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RESEARCH PAPER

Molecular characterization and systemic induction of single-chain ribosome-inactivating proteins (RIPs) in sugar beet (*Beta vulgaris*) leaves

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Abstract

Sugar beet (*Beta vulgaris* L.) leaves contain virus-inducible type 1 (single chain) ribosome-inactivating proteins that have been named beetins. The structural and functional characterization, the cellular location, and the potential role of beetins as antiviral agents are reported here. Beetins are formed of a single polypeptide chain with a varying degree of glycosylation and strongly inhibited *in vitro* protein synthesis in rabbit reticulocyte lysates (IC_{50}=1.15 ng ml^{-1}) and a *Vicia sativa* L. cell-free system (IC_{50}=68 ng ml^{-1}) through the single depurination of the large rRNA. Beetins trigger the multidepurination of tobacco mosaic virus (TMV) genomic RNA which underwent extensive degradation upon treatment with acid aniline. Beetins are extracellular proteins that were recovered from the apoplastic fluid. Induction of sugar beet RIPs with either H_2O_2 or artichoke mottled crinkle virus (AMCV) was observed in leaves distant from the site of application of such elicitors. The external application of purified beetin to sugar leaves prevented infection by AMCV which supports the preliminary hypothesis that beetins could be involved in plant systemic acquired resistance subjected to induction by phytopathogens.

Key words: Beetin, *Beta vulgaris*, protein synthesis inhibitor, ribosome-inactivating protein, sugar beet.

Introduction

Ribosome-inactivating proteins (RIPs) are catalytic translation inhibitors that are present in a number of plants and some bacteria and that act by arresting protein synthesis by eukaryotic and, in some cases, prokaryotic ribosomes (Barbieri *et al.*, 1993; Hartley and Lord, 2004*a*, *b*; Stirpe, 2004). On the basis of the available evidence, they have been proposed as anti-pathogenic proteins (Barbieri *et al.*, 1993; Hartley *et al.*, 1996; Wang and Tumer, 2000; Nielsen and Boston, 2001; Park *et al.*, 2002; Hartley and Lord, 2004*a*, *b*; Stirpe, 2004). Interest in RIPs is currently increasing due to their use as the toxic moiety of immunotoxins and conjugates for the experimental therapy of cancer (Barbieri *et al.*, 1993; Girbés *et al.*, 1996*a*, 2003; von Mehren *et al.*, 2003).

RIPs may be classified as type 1 and type 2 RIPs. Type 1 RIPs are single-chain proteins with N-glycosidase activity and are the most widely distributed proteins (Barbieri *et al.*, 1993; Hartley and Lord, 2004*a*, *b*; Stirpe, 2004). Type 2 RIPs are two-chain proteins linked by disulphide bonds with an A chain, functionally identical to a type 1 RIP, and a B chain, which is a lectin and is usually specific for D-galactose and derivatives (Barbieri *et al.*, 1993). In recent years, *Sambucus* species have been found to contain a complex mixture of type 1 and a special type 2 RIPs (Girbés *et al.*, 2003). The *Sambucus* type 2 RIPs are noteworthy in that they are several orders of magnitude less toxic to animals than ricin (Lord *et al.*, 1994), and are therefore referred to as non-toxic type 2 RIPs. Among these proteins ebulin l (Girbés *et al.*, 1993*b*) and nigrin b (Girbés

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et al., 1993*a*) are the best known. To date some other non-toxic type 2 RIPs have been found (Stirpe, 2004).

Concerning the mechanisms of action, RIPs inactivate ribosomes via the single depurination of the large rRNA which, upon treatment with acid aniline, releases what is known as the RIP diagnostic fragment (Barbieri *et al.*, 1993). Some RIPs also act on nucleic acid other than rRNA, such as salmon sperm DNA (Barbieri *et al.*, 1994, 2004) or genomic viral RNA (Barbieri *et al.*, 1994; Girbés *et al.*, 1996*b*), and even on synthetic polynucleotides, thus leading to their proposal as polynucleotide: adenosine *N*-glycosidases (Barbieri *et al.*, 1996).

RIPs have been proposed as antiviral agents in plants (Taylor *et al.*, 1994; Hong *et al.*, 1996; Krishnan *et al.*, 2002). In this sense, it has been shown that RIPs display preventive effects on tobacco mosaic virus (TMV) infection and propagation upon external application (Taylor *et al.*, 1994). On the other hand, transgenic tobacco plants carrying the gene coding for a type 1 RIP, such as pokeweed antiviral protein (PAP), display resistance to viral infection through the expression of very low amounts of PAP due to toxicity problems (Lodge *et al.*, 1993). Regarding the type 2 RIPs, the type 2 RIP SNA I′ exhibits *in planta* antiviral activity in transgenic tobacco (Chen *et al.*, 2002).

A number of reports indicate that many RIPs are inducible by different events such as senescence (Stirpe *et al.*, 1996), mechanical stress (Song *et al.*, 2000), and environmental stress (Rippmann *et al.* 1997). Both viral infection and molecular mediators of viral infection such as H_2O_2 and salicylic acid trigger the expression of two single-chain RIPs named beetins 27 and 29 (Girbés *et al.*, 1996*b*). A salicylic-independent systemic induction of type 1 RIPs has also recently been described (Song *et al.*, 2000; Zoubenko *et al.*, 2000). In this work, the molecular and functional characterization of beetin (BE) is reported. A cDNA fragment containing what seems to be BE has previously been cloned and expressed in *Escherichia coli* and the corresponding protein was named betavulgin (Hornung *et al.*, 1996). As shown here, the sugar beet RIP is an extracellular type 1 RIP that is induced at sites distant from the point of application and, once applied externally, it protects sugar beet leaves against further viral infection.

Materials and methods

Materials

Current chemicals and biochemicals were of the highest purity available and were from sources described previously (Arias *et al.*, 1992, 1993, 1994). Immobilon membranes were purchased from Millipore Ibérica (Madrid, Spain). [^{35}S]Promix (1114 Ci mmol^{-1}) and L-[^{3}H]valine (sp. act. 33 Ci mmol^{-1}) were purchased from Amersham Biosciences Europe GmbH (Barcelona, Spain). The FPLC system and all chromatographic supports and columns were purchased from Amersham Biosciences Europe GmbH (Barcelona, Spain). Polyclonal rabbit anti-BE 27/29 antibodies were prepared and purified according to standard protocols as described by Citores *et al.* (1994, 1996). Sugar beet (*Beta vulgaris* L. spp. *vulgaris*) leaves infected in the field with beet mild yellowing virus (BMYV) were collected and stored at −20 °C until needed. Control sugar beet plants were grown in the laboratory under controlled temperature and humidity conditions to give maximal growth without pathogen contamination.

Isolation of BE from field-grown virus-infected sugar beet leaves

BE from sugar beets was isolated following a general procedure for the isolation of single-chain RIPs (Arias *et al.*, 1994). Sugar beet leaves were ground and extracted essentially as described previously. 300 g of sugar beet leaves infected with BMYV harvested in infected fields were cut into small pieces and then ground in a blender and extracted overnight with 2.4 l of 5 mM sodium phosphate (pH 7.4) buffer containing 140 mM NaCl. The extract was acidified to pH 4 with glacial acetic acid and the resulting suspension was centrifuged at 14 300 *g* for 45 min at 0 °C and defatted by filtration through a cheesecloth. This extract was chromatographed with SP-Sepharose Fast Flow (5×4.8 cm) equilibrated with 10 mM Na-acetate (pH 4). The unbound material was eluted, the column was washed, and the retained protein was eluted with 5 mM sodium phosphate (pH 7.4) buffer containing 0.5 M NaCl. The protein solution was dialysed overnight against water and was further chromatographed with SP-Sepharose Fast Flow using a linear gradient of 30–200 mM NaCl in 5 mM sodium phosphate (pH 7.4) buffer. Protein fractions were assayed for protein synthesis inhibition as described elsewhere (Arias *et al.*, 1994). The fractions inhibiting protein synthesis were pooled and subjected to chromatography with Superdex 75 HiLoad 26/60. The protein peaks showing inhibitory activity on protein synthesis were pooled and dialysed against water.

Induction of BE by treatment of sugar beet leaves with elicitors

Treatment of leaves from laboratory-grown sugar beets with either H_2O_2 or salicylic acid was carried out by spraying dilute solutions (5 mM) every 24 h over 3 d. Crude protein extracts from beet leaves were prepared by grinding 1 g of control or either H_2O_2- or salicylic acid-treated leaves in a mortar with liquid nitrogen and 100 mg of the resulting powder were extracted overnight at 4 °C with 10 vols of a solution containing 140 mM NaCl and 5 mM sodium phosphate (pH 6.6). Then the extracts were centrifuged at 13 000 rpm and the clarified supernatant was stored at −20 °C until needed.

Protein synthesis in cell-free translation systems and in HeLa cell cultures

Cell-free translation was carried out with rabbit reticulocytes, rat liver, *Vicia sativa* L. and *Triticum aestivum* L. lysates prepared in the laboratory and described by Girbés *et al.* (1993*b*) and Arias *et al.* (1993). Protein synthesis in HeLa cells was studied using 1 μCi μl^{-1} of [^{35}S]Promix as detailed by Citores *et al.* (2002). The inhibitory effects were represented as the IC_{50} value, which is defined as the amount of inhibitory protein that gives 50% of inhibition of protein synthesis.

Molecular cloning from cDNA and genomic DNA fragments encoding BE and expression of recombinant BE in Escherichia coli

The cDNA coding for BE was amplified by PCR using two oligonucleotides deduced from the betavulgin sequence (Hornung, 1996) namely NBA [5′-GTAGTTTATGCACCATGGGGGCAGA-TGTAACTTTT-3′] as the N-terminal primer that contains a

restriction site for *Nco*I and XB [5′-CACAAGTAATTAGTCGAG-CTAAGGTACATAGCTTAGGATTCC-3′] as the C-terminal primer that contains a restriction site for *Xho*I. This primer was designed with a stop codon at the end of the coding region. The amplified DNA was inserted into the *E. coli* expression vector pET25(b+) at the 3′ position with respect to the *Pel*B signal sequence. The expression of the recombinant protein was carried out by transformation with pET-B into the *E. coli* strain BL21(DE3)pLys, which contains the T7 lysozyme gene. A transformant colony was grown overnight at 37 °C in LB medium supplemented with ampicilin (200 μg ml^{-1}) and 1% (w/v) glucose. The expression of the recombinant protein was induced by adding IPTG to the medium at a concentration of 0.4 mM when the OD_{600} of the culture reached 0.4. *E. coli* cells were harvested after 6 h of incubation and suspended in 0.2 vols of buffer containing 10 mM TRIS–HCl (pH 8.0) and 150 mM NaCl and frozen at −20 °C overnight. The lysogeny of the cells was promoted by thawing at 37 °C, followed by incubation at 22 °C for 5 min. The cell lysate was passed through of a syringe to break the DNA. The cell lysate was then centrifuged at 27 000 *g* for 30 min at 4 °C and the soluble and insoluble fractions were separated through a syringe. Genomic DNA was purified from leaves of *Beta vulgaris* using a 'Genomic Prep-Cell and tissue DNA isolation Kit' (Amersham Biosciences Europe GmbH, Barcelona, Spain). The PCR product of the genomic beetin sequence was obtained using two primers corresponding to the 3′ and 5′ terminal sequence of the cDNA cloned previously (Hornung *et al.*, 1996).

28 S rRNA N-glycosidase assay

The N-glycosidase activity of BE was assayed in 100 μl samples of rabbit reticulocytes lysate, 100 μl of S-30 *Vicia sativa* lysate (Arias *et al.*, 1992, 1993), or 5 μg of TMV RNA, which were incubated with the corresponding protein: either purified BE or total proteins from the soluble fraction containing the recombinant BE. After treatment, the RNA was analysed by extraction, phenolization, ethanol precipitation, and RNA electrophoresis as described elsewhere (Girbés *et al.*, 1993*b*). The depurination assay of the 23 S rRNA of *E. coli* BL21(DE3)pLysS-pETB that expresses the recombinant beetin (rBE) was carried out by the treatment of 5 μg of the total bacterial RNA with aniline and electrophoresed as indicated above.

Deglycosylation of BE forms and mass spectrometry analysis

Deglycosylation of BE forms was carried out as follows: 1 mg of a lyophilized mixture of BE forms (peak I in Superdex 75) was reacted with 2.5 ml of HF acid in a Kal-F reactor for 3 h at 20 °C. Then, the acid phase was evaporated off and the protein was dissolved in 5 ml of water. After lyophilization, the protein was dissolved in 1 ml of a solution of 0.1 M acetic acid. One aliquot of 1 μl of this solution was analysed by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) using a spectrometer (Voyager-DE STR, Biospectrometric Work station, Applied Biosystems). The matrix used was sinapinic acid and this was calibrated externally with enolase as standard.

Other procedures

N-terminal amino acid analysis and SDS–PAGE of proteins were carried out as described by Arias *et al.* (1994). Western blot analysis was carried out as described by Citores *et al.* (1998). The presence of glycan chains in BE was studied using the Glycan Detection Kit from Boehringer Ingelheim España SA (Barcelona, Spain). The infection of *Beta vulgaris* with the artichoke mottle crinkle virus (AMCV) was carried out using a crude extract of *Nicotiana clevelandii* previously infected with the same virus essentially as described elsewhere (Tavladoraki *et al*., 1993).

Results

Isolation, characterization and N-terminal amino acid sequence of BE

Sugar beet leaves highly infected with viruses harvested in the field were used. Dialysed crude protein extracts were concentrated by chromatography through SP-Sepharose and further resolved by SP-Sepharose chromatography using a 30–200 mM NaCl gradient. Fractions were collected and assayed for the inhibition of protein synthesis in a rabbit reticulocytes lysate system. As shown in Fig. 1A, two zones of the gradient contained strong inhibitory activity at a dilution of the crude protein extract of 1:5000. At a dilution of 1:50 000 the inhibition was only seen in those fractions corresponding to peak II, which seems to concentrate the inhibitory activity. The fractions corresponding to each peak were pooled and subjected to a second chromatography step

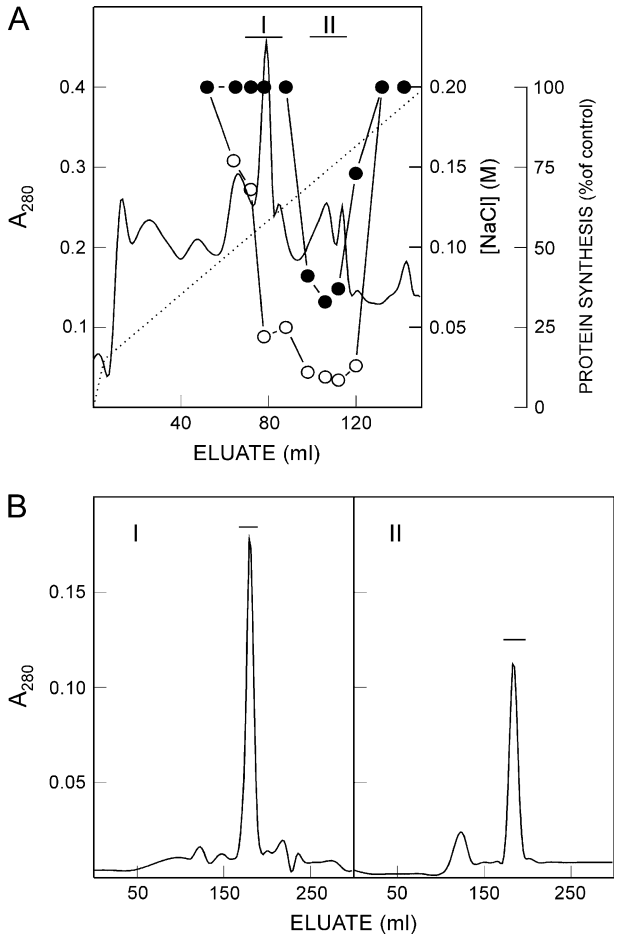

Fig. 1. Isolation of BE 27 and 29 type 1 RIPs by SP-Sepharose FF (A) and gel filtration (B) chromatography. (A) SP-Sepharose FF chromatography was performed as indicated in the Materials and methods. (B) Fractions I and II from the previous chromatography were subjected to Superdex 75 HiLoad 26/60 chromatography. Horizontal bars indicate the fractions that inhibited protein synthesis and that were pooled and subjected to SDS–PAGE analysis. Symbols: A_{280} (line), [NaCl] (dotted line), inhibition of protein synthesis at 1:50 000 (filled circle) and 1:5000 (open circle) dilutions.

by gel filtration through Superdex 75 HiLoad 26/60. The protein pool corresponding to each peak contained major sharp bands which correspond to the inhibitory proteins (Fig. 1B), as will be shown later. SDS–PAGE of proteins in peaks I and II, either in the presence or in the absence of 2-mercaptoethanol (2ME), indicated that peak I protein purified by the gel filtration step contained a band protein with an apparent M_r of 27 000 and a minor band with an apparent M_r of 29 000 (Fig. 2A) and peak II protein only contained the protein with M_r 27 000. For comparative purposes, a type 1 RIP, i.e. PAP (Irvin, 1983), whose position in the gel is only slightly affected by the presence of 2ME, and a type 2 RIP, i.e. nigrin b (Girbés *et al.*, 1993*a*), which in the presence of 2ME is dissociated into its two subunits, were also run. It was not possible to separate both forms BE27 and BE29 in peak I by mild methods, most probably because both forms represent different states of glycosylation of the same polypeptide chain.

As shown in Fig. 2B, mass spectrometry analysis revealed that BE27 has a molecular weight of 27 592 (Fig. 2Ba), which fits well with the value of 27 000 obtained by SDS–PAGE. The mixture of BE27 and BE29, corresponding to peak I in Fig. 1, has a mixture of native proteins with molecular weights ranging from 27 606 to 29 802 (Fig. 2Bb). Chemical treatment of the mixture to promote deglycosylation cleaved the polypeptide chain between Pro92 and Asp93, giving two fragments with M_r values of 10 141 and 17 445, respectively (Fig. 2Bc). It is assumed that the MALDI/TOF-MS technique has a mass error in the range of 0.1–0.5% (e.g. up to a 100 Da error for a 20 000 Da protein) (Hillenkamp *et al.*, 1991). Therefore the small differences in the mass spectrometry values obtained here fall within the error of the technique. To assess whether BE29 and BE27 are related proteins, their N-terminal amino acid sequences were analysed. Both proteins show the sequence H_2N-ADVTFDLETASKTKYGTFLSNLRNI ... which

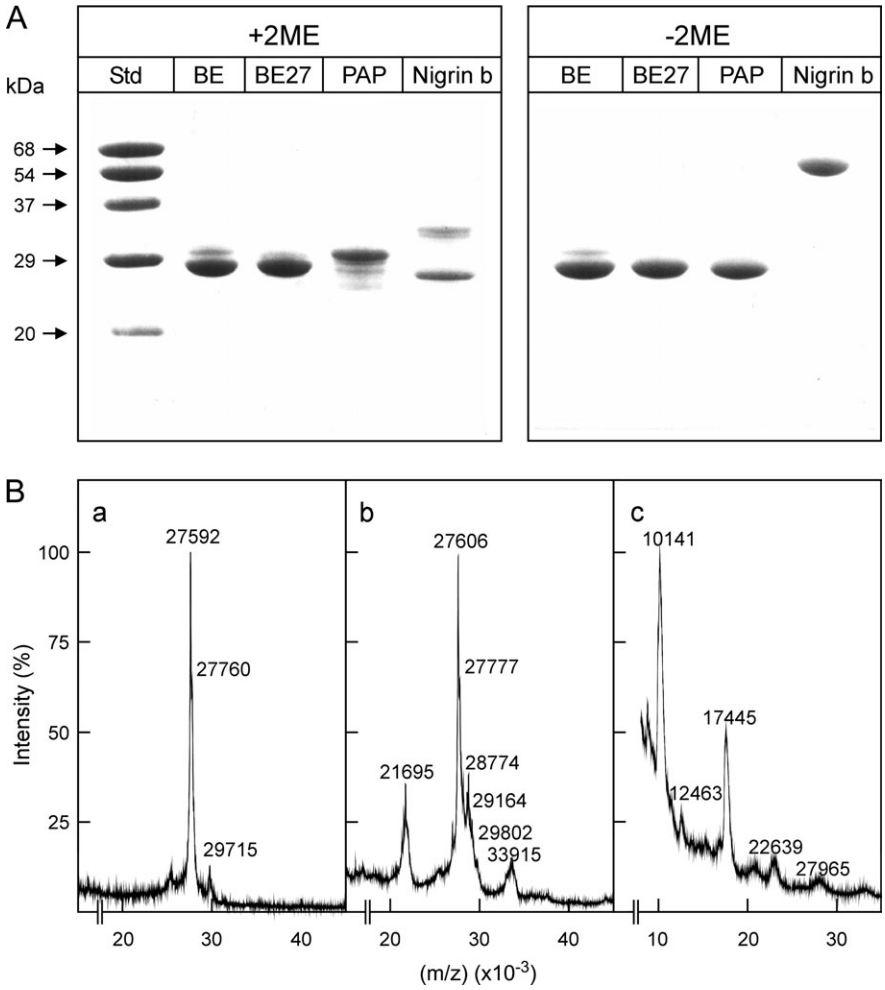

Fig. 2. Structural analysis of BE. (A) Analysis of BEs by SDS-PAGE: protein fractions (13 μg per well) from Superdex 75 26/60 HiLoad chromatography were electrophoresed in the absence or the presence of 2ME. (B) MALDI-TOF mass spectrum obtained from BE27 and BE29: protein peaks were assigned molecular weights using cytochrome *c* and bovine serum albumin as external standards; a, native BE27; b, a mixture of BE27 and BE29 as eluted in peak I; c, deglycosylated protein from the mixture in (b).

indicates that they are closely related. In addition, both proteins were found glycosylated (data not shown).

Depurinating activities of BE on mammalian and plant ribosomes and TMV RNA

Studies of the inhibition by BE27 of protein synthesis indicated that the most sensitive cell-free system was that of rabbit reticulocyte lysates (IC_{50}=1.15 ng ml^{-1}). The IC_{50} values for rat liver, *Vicia sativa* L., and *Triticum aestivum* L. cell-free systems were 68, 617, and 1318 ng ml^{-1}, respectively. By contrast, cultured HeLa cells proved to be very insensitive to BE (IC_{50} >100 μg ml^{-1}) unlike its sensitivity to the highly toxic type 2 ricin (IC_{50}=0.07 ng ml^{-1}).

As shown in Fig. 3, BE was able to depurinate both mammalian and *V. sativa* rRNA which, upon treatment with acid aniline, released RNA fragments that are diagnostic for RIP action on ribosomes (Barbieri *et al.*, 1993). The potential effects of BE on TMV genomic RNA was investigated further. As also shown in Fig. 3, BE promoted an extensive depurination of TMV genomic RNA which, upon treatment with acid aniline, led to the complete degradation of the polyphosphate RNA backbone. According to this study's results the efficiency of BE seemed to be of at least the same order on both rabbit and *V. sativa* ribosomes as that on TMV genomic RNA. This direct action of BE on genomic viral RNA is in agreement with previous data reported for other RIPs (Barbieri *et al.*, 1996).

Molecular cloning of genomic DNA encoding for BE and the presence of introns

Samples of sugar beet DNA were isolated from leaves and amplified by PCR using primers corresponding to the 5′ and 3′ terminal sequences of a cDNA cloned previously (Hornung *et al.*, 1996). The PCR product was cloned into the PCR 2.1 vector, which was propagated and sequenced by the dideoxy method. The genomic DNA clone has no introns in the BE gene and contained an ORF encoding a protein of 149 amino acids accounting for a theoretical M_r of 27 557 (data not shown). This is consistent with the apparent M_r of BE27 calculated from the SDS-PAGE analysis (Fig. 2A). The amino acid sequence contains the conserved amino acids characteristic of the active site of type 1 RIPs (Barbieri *et al.*, 1993) and the C-terminal region of the protein contains two putative glycosylation sites. The BE amino acid sequence is identical to that of betavulgin, described previously (Hornung *et al.*, 1996). BE shares sequence homologies with several well-known RIPs, especially those exhibiting anti-HIV-1 activity namely: PAP-II (32%; Poyet *et al.*, 1994), MAP 30 (27%; Lee-Huang *et al.*, 1990), TAP 29 and trichosanthin (28%; Lee-Huang *et al.*, 1991*a*), gelonin (25%; Lee-Huang *et al.*, 1991*b*), and DAP-32 (25%; Lee-Huang *et al.*, 1991*b*).

Expression in Escherichia coli *and enzymatic activity of rBE*

The gene coding for BE inserted into pET25 was expressed in *E. coli* grown at 37 °C. pET25-transformed *E. coli* strains were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), allowed to grow, and after either 3 h or 6 h the cultures were lysed. The total soluble protein fraction from lysates was analysed by SDS–PAGE and western blotting. As shown in Fig. 4A, the blots probed with polyclonal rabbit anti-BE revealed that the recombinant bacterial strain carrying pET25(b+) containing the BE-cDNA, synthesize two protein bands of apparent M_r values of 27 000 and 29 000 most probably without and with the leader sequence, respectively. By contrast, bacteria carrying pET25(b+) without BE-cDNA did not produce reactive protein bands. The growth of transformed bacteria expressing recombinant BE (rBE) was arrested for 3 h, after which the growth

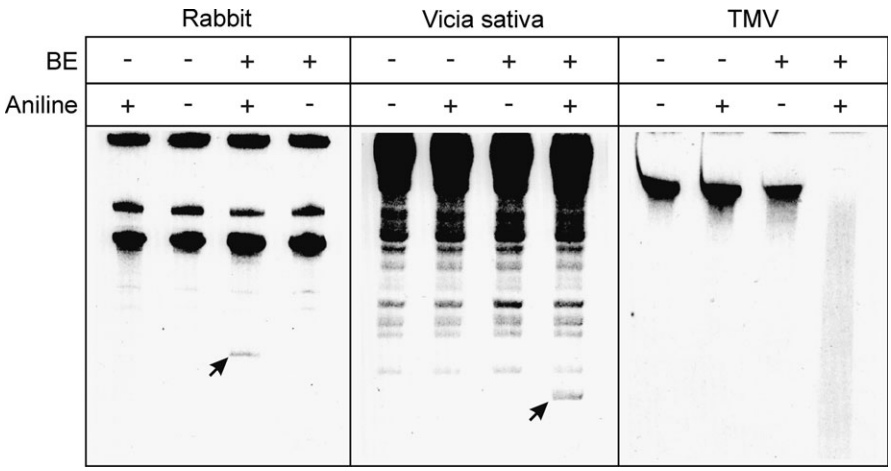

Fig. 3. N-glycosidase activity of BE27. rRNA N-glycosidase activity was assayed as indicated in the Materials and methods. The amount of BE27 added to each reaction mixture was 6 μg. Each lane contained 3 μg of RNA. Arrows indicate the RNA fragment released as a consequence of RIP action upon acid aniline treatment.

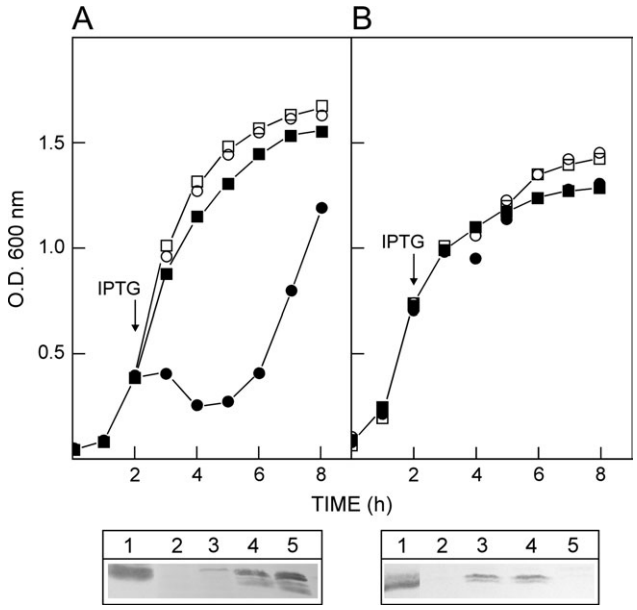

Fig. 4. Growth curves of *E. coli* BL21(DE3)pLysS control and BE-expressing cells. (A) *E. coli* was grown in LB media containing ampicilin 100 μg ml^{-1} at 37 °C and optical density was measured. Symbols: (open squares), non-induced BL21(DE3)pLysS-pET25b+; (solid squares), BL21(DE3)pLysS-pET25b+ induced with IPTG 1 mM; (open circles), non-induced BL21(DE3)pLysS-pETB; (solid circles), BL21(DE3)-pLysS-pETB induced with IPTG 1 mM. For western blot analysis aliquots of 1 ml of growing cultures were centrifuged at 4000 rpm at 22 °C for 10 min. The pellet was suspended in 75 μl of PBS buffer. Samples of 10 μl of crude protein extracts were electrophoresed and blotted as indicated in the Materials and methods. Lane 1, 250 ng of native BE27; lane 2, BL21(DE3)pLysS-pET25b+ 3 h after induction with IPTG 1 mM; lane 3, non-induced BL21(DE3)pLysS-pETB; lane 4, BL21(DE3)pLysS-pETB 3 h after induction with IPTG 1 mM; lane 5, BL21(DE3)pLysS-pETB 6 h after induction with IPTG 1 mM. (B) *E. coli* was grown in LB media containing ampicilin 100 μg ml^{-1} at 23 °C and optical density was measured. Symbols: (open squares), non-induced BL21(DE3)pLysS-pET25b+; (solid squares), BL21(DE3)pLysS-pET25b+ induced with IPTG 0.4 mM; (open circles), non-induced BL21(DE3)pLysS-pETB; (solid circles), BL21(DE3)pLysS-pETB induced with IPTG 0.4 mM. For western blotting, 10 μl of crude protein extract obtained as indicated above from an *E. coli* culture was added to each electrophoresis well. Lane 1, 250 ng of native BE27; lane 2, crude protein extract from BL21(DE3)pLysS-pET25b+ 3 h after induction with IPTG 0.4 mM; lane 3, crude protein extract from BL21(DE3)pLysS-pETB 3 h after induction with IPTG 0.4 mM; lane 4, BL21(DE3)pLysS-pETB 6 h after induction with IPTG 0.4 mM; lane 5, non-induced BL21(DE3)pLys-pETB.

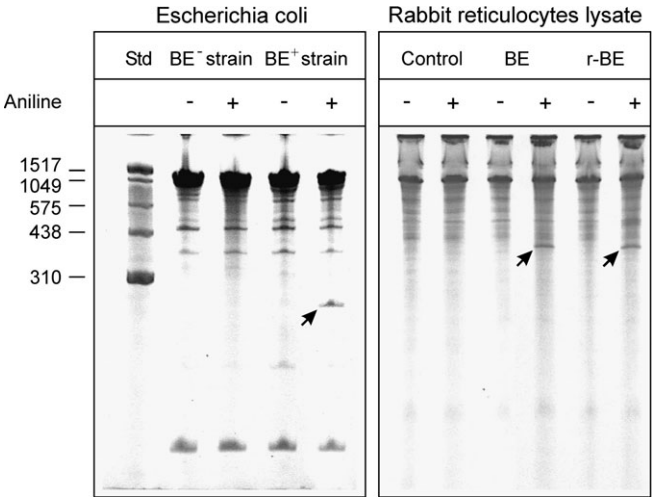

Fig. 5. N-glycosidase activity of rBE. Depurination activity was measured as indicated in the Materials and methods. Left: *in vivo* depurination of *E. coli* 23S rRNA; each lane corresponds to 3 μg of total RNA. The arrow shows the fragment split by aniline treatment. Numbers on the left indicate the size of the standards (Std) in nucleotides. Right: depurination of rabbit reticulocyte lysates; r-BE is a soluble crude protein extract from BL21(DE3)pLysS-pETB(rBE). The arrows show the fragment split by aniline treatment.

restarted at the same rate as before induction with IPTG, probably by the loss of the plasmid. As shown in Fig. 4B this difficulty can be avoided by growing the bacterium at a lower temperature (23 °C) and using a lower concentration of IPTG (0.4 mM). The arrest of growth, concomitant with BE expression, is most probably related to the strong toxicity of rBE against bacterial ribosomes, as has been described for other RIPs such as MAP (Habuka *et al.*, 1990), PAP (Poyet *et al.*, 1994), or trichosanthin (Shaw *et al.*, 1991). As shown in Fig. 5, bacterial ribosomes from strains carrying pET25(b+) with BE-cDNA are sensitive to the recombinant BE since treatment of the corresponding rRNA with acid aniline promoted the release of the RIP-diagnostic RNA fragment. This seems to be a clear consequence of the accumulation of rBE in an active form, even during the process of polypeptide chain growth and folding. Due to the strong effects of accumulated rBE inside the cells it was impossible to isolate enough rBE from the bacterial cultures to carry out conventional procedures of isolation and chemical and physical characterization. The total soluble protein of the *E. coli* BE+ strain displayed very strong translation inhibitory activity, with an IC_{50} close to 5 ng ml^{-1} in rabbit reticulocytes lysates.

Subcellular location, systemic induction, and topical antiviral action of BE

It has been described that pokeweed antiviral protein (PAP) is located outside the cell, between the plasma membrane and the cell wall (Ready *et al.*, 1986). To ascertain the location of BE forms, the apoplastic fluid from virus-infected plants harvested in the field were isolated and the presence of BE was studied by western blot analysis using anti-beetin antibodies. To allow a comparison the same amount of crude protein extract was loaded onto each gel lane. As shown in Fig. 6a, induction of sugar beet leaves with H_2O_2, salicylic acid, or AMCV led to the induction of BE forms. Analysis of the apoplastic fluid revealed the presence of BE27 (major protein form) and some label corresponding to BE29 (minor protein form), while no protein label was found in the extract prepared from the rest of the tissue previously depleted of apoplastic fluid by vacuum infiltration with buffer (Fig. 6b). The BE27 purified

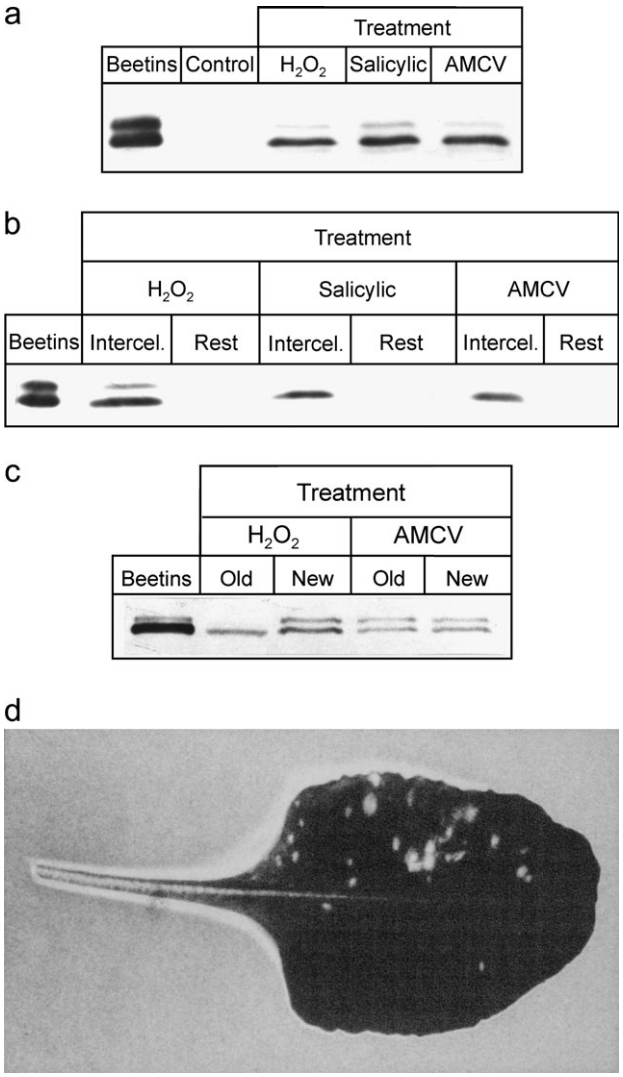

Fig. 6. Subcellular location and systemic induction of BEs. The presence of BEs was analysed by western blot, as indicated in the Materials and methods. (a) Crude extracts of sugar beet plants grown in the laboratory and treated with chemical elicitors: either H_2O_2 and salicylic acid or AMCV particles; (b) apoplastic fluid of sugar beet leaves obtained as indicated in the Materials and methods and the rest of the leaf; (c) old and new leaves of laboratory-grown sugar beet plants induced either with H_2O_2 or AMCV in old leaves; (d) effects of the external application of BE on a half control leaf heavily infected with AMCV. In (a), (b), and (c), 80 μg of crude protein extract were subjected to electrophoresis. 250 ng of purified beetins were used as standard. In (d), 50 μl of a crude extract of *N. clevelandii* containing viral particles were applied to one-half of the leaf and 50 μl of the same solution, but containing 10 μg ml^{-1} of purified beetins, were applied to the other half.

from the apoplastic fluid retained full translational inhibitory activity (data not shown).

In order to gain further insight into the role played by BE induction, it was investigated whether that process is merely a local consequence of virus attack or, instead, if it is of a systemic nature. As also shown in Fig. 6c, induction with either H_2O_2 or AMCV not only promoted BE expression at the site of application (old leaves), but also at distant sites (new leaves). The onset and the intensity of induction seemed to be the same in the old and new leaves. To the best of the authors' knowledge, this is the first time that this systemic induction of type 1 RIPs in response to viral insult or chemical stimulation has been described and it could play an important role in the protection of sugar beet against viruses, at least in the early stages of infection. In addition, the direct antiviral action derived from topical application of BE against infection by AMCV was studied and it was found that the simultaneous application of BE together with viral particles strongly prevented the infective process as assessed by visual inspection (Fig. 6d). These application-dependent antiviral effects are in close agreement with the data reported for other type 1 RIPs (Irvin, 1983; Chen *et al.*, 1992).

Discussion

Previous screening work showed that sugar beet contained proteins that, according to their physical and chemical characteristics, could be classified as RIPs (Gasperi-Campani *et al.*, 1985). Further research confirmed that sugar beet contains at least two RIPs that are subject to induction by viral infection and the molecular mediators of viral infection either, H_2O_2 or salicylic acid (Girbés *et al.*, 1996*b*). Shortly afterwards, a report describing the sequence of a fragment of cDNA from mangold (*Beta vulgaris*) coding an RIP named betavulgin was published (Hornung *et al.*, 1996). BEs have been characterized here at the molecular level and a genomic DNA fragment coding for the BE polypeptide chain has also been characterized, thus allowing new insights to be gained into these proteins as well as the nature of their induction. BEs isolated from sugar beet fulfil all the requirements in order to be considered as single chain (type 1) RIPs, highly toxic to mammalian ribosomes, but non-toxic to intact cultured mammalian cells. BE belongs to the type 1 RIPs and acts on plant ribosomes. BE has glycan chains and displays two main forms with apparent M_r values of 27 000 (BE27) and 29 000 (BE29). BE29 seems to be far more glycosylated than BE27. In fact, from the mass spectrometry analysis it can be suggested that BE27 is a non-glycosylated form of BE since the molecular weight 27 592 is almost completely coincident with 27 556 estimated from the amino acid sequence.

BE was immunodetected only in the apoplastic fluid (Fig. 6b). However, the finding that no immunoreactive material was detected in the leaf deprived of the apoplastic fluid by vacuum infiltration would mean that BE is concentrated essentially in the intercellular fluid and that the BE contained in the rest of the leaf could be below the limit of detection rather that being absent.

The expression experiments indicated that BE is very active against *E. coli* ribosomes since they become depurinated as the bacteria grows and BE is being produced and accumulated inside the cells. Such a phenomenon has been

already described for other very active type 1 RIPs (Habuka *et al.*, 1990; Shaw *et al.*, 1991; Poyet *et al.*, 1994). This is the reason why, to date, it has been very difficult to produce workable amounts of rBE. In addition, rBE seems to be more active than native BE.

The topical antiviral activity of BE is consistent with the current antiviral role proposed for RIPs (Barbieri *et al.*, 1993; Lord *et al.*, 1994; Stirpe, 2004). Nonetheless, since the local lesion assay requires the abrasion of the leaf surface with carborundum, the possibility that BE trigger the inactivation of the ribosomes of the damaged cells hence inhibiting viral replication cannot be ruled out.

These induction experiments indicated that the enzyme under consideration is induced by phenomena such as chemical elicitors and viral infection acting at sites distant from the induction sites and therefore it could be considered as a component of the plant systemic acquired resistance system together with enzymes such as glucanases and chitinases (Enyedi *et al.*, 1992*a*, *b*). On these grounds, and since BE displays a strong depurinating activity against both plant ribosomes and TMV genomic-RNA, it would be a good candidate for the construction of transgenic plants resistant to RNA-virus infection bearing the BE gene under the control of a virus-inducible promoter. Perhaps the same promoter operating in sugar beet for BE expression could be useful for such a purpose.

The induction of type 1 RIPs is a phenomenon that has only been unravelled in recent years. It has been described that salt stress (0.5 M NaCl) strongly promotes the expression of a type 1 RIP by *Mesembryanthemum crystallinum* (common ice plant) (Rippmann *et al.*, 1997). Jasmonate can also induce RIP expression in barley (Chaudry *et al.*, 1994) and in *Phytolacca insularis* (Song *et al.*, 2000). However, these two RIPs are not induced by salicylic acid, in contrast to BE. This opens the possibility that at least two pathway types may be operating to promote type 1 RIP induction.

The evidence available clearly suggests that RIP induction could play a role as a mechanism of plant protection. In addition, recent studies have indicated that the systemic resistance-inducing proteins CA-SRI from *Clerodendrum aculeatum* (Kumar *et al.*, 1997) and CIP-29 and CIP-34 from *Clerodendrum inerme* (Olivieri *et al.*, 1996) are true single-chain RIPS. This opens the question of whether some RIPs could really act as intermediate elements of systemic acquired resistance, as may be deduced from the data published by Kumar *et al.* (1997) and Olivieri *et al.* (1996), or instead they would be the result of the induction processes (Girbés *et al.*, 1996*b*). In other words, all these findings again raise the as yet unanswered question of whether RIPs are the cause or the result of the induction process. Another question that awaits an explanation concerns the fact that BE is commonly isolated from field-grown beets heavily infected with BMYV. If BE displays antiviral activity how can the viral particles infect the plants upon de-induction of BE? It is possible that BE induction would control moderate infection processes but not the heavy ones. Further studies will address these issues as well as the improvement of BE expression by a reduction of its cytotoxicity in order to produce workable amounts of rBE.

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