is as active as MU1 (Arias et al., 2003). Furthermore, the rMU1-dependent inhibition of translation, a consequence of such *N*-glycosidase activity, strongly resembled that found for the native protein, thereby suggesting that the glycan chains present in MU1 do not contribute to the enzymatic activity. This represents a clear advantage over native glycosylated RIPs, since usually the sugar part of the proteins elicits immunological responses that trigger the neutralization of the corresponding RIP-containing immunotoxin or conjugate, thus reducing their effectiveness in cancer patients (Szatrowski et al., 2003). Taking into account the immunological response because of the appearance of anti-RIP antibodies, a number of different RIPs would probably be available for targeting therapy.

The disadvantage of entrapping rMU1 within the insoluble protein bodies may be overcome by solubilization and refolding. In contrast to a number of recombinant RIPs that display varying degrees of activity, the insoluble form of rMU1 recovered was fully active as compared with both the soluble rMU1 and the native MU1. The yields of rMU1 are among the most striking reported to date and can easily be scaled up.

Active recombinant RIPs may be used for the preparation of both chemical and recombinant immunotoxins. Preliminary experiments indicated that derivatization of rMU1 with succinimidyl-pyridyl-dithiopropionate, commonly used to link a RIP to a carrier protein, i.e. a monoclonal antibody (Girbés et al., 1996b; Kreitman, 2000; von Mehren et al., 2003), or transferrin (Citores et al., 2002) does not impair the enzymatic activity of rMU1. Therefore, this protein may be used to build the above-mentioned chemical constructs for targeting therapy.

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