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Engineered resistance against proteinases

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Exogenous proteinase inhibitors are valuable and economically interesting protective biotechnological tools. We examined whether small proteinase inhibitors when fused to a selected target protein can protect the target from proteolytic degradation without simultaneously affecting the function and activity of the target domain. Two proteinase inhibitors were studied: a Kazal-type silk proteinase inhibitor (SPI2) from *Galleria mellonella*, and the *Cucurbita maxima* trypsin inhibitor I (CMTI I). Both inhibitors target serine proteinases, are small proteins with a compact structure stabilized by a network of disulfide bridges, and are expressed as free polypeptides in their natural surroundings. Four constructs were prepared: the gene for either of the inhibitors was ligated to the 5′ end of the DNA encoding one or the other of two selected target proteins, the coat protein (CP) of Potato potyvirus Y or the *Escherichia coli* β-glucuronidase (GUS). CMTI I fused to the target proteins strongly hampered their functions. Moreover, the inhibitory activity of CMTI I was retained only when it was fused to the CP. In contrast, when fused to SPI2, specific features and functions of both target proteins were retained and the inhibitory activity of SPI2 was fully preserved. Measuring proteolysis in the presence or absence of either inhibitor, we demonstrated that proteinase inhibitors can protect target proteins used either free or as a fusion domain. Interestingly, their inhibitory efficiency was superior to that of a commercial inhibitor of serine proteinases, AEBSF.

Keywords: proteinase inhibitors, protein protection, fusion proteins

INTRODUCTION

Proteolysis, the hydrolytic cleavage of peptide bonds, is responsible for many specialized intra- and extra-cellular biological processes. Enzymes responsible for proteolysis (designated proteinases or proteases) are encoded by approx. 2% of all genes and are essential for the survival of all organisms (Barrett *et al.*, 2001). Although unquestionably indispensable, they are potentially very damaging to the cell and therefore mechanisms for their control have evolved. One of these mechanisms is based on inactivation of proteinases by complex formation with polypeptide inhibitors (Laskowski & Kato, 1980; Enghild *et al.*, 1990). Two functional groups of proteinase inhibitors can be distinguished, one that reacts with the target proteinase through an irreversible "trapping" mechanism, the other using tight-binding reactions (Rawlings *et al.*, 2004). Inhibitors of the latter group are rather small proteins (27–200 amino acids) and

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Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; BSA, bovine serum albumin; CMTI I, *Cucurbita maxima* trypsin inhibitor I; CMTI::CP, protein fusion of CMTI I and CP; CMTI::GUS, protein fusion of CMTI I and GUS; CP, coat protein; GUS, β-glucuronidase; His-tag, tag of 6 histidine residues; IC_{50}, inhibitor concentration required for 50% inhibition of proteinase activity; IPTG, isopropyl β-D-thiogalactoside; K_i, inhibitory dissociation constant; K_m, Michaelis constant; MU, 4-methylumbelliferone; MUG, 4-methylumbelliferyl-β-D-glucuronide; NH_2-TNBS, amino groups covalently attached to TNBS; NS-EM; negative stain electron microscopy; PMSF, phenylmethylsulfonyl fluoride; PVY, Potato potyvirus Y; SPI2, silk proteinase inhibitor; SPI::CP, protein fusion of SPI2 and CP; SPI::GUS, protein fusion of SPI2 and GUS; TNBS, trinitrobenzene sulfonic acid; VLPs, virus-like particles.

in most cases are specifically directed against proteinases of one mechanistic class (serine, cysteine, aspartic or metalloproteases; Bode & Huber, 2000). Moreover, in most cases these inhibitors contain a characteristic pattern of disulfide bonds that stabilize the structure of the inhibitor. Historically the topological relationships between these disulfide bridges have been used as an auxiliary criterion for inhibitor classification (Laskowski & Kato, 1980; Rawlings *et al.*, 2004). The majority of presently known inhibitors are directed towards serine proteinases and interact with the proteinase *via* an exposed reactive-site loop in a so-called canonical or substrate-like manner (Laskowski & Kato, 1980; Bode & Huber, 1992; Laskowski *et al.*, 2000). The binding loop of the inhibitor on which the reactive site residue P1 is exposed is inserted into the cleft of the active site of the proteinase as would be a substrate, with the scissile peptide bond approaching and blocking the catalytic proteinase residue in a noncovalent, near-Michaelis-like geometry (Bode & Huber, 1992; 2000).

Hundreds of proteinase inhibitors are now known and are the subject of an overwhelming number of research communications. Their potential for application in medicine, agriculture or biotechnology makes them objects of special interest (Xiao *et al.*, 1999; Shi *et al.*, 2001; Mandal *et al.*, 2002; Nadler, 2003). Moreover, since proteinases are essential in the virus replication cycle, their inhibitors are attractive candidates for the design of new antiviral strategies (Kondo *et al.*, 1992; Cimerman *et al.*, 1996; Gutierrez-Campos *et al.*, 1999; Takahashi *et al.*, 1999). Last but not least, exogenous proteinase inhibitors can be considered as valuable and economically important protective tools, for example in bioreactors, where they can enhance the yield and prolong the life of desired protein products.

Our attention turned to two very small proteins that are proteinase inhibitors, 1) a silk proteinase inhibitor (SPI2) of the Kazal-type, recently described as a natural component of the silk of *Galleria mellonella* (Nirmala *et al.*, 2001), and 2) the *Cucurbita maxima* trypsin inhibitor I (CMTI I) of the squash inhibitor family (Bolewska *et al.*, 1995). SPI2 is the shortest known single-domain member of the Kazal family of proteinase inhibitors (36 amino acids, 3.9 kDa) with unique structural features. Each typical Kazal-type domain includes six conserved cysteines (I to VI) that form three intra-domain disulfide bridges dictating the formation of three rings designated A, B and C (Laskowski & Kato, 1980; Nirmala *et al.*, 2001). SPI2 is atypical, as cysteines I and V are absent precluding the formation of the A-ring and providing an increased flexibility to the C-ring. In contrast, the B-ring with its P1 reactive site residue is highly conserved suggesting that it is probably crucial for the inhibitory activity (Nirmala *et al.*, 2001). SPI2 has been shown to be active against fungal and bacterial serine proteinases with the strongest inhibition directed against proteinase K from the mould *Tritirachium album*, and subtilisin from *Bacillus subtilis*; its activity against trypsin is relatively weak (Nirmala *et al.*, 2001). CMTI I is one of the best known representatives of the family of squash proteinase inhibitors (Leluk *et al.*, 1983; Bode *et al.*, 1989; Otlewski & Krowarsch, 1996) that are composed of 27–33 amino acids and possess very rigid structures. Similarly to the members of the Kazal family, the most important structural feature of squash inhibitors is the tight cross-linking of the polypeptide chain by three disulfide bridges (Bode *et al.*, 1989; Holak *et al.*, 1989; Stachowiak *et al.*, 1990). The squash inhibitors strongly inhibit bovine trypsin and a number of medically important serine proteinases (Otlewski *et al.*, 1990; Hayashi *et al.*, 1994).

In the present study, we investigated whether these small polypeptide inhibitors fused to a chosen target protein could protect the target protein against proteolysis without influencing the function and activity of the target domain. As target proteins, the coat protein (CP) of Potato potyvirus Y (PVY; Shukla *et al.*, 1988; Shukla & Ward, 1989) and bacterial β-glucuronidase (GUS; Gallagher, 1992) were selected. These targets reflect two general protein features – the ability to form supramolecular structures spontaneously, and enzymatic catalysis, respectively. It has been shown that potyviral CPs when purified from intact virions or expressed in *Escherichia coli*, yeast or a baculovirus system form long rod-shaped virus-like particles (VLPs) easily visualized by electron microscopy (EM; McDonald *et al.*, 1976; McDonald & Bancroft, 1977; Jagadish *et al.*, 1991; Edwards *et al.*, 1994). Additionally, a model for these rod-shaped particles has been proposed (Shukla & Ward, 1989; Jagadish *et al.*, 1996) and the conditions of assembly and disassembly of potyvirus VLPs defined (McDonald & Bancroft, 1977; Palucha *et al.*, 2005). GUS found in bacteria and in vertebrates specifically hydrolyses β-linked D-glucuronides to D-glucuronic acid and aglycones (Gallagher, 1992; Wenzl *et al.*, 2005). The GUS used here is encoded by the *E. coli gusA* gene, it is a stable enzyme that has desirable properties for the construction and analysis of gene fusions (Gallagher, 1992). Both the structure-forming capacity of the CP and the catalytic activity of GUS are easy to analyse; CP oligomerizes forming defined VLPs, whereas GUS shows an easily measurable enzymatic activity.

Here we characterized the recombinant proteins and compared their expression efficiency, their structural ability to form VLPs and their enzymatic activity. In proteolysis experiments (using a serine proteinase, either proteinase K or trypsin) we demonstrated that when fused to the target domains,

SPI2 or CMTI I generally preserved their inhibitory capacities. In addition, their inhibitory efficiency was superior to that of the commercial inhibitor of serine proteinases AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride), a water-soluble analogue of PMSF (phenylmethylsulfonyl fluoride).

MATERIALS AND METHODS

Plasmid construction. Recombinant DNA manipulations were carried out using standard protocols (Sambrook & Russell, 2001). The genes encoding the target proteins were prepared as follows. Total RNA was extracted from the PVY strain Wilga-infected potato leaf tissue using the RNeasy Plant Mini Kit (Qiagen) and used as template in first strand cDNA synthesis with oligo$(dT)_{21}$ as primer and the SuperScript II reverse transcriptase (Invitrogen), as recommended by the supplier. The first strand cDNA of the PVY, and plasmid pFF19G containing the GUS gene (Timmermans *et al.*, 1990) served as templates for PCR reactions with primers corresponding to the DNA regions encoding the N- and C-termini of CP and GUS, respectively. The CP coding sequence was amplified with CP_1 and CP_2 as forward and reverse primers, respectively, and the GUS gene was amplified with GUS_1 and GUS_2 as forward and reverse primers, respectively (Table 1). The primers used were extended to introduce an *Nco*I site with an ATG codon and an *Xho*I site at the 5′ and 3′ end of the amplified products, respectively. About 0.1 μl of either the first-strand SuperScript II reaction, or about 0.5 μg of pFF19G was amplified in a 50 μl reaction mixture with *Pfu* DNA polymerase (Promega) as recommended by the supplier. The annealing temperature was 58°C (for the CP primer pair) or 59°C (for the GUS primer pair). The resulting PCR products were purified by electrophoresis on 1% agarose gels, extracted using the Gel Extraction Kit (Qiagen), and cloned into the *Sma*I site of the pBluescript II KS plasmid (pBl, Stratagene). Subsequently, the inserts were cleaved from the pBl recombinant vectors with *Xho*I and *Nco*I (Fermentas), purified on a 1% agarose gel, extracted as above and cloned between the *Xho*I and *Nco*I sites of the pET28a plasmid (Novagen). The resulting recombinant plasmids are designated pCP and pGUS.

Two vectors, pPICZαB/rSPI2 (a kind gift of Krystyna Grzelak, IBB PAS) and pAED4 (containing the CMTI I gene; a kind gift of Andrzej Bierzynski; Bolewska *et al.*, 1995) served as templates for PCR reactions with primers corresponding to the DNA regions encoding the N- and C-termini of the SPI2 and CMTI I proteinase inhibitors and extended to introduce an *Nco*I site into both ends of the amplified products. The primers used in PCR amplification of the SPI2 gene were SPI_1 and SPI_2 as forward and reverse primers, respectively (Table 1), and those used to amplify the CMTI I gene were $CMTI_1$ and $CMTI_2$ as forward and reverse primers, respectively (Table 1). PCR was carried out as indicated above for CP and GUS with the annealing temperatures 57°C (for the SPI2 pair) or 59°C (for the CMTI I pair). The resulting products were purified by electrophoresis on 1.5% agarose gels, extracted with the Gel Extraction Kit, and cloned into the *Sma*I site of pBl. The pBl plasmid containing the SPI2 or CMTI I sequence was digested with *Nco*I. The inserts were purified as indicated above and cloned into the *Nco*I site of the previously obtained pCP and pGUS. The resulting recombinant plasmids were pSPI::CP, pCMTI::CP, pSPI::GUS and pCMTI::GUS (Fig. 1). The orientation of the inserts was verified by PCR with ET2188 (complementary to the region upstream of the T7 promoter; Table 1) as forward primer, and SPI_2 or $CMTI_2$ as reverse primer. All inserts and fusion genes were sequenced and proven to be correct. The molecular mass of the expected proteins was predicted on the basis of their amino-acid composition using ProtParam from the ExPASy Proteomics Server.

Expression in *E. coli* and purification of recombinant proteins. The *E. coli* strain BL21(DE3)pLysS (Novagen) was transformed with the recombinant plasmids according to the Novagen manual. Since

Table 1. List of primers.

Primer	Sequence (5′ to 3′)	Restriction site
CP_1	GGACCATGGAAGCAAATGACACAATC	*Nco*I
CP_2	GACCTCGAGCATGTTCTTGACTCC	*Xho*I
SPI_1	GGACCATGGCCGCAGTTTGCACCACCGAGT	*Nco*I
SPI_2	GACCCATGGAACATTCACCTTCATGATC	*Nco*I
GUS_1	GACCATGGTGTTACGTCCTGTAGAAAC	*Nco*I
GUS_2	GACTCGAGTTGTTTGCCTCCCTGCT	*Xho*I
$CMTI_1$	GACCATGGCGCGTGTTTGCCCGCGTATCCT	*Nco*I
$CMTI_2$	GACCATGGCACCGCAGTAACCGTGTTCCA	*Nco*I
ET2188	GATCTCGATCCCGCGAAAT	

*Nco*I and *Xho*I sites are underlined.

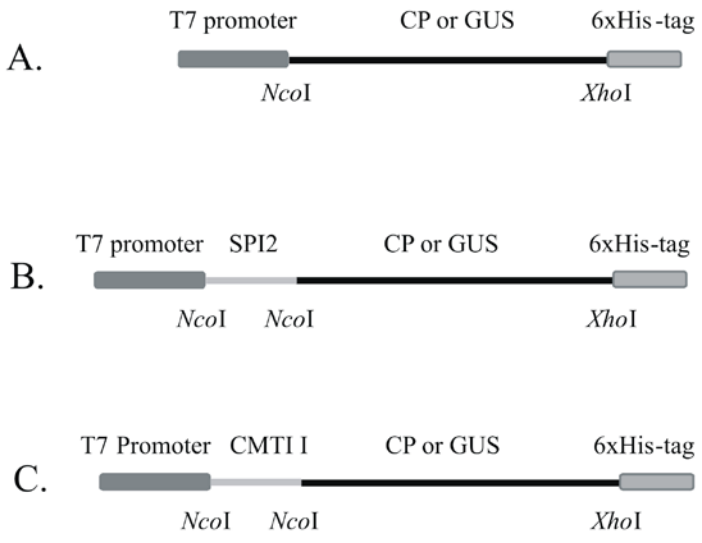

Figure 1. Schematic representation of the DNA constructs.
A. pCP or pGUS; **B**. pSPI::CP or pSPI::GUS; **C**. pCMTI::CP or pCMTI::GUS; tagged with a 6xHis-tag at the C-terminus. CP, PVY coat protein (267 amino acids); GUS, β-glucuronidase (600 amino acids); SPI2, silk proteinase inhibitor (36 amino acids); CMTI I, *C. maxima* trypsin inhibitor (31 amino acids). Not to scale.

it was difficult to obtain satisfactory induction levels of CMTI::CP in the BL21(DE3)pLysS strain, the BL21(DE3) strain was used for the expression of CMTI::CP. Bacterial cultures were grown at 37ºC in LB medium containing 25 μg/ml of kanamycin. When the OD_{600} reached 0.6–0.9, expression of the recombinant proteins was induced with 0.4 mM isopropyl β-D-thiogalactoside (IPTG) and the cultures were further incubated at 26–28ºC. After addition of IPTG, growth of the transformed cells was monitored at OD_{600} every hour. To follow the expression of the recombinant proteins, equal amounts of cell extracts (corresponding to 1 ml of culture cells, OD_{600} = 0.5) were electrophoresed on a 12% polyacrylamide-SDS gel, stained with Coomassie Brilliant Blue and the protein expression level was quantified by computer densitometry of the scanned images using specialized software (ZERO-Dscan of Scanalytics, Inc.). Finally, 4–6 h after induction, the cells were harvested by centrifugation, lysed and the recombinant proteins were purified under native conditions on Ni-NTA agarose resin according to the QIA expressionist manual (Qiagen). The column fractions were analysed by SDS/PAGE and the concentration of the eluted proteins was determined using the Protein Assay kit (Bio-Rad) with bovine serum albumin (BSA) as the standard. Subsequently, purified recombinant proteins were dialyzed overnight at 4ºC against 10 mM sodium phosphate buffer, pH 7.5. In the case of the CP and its SPI2 fusion, the dialyzed proteins were concentrated by 2 h ultracentrifugation (173 000 × *g*, 4ºC) and the pellets redissolved in 10 mM sodium phosphate buffer, pH 7.5, for EM imaging.

Negative stain electron microscopy. Samples of CP, SPI::CP and CMTI::CP fusions at approx. 0.06 mg/ml suspended in 10 mM sodium phosphate buffer, pH 7.5, were adsorbed to a carbon-mica interface. The carbon film with adsorbed protein was floated onto a solution of 1% uranyl acetate and the film was picked up by a copper grid and air-dried (Valentine *et al.*, 1968). Micrographs were taken with a JEOL 1200 EX II microscope at a nominal magnification of 40 000 times.

GUS fluorometric assay. The activity of recombinant GUS, SPI::GUS and CMTI::GUS was measured as described (Rao & Flynn, 1990). The test is based on the fluorescence of 4-methylumbelliferone (MU), which is a product of the enzymatic hydrolysis of 4-methylumbelliferyl-β-D-glucuronide (MUG). The assays were performed using microtiter plates in 50 μl reaction mixtures containing 50 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% sarcosyl, 8.3 to 9.8 mM β-mercaptoethanol (depending on the volume of MUG added to the reaction mixture), 220 pM (0.011 pmoles) recombinant GUS or one of its fusions, and either 7 mM MUG in time course assays or 0.8 to 7.2 mM MUG to determine Michaelis constants (K_m) and maximal velocities (V_{max}). The control assay was performed without GUS or its fusions. The reaction mixtures were incubated at 37ºC for the times indicated and terminated by addition of 150 μl of 0.2 M Na_2CO_3. The mixtures for standard curve determinations contained 200 μl of 0.2 M Na_2CO_3 and MU concentrations ranging between 0.05 and 14 μM. Measurements were performed in a Synergy™ HT multi-detection microplate reader (Bio-Tek Instruments) with the excitation and emission wavelengths set at 380 and 485 nm, respectively. The results are expressed in pmoles of MU, calculated on the basis of the MU standard curve. The K_m and V_{max} values were determined by the nonlinear least-squares fit using the SigmaPlot software (Systat).

Proteinase assays. Proteinase K (Fermentas) or trypsin (bovine pancreas, Boehringer Mannheim) were used as target enzymes of the proteinase inhibitors and their fusions. Inhibition of proteinase K and trypsin activity was measured using azocoll (Calbiochem), a red dye-labeled standard proteinase substrate (Chavira *et al.*, 1984). The assay was modified and adapted for measurements with a microtiter plate photometer. Azocoll was treated as described (Nirmala *et al.*, 2001) and used for the assay (100 µl of 50 mM Tris/HCl, pH 7.5 — buffer A, containing 15 mg/ml of azocoll per well of a flat-bottomed microtiter plate). Subsequently, portions of 100 µl of proteinase K (8.6 pmoles) or trypsin (10.4 pmoles) in buffer A and either 100 µl of SPI2 and its fusions or CMTI I and its fusions or AEBSF (Sigma) diluted in buffer A were added to each well. In negative controls (no proteolytic activity) the proteinase was replaced by buffer A, and in positive controls (no inhibitory activity) the inhibitor was replaced by buffer A. The microtiter plates were incubated with shaking at 37°C for 2 h and centrifuged (1800 r.p.m., 2 min, 4°C) to pellet azocoll. The clear supernatant (150 µl) was transferred to another microtiter plate. The absorbance of the azo-dye liberated by the proteolytic activity of proteinase K or trypsin was measured at 492 nm in a Synergy™ HT multi-detection microplate reader (Bio-Tek Instruments). The difference in absorbance between the positive and negative controls was taken as representing 100% proteinase activity. For each sample, a plot of percentage of proteinase activity *versus* concentration of proteinase inhibitor was established to calculate the IC_{50} (concentration of the inhibitors corresponding to 50% inhibition of proteinase K or trypsin activity) values. The inhibitory activity titre of the samples assayed was calculated from triplicate measurements.

Determination of the inhibitory dissociation constants. The inhibition of proteinase K and trypsin activity was measured spectrophotometrically with succinylated casein (Calbiochem) as substrate. The increase in concentration of amino groups (NH_2) appearing by hydrolysis of the succinylated casein was measured with trinitrobenzene sulfonic acid (TNBS; Sigma). TNBS reacts with exposed NH_2 groups forming an adduct that absorbs at 405 nm. It has been suggested that this method can be used for kinetic analyses of proteinase activity (Surovtsev *et al.*, 2001). The kinetic constants were determined by incubating the enzymes in the absence or presence of two concentrations of SPI2, SPI::CP and SPI::GUS (for proteinase K) or CMTI I and CMTI::CP (for trypsin), with increasing substrate concentrations. The average molecular mass of succinylated casein was taken as 24.3 kDa. The assays were performed in triplicate using microtiter plates with 250 µl reaction mixtures containing 50 mM borate buffer, pH 8.0 (buffer B), five succinylated casein concentrations (0.5 to 5.1 µM) in the proteinase K assay or four succinylated casein concentrations (1.0 to 5.1 µM) in the trypsin assay, two inhibitor concentrations for each protein assayed (0.04 and 0.06 µM SPI2 or SPI::CP, 0.05 and 0.09 µM SPI::GUS, 0.002 and 0.004 µM CMTI I, or 0.5 and 0.8 µM CMTI::CP), 0.006% TNBS and 250 ng of proteinase K (8.6 pmoles) or trypsin (10.4 pmoles). In negative controls, succinylated casein was replaced by buffer B. The microtiter plates were shaken at 37°C for 30–40 min. The absorbance of TNBS covalently attached to NH_2 (NH_2-TNBS) resulting from the proteolytic activity of proteinase K or trypsin was measured in a multi-detection microplate reader at 405 nm. The results were expressed in µmoles/min of NH_2-TNBS produced. The NH_2-TNBS extinction coefficient (20 000 M^{-1} cm^{-1}) was used as determined by Mathrubutham *et al.* (http://www.piercenet.com/files/quanticleave_poster.pdf). Initial rate studies and dissociation constant determinations were performed by the nonlinear least-squares fit using the SigmaPlot software (Systat) according to the equation: $v = V_{max}/(1+(K_m/S)(1+I/K_i))$ where K_m is the Michaelis constant, V_{max} is the maximum reaction velocity at saturating substrate concentration S, K_i is the dissociation constant for the enzyme–inhibitor complex, and I is the inhibitor concentration.

Comparative proteolysis. I. Recombinant GUS and its fusion to SPI2 were submitted to proteinase K digestion. In the reaction mixtures 970 nM SPI::GUS or GUS was digested in the presence of proteinase K. The molar ratio between the SPI::GUS fusion and proteinase K was about 12 times higher than that allowing 50% reduction of proteinase K activity. The incubations were performed for 2.5 h at 37°C with vigorous shaking in 50 µl of buffer A. Negative controls were reactions without proteinase K. Subsequently, the reactions were diluted about 3500 times and the GUS activity was verified by fluorometric assay.

II. Recombinant CP, its fusion to SPI2 and BSA (used as internal control) were submitted to proteinase K digestion in the absence or presence of SPI2 alone or AEBSF (Sigma). In the reaction mixtures the molar ratio between the inhibitors and proteinase K was about 12 times higher than that allowing 50% reduction of proteinase K activity. The incubations were performed for 2.5 h at 37°C with vigorous shaking in 15 µl of buffer A. Negative controls were reactions without proteinase K. Subsequently, the digestion results were analysed by SDS/PAGE.

RESULTS

Protein expression and purification

The recombinant proteins CP, GUS and the four fusion proteins SPI::CP, CMTI::CP, SPI::GUS and CMTI::GUS, tagged with 6 histidine residues (His-tag) at their C-terminus (Fig. 1), were expressed in *E. coli* and purified under native conditions using Ni-NTA agarose affinity chromatography. The expected molecular masses of the recombinant CP and its fusions SPI::CP and CMTI::CP were 31.2, 35.4 and 34.7 kDa, whereas the expected molecular masses of the recombinant GUS and its fusions SPI::GUS and CMTI::GUS were 69.6, 73.9 and 73.1 kDa, respectively. The molecular masses of all recombinant proteins obtained by SDS/PAGE were as expected (not shown).

Growth of the transformed cells was monitored after addition of IPTG by measuring the OD_{600} (Fig. 2A, C). Untransformed control cultures were treated with the same concentrations of IPTG. Growth of the cells expressing the CP or its fusions declined progressively after 2 h of IPTG induction, while GUS or its fusions did not significantly influence the rate of transformant growth (Fig. 2A, C). Although the CMTI::CP production level was slightly lower than that of CP or SPI::CP, it seemed that the growth of these three transformants inversely correlated with overproduction of CP variants (Fig. 2A, B). The growth rate of cells overproducing recombinant GUS or its fusion to proteinase inhibitors was comparable to that of the control culture and did not seem to be negatively correlated with expression of the foreign proteins (Fig. 2C, D).

Characterization of purified recombinant proteins

The preservation of structural and physiological features of purified recombinant CP and its fusions with the proteinase inhibitors was verified by negative stain electron microscopy (NS-EM). Analysis of CP and SPI::CP revealed the presence of characteristic rod-shaped VLPs (Fig. 3A–D). No sign of helical packing in Fourier transform from NS-EM images was detected suggesting that in

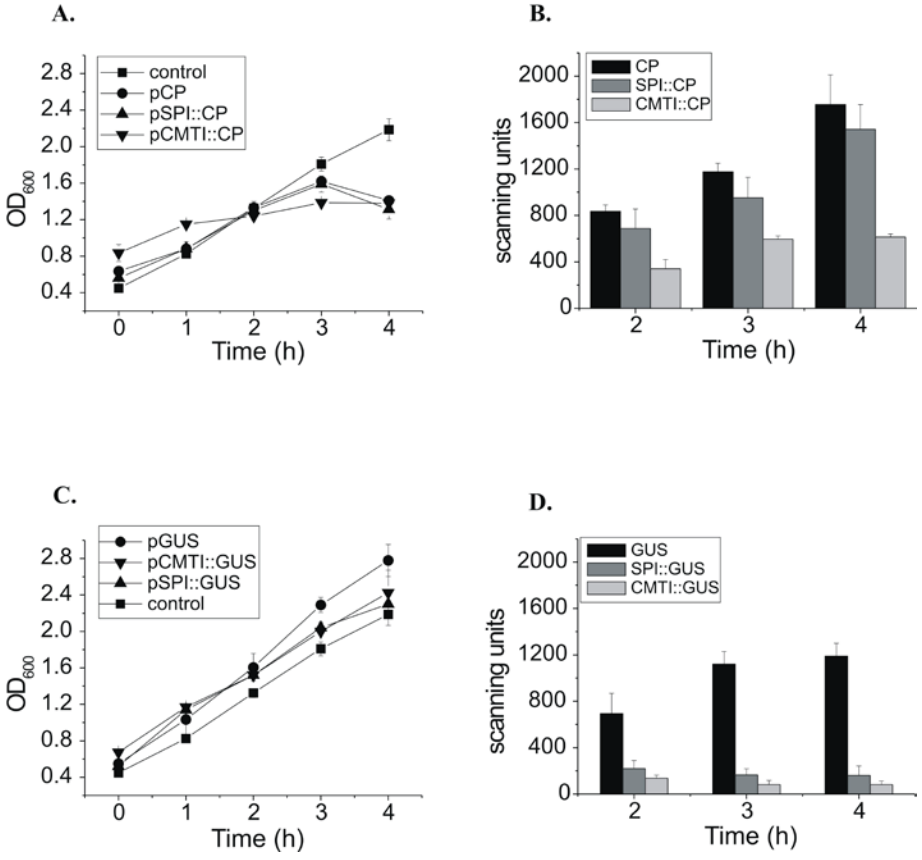

Figure 2. Growth of bacterial cultures in relation to the level of expression of recombinant proteins.
A and **C**. Growth of bacterial cultures after IPTG induction for the expression of the recombinant proteins. *E. coli* cells were transformed with pCP, pSPI::CP or pCMTI::CP (A) or pGUS, pSPI::GUS or pCMTI::GUS (C); controls were untransformed cells. **B** and **D**. Expression level of recombinant proteins: CP, SPI::CP or CMTI::CP (B); GUS, SPI::GUS or CMTI:: GUS (D) at the times indicated after IPTG induction. The same number of cells collected at one-hour intervals was analysed by SDS/PAGE. The intensity (scanning units) of Coomassie-stained bands of the expressed proteins was calculated using the ZERO–Dscan (Scanalytics, Inc.) program.

both cases the VLPs are stacked disks with a constant, identical diameter (M. Milner, J. Conway and A. Hoenger, unpublished results). Morphologically, the particles resembled VLPs of other potyviruses such as Johnsongrass mosaic virus, i.e. highly heterogeneous in length but of constant diameter (McDonald & Bancroft, 1977; Jagadish *et al.*, 1991). At pH 6 or below, the VLPs assembled from recombinant CP or SPI::CP tended to disaggregate and at pH 4 disappeared completely, similarly to what had been described for wild type PVY CP (McDonald & Bancroft, 1977). Similarly, prolonged sonication destabilized the structure of both types of VLPs (not shown). As opposed to SPI::CP, self-assembly of CMTI::CP into VLPs did not occur and only a few short and badly ordered aggregates could be detected (Fig. 3E, F).

To evaluate the enzymatic activity of GUS, SPI::GUS and CMTI::GUS, the proteins were tested employing MUG as a substrate, and the appearance of the reaction product MU was measured spectrofluorimetrically. All three recombinant proteins were enzymatically active, CMTI::GUS possessing the lowest activity (Table 2; Fig. 4A). The linearity of increasing product (MU) concentrations as a function of time was confirmed by time course assays (Fig. 4). To ensure maximum saturation of the enzyme with the substrate, the substrate concentration for the time course assays was based on preliminary kinetic data as 7 times the K_m. Nonlinear regression on MUG saturation curves was performed to calculate the K_m and V_{max} of GUS and its fusions (Table 2). The K_m values for the three enzymes were quite similar but the values

Table 2. Comparison of enzymatic properties of GUS and its fusions: SPI::GUS and CMTI::GUS.

	GUS	SPI::GUS	CMTI::GUS
K_m [mM]	0.87 ± 0.095	0.89 ± 0.05	1.05 ± 0.07
V_{max} [nmoles/min]	52.83 ± 1.33	41.32 ± 0.56	8.8 ± 0.14
V_{max}/K_m	60.72	46.43	8.38

Each protein was tested with MUG as substrate, and the MU product was measured spectrofluorimetrically (see Materials and Methods). The kinetic constants and maximal velocities were determined by the nonlinear least-squares fit using SigmaPlot software (Systat).

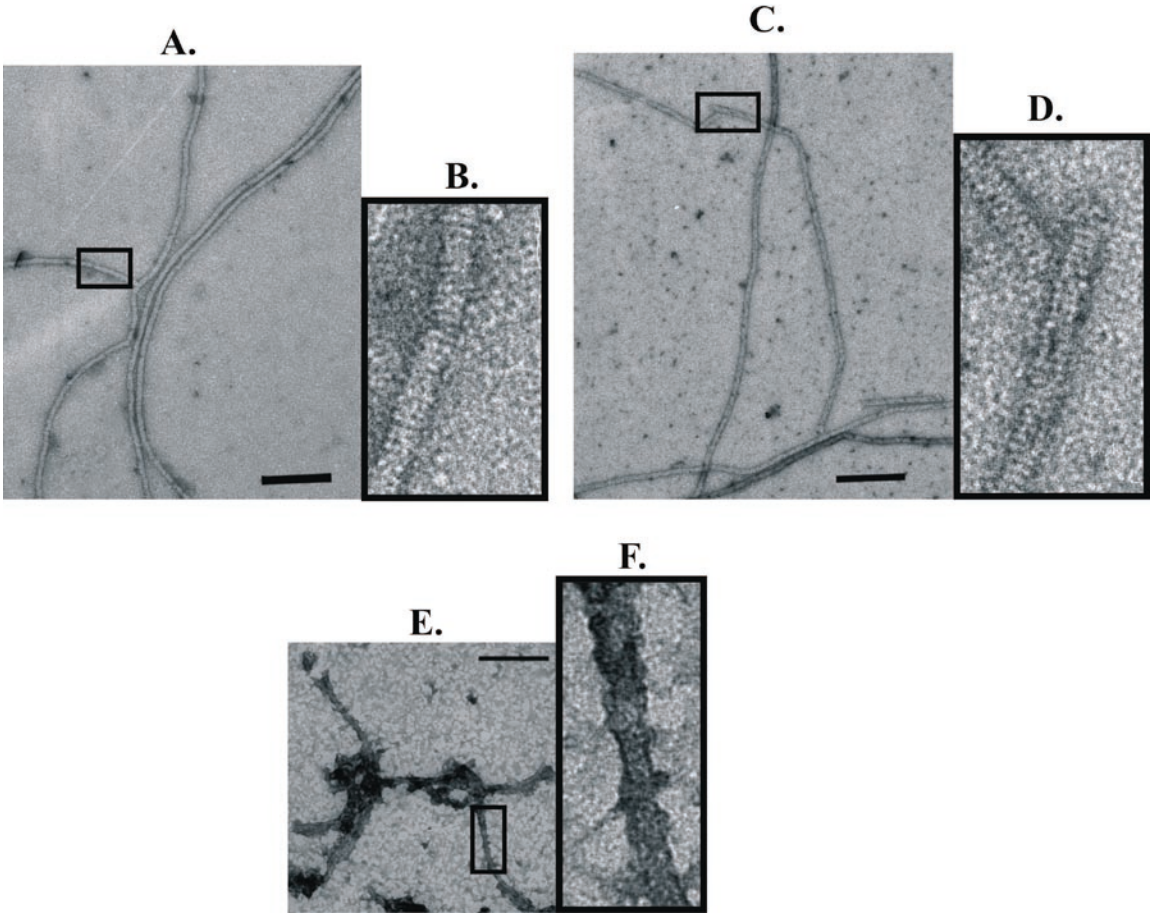

Figure 3. Electron microscopy (negative stain) of recombinant CP, SPI::CP and CMTI::CP particles.
A, **C** and **E**. CP VLPs, SPI::CP VLPs and CMTI::CP, respectively. Bar represents 200 nm. **B**, **D** and **F**, digital magnification (6.5 times) of the fragments marked by rectangles in **A**, **C**, **E**, respectively.

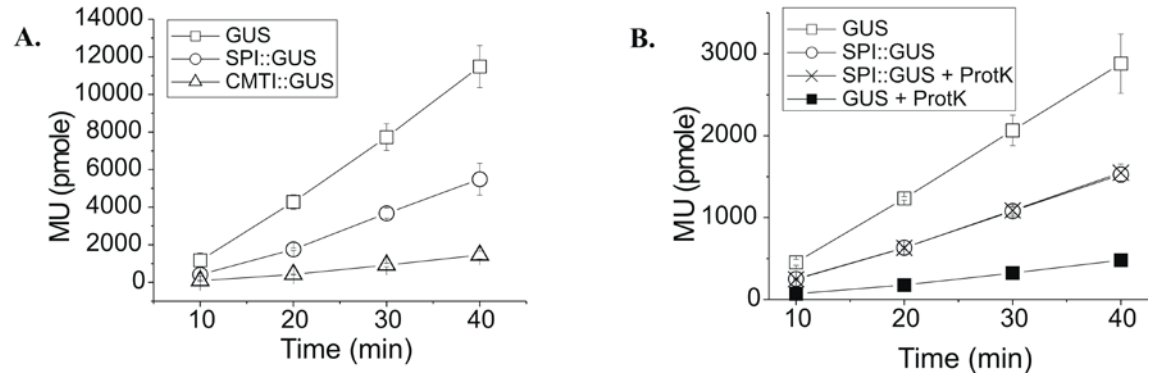

Figure 4. Enzymatic activity of GUS and its fusions.
A. Each protein (0.011 pmole) was tested with 7 mM MUG as substrate and the appearance of MU was measured spectrofluorimetrically at 10 min intervals (Materials and Methods). **B**. Enzymatic activity of 0.011 pmoles of SPI::GUS or GUS after 2.5 h incubation with or without proteinase K.

of maximal velocities were significantly lower for the fusion proteins, the lowest V_{max} being for CMTI::GUS. Although the ratio of V_{max} to K_m is not an ideal tool, it can be used to compare enzymatic efficiency. The V_{max}/K_m ratio suggested that the fusion with proteinase inhibitors changed the enzymatic activity of the GUS target protein especially when fused to CMTI I as clearly confirmed by time course assays (Table 2; Fig. 4A).

In conclusion, when fused to SPI2, specific features and functions of both target proteins were retained, with some reduction of the target protein enzymatic activity. On the contrary, fusion with CMTI I resulted in structural changes of the CP impeding VLP formation as well as a significant inhibition of GUS activity.

Inhibitory properties of recombinant proteins

SPI2 is known to inhibit fungal and bacterial serine proteinases with the strongest inhibition directed against proteinase K (Nirmala *et al.*, 2001). Likewise, CMTI I is active against trypsin and some medically important serine proteinases (Otlewski *et al.*, 1990; Hayashi *et al.*, 1994). Considering its availability, proteinase K was chosen to study the inhibitory capacity of SPI2 analysed alone or fused to CP or to GUS. Similarly, CMTI I and its both fusions were analysed in reactions with trypsin. The inhibitory activities of the recombinant proteins towards proteinase K or trypsin were determined by quantification of IC_{50} values using azocoll (Tables 3 and 4). The IC_{50} is the concentration of inhibitor required for 50% reduction of proteinase activity. The IC_{50} values of both fusions with SPI2 (23.6 and 80.6 nM for SPI::CP and SPI::GUS, respectively) were in a similar, nanomolar range as the values obtained for SPI2 alone (18.3 nM). Importantly, the three proteins SPI2, SPI::CP and SPI::GUS were much stronger inhibitors of proteinase K than the commercially available AEBSF (2.75×10^6 nM). Even the highest IC_{50} value obtained for SPI::GUS (80.6 nM) was about thirty thousand times lower than that for AEBSF (2.75×10^6 nM). Similarly, the IC_{50} of proteins fused to CMTI I were

Table 3. Inhibition of proteinase K activity by SPI2 and its fusions *versus* that by AEBSF.

	IC_{50} [nM]	K_i [nM]
SPI	18.3±1.7	80±3.5
SPI::CP	23.6±3.2	90±5.4
SPI::GUS	80.6±4.2	195±20
CP	no inhibition	
GUS	no inhibition	
AEBSF	$(2.75\pm0.12)\times10^6$	

IC_{50}, concentration (nM) of inhibitor required for 50% reduction of proteinase activity. Proteinase K activity was measured as described in Materials and Methods with 28.7 nM proteinase K. The inhibition of proteinase K activity was observed with neither CP (up to 4200 nM) nor with GUS (up to 166 nM). The inhibitory dissociation constants (K_i) were determined by the nonlinear least-squares fit using SigmaPlot software (Systat).

Table 4. Inhibition of trypsin activity by CMTI I and its fusions *versus* that by AEBSF.

	IC_{50} [nM]	K_i [nM]
CMTI	4.8±0.47	5.3±0.8
CMTI::CP	468.5±79.6	743.2±85
CMTI::GUS	no inhibition	
CP	no inhibition	
GUS	no inhibition	
AEBSF	3117.6±116.5	

IC_{50}, concentration (nM) of inhibitor required for 50% reduction of proteinase activity. Trypsin activity was measured as described in Materials and Methods with 34.7 nM trypsin per reaction. The inhibition of trypsin activity was observed with neither CMTI::GUS (up to 2800 nM), CP (up to 500 nM) nor GUS (up to 2800 nM). The inhibitory dissociation constants (K_i) were determined by the nonlinear least-squares fit using SigmaPlot software (Systat).

compared to the IC_{50} calculated for non-fused CMTI I and AEBSF (Table 4). For CMTI::GUS, inhibition of trypsin activity was not observed. Although the IC_{50} for CMTI::CP (468.5 nM) was about one hundred times higher than the IC_{50} for CMTI I alone (4.8 nM), it was still about six times lower than the IC_{50} for AEBSF (Table 4). Inhibition of proteinase K or trypsin was not observed for either target protein used alone — CP or GUS (Tables 3 and 4).

Inhibition kinetics experiments allowed us to compare the inhibition mechanisms of SPI2 and CMTI I alone or fused to either target protein. Values of the apparent catalytic constants (not shown) and inhibition parameters (inhibitory dissociation constants — K_i) for SPI2 and its fusions, or CMTI I and its fusions (Tables 3 and 4) were determined using succinylated casein as substrate and proteinase K or trypsin, respectively. Calculation of the best estimate of the K_i for the reversible enzyme-inhibitor complex was performed by nonlinear regression analysis. Initial kinetic assessment, at two inhibitor concentrations, illustrated by Lineweaver-Burk (Figs. 5 and 6) or Hanes-Wolf (not shown) plots indicated the same competitive-type inhibition for the SPI2 and CMTI I inhibitors and their fusions. The K_i values were generally in agreement with the IC_{50} values obtained and testified that the fusion proteins appear to be rather strong inhibitors. Nevertheless, a change of the K_i values of SPI::GUS and especially of CMTI::CP in comparison to the K_i of SPI2 and CMTI I suggested that fusion of the inhibitor with the target protein reduced the strength of the interactions between the inhibitory domain of the fusion protein and the cognate proteinase (Tables 3 and 4).

To further test whether fusion with small proteinase inhibitors might influence the stability of the target proteins during the enzymatic reaction, comparative proteolytic digestions were performed. The results of proteinase K digestions were monitored first by spectrofluorimetric evaluation of the enzymatic activity of GUS and its SPI2 fusion (Fig. 4B) and were also visualised by SDS/PAGE (Fig. 7). In all experiments, to achieve efficient proteinase K inhibition, the molar ratio between the inhibitors and proteinase K was about 12 times higher than that allowing 50% reduction of proteinase K activity. In such conditions the enzymatic activity of GUS was lost but the activity of SPI::GUS was

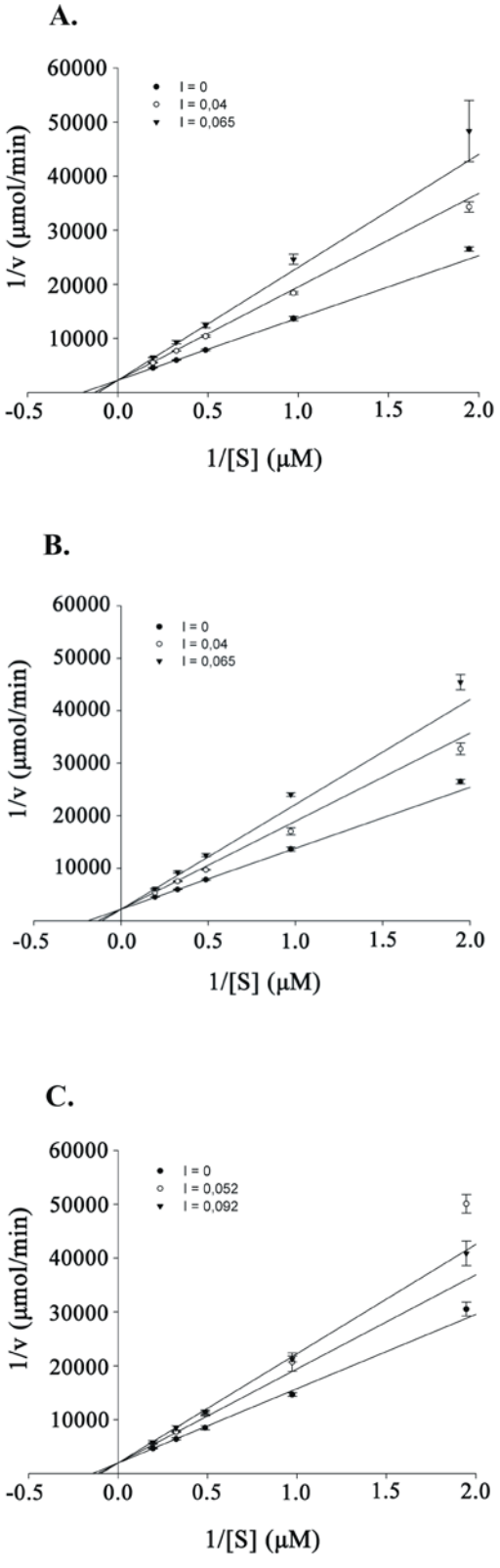

Figure 5. Lineweaver–Burk plots of the inhibition of proteinase K by SPI2 and its fusions.
A. Proteinase K activity was measured in the absence (●) or presence of 0.04 µM (○) or 0.065 µM (▼) SPI2. **B**. Proteinase K activity was measured in the absence (●) or presence of 0.04 µM (○) or 0.065 µM (▼) SPI::CP. **C**. Proteinase K activity was measured in the absence (●) or presence of 0.052 µM (○) or 0.092 µM (▼) SPI::GUS. The reciprocal of the velocity of substrate hydrolysis (1/v) for each inhibitor concentration was plotted against the reciprocal of substrate concentration (1/[S]); each point represents the mean value of three experiments and "I" indicates the inhibitor concentration. The straight lines indicate the best fit for the data obtained by linear regression analysis (Sigma Plot, Jandel Scientific, San Rafael, CA, USA).

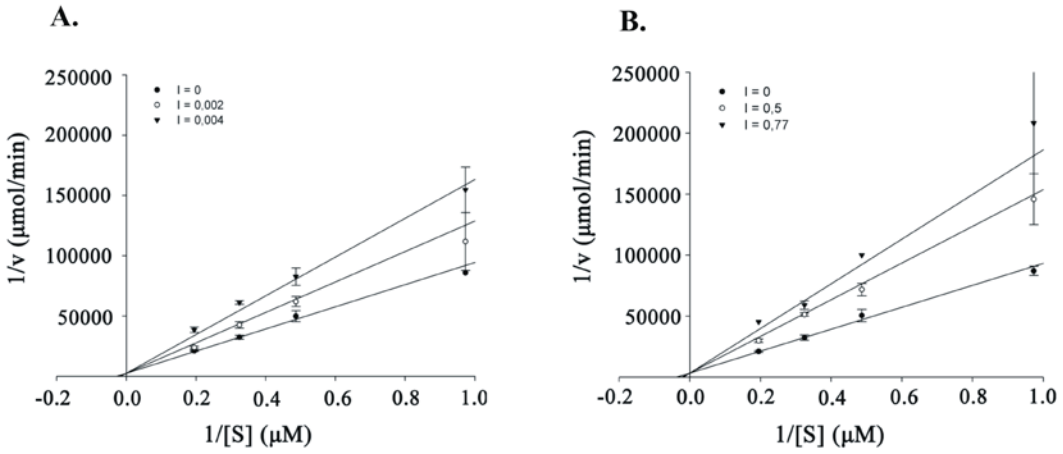

Figure 6. Lineweaver–Burk plots of the inhibition of trypsin by CMTI I and CMTI::CP.
A. Trypsin activity was measured in the absence (●) or presence of 0.002 µM (○) or 0.004 µM (▼) CMTI I. **B**. Trypsin activity was measured in the absence (●) or presence of 0.5 µM (○) or 0.77 µM (▼) CMTI::CP. The reciprocal of the velocity of substrate hydrolysis (1/v) for each inhibitor concentration was plotted against the reciprocal of substrate concentration (1/[S]); each point represents the mean value of three experiments and "I" indicates the inhibitor concentration. The straight lines indicate the best fit for the data obtained by linear regression analysis (Sigma Plot, Jandel Scientific, San Rafael, CA, USA).

totally retained (Fig. 4B). In further analyses the second target protein — CP, its fusion to SPI2 and an independent protein — BSA were submitted to proteinase K digestion in the absence or presence of SPI2 or AEBSF (Fig. 7). CP or BSA (Fig. 7, lanes 7, 6) were readily hydrolysed by proteinase K. In contrast, when SPI2 was added to the digestion mixtures the CP and BSA (Fig. 7, lanes 9, 8) were protected from proteolysis. When lower amounts of SPI2 were tested, inhibition of proteinase K activity was partial or not observed (not shown). Similarly, inhibition of proteinase K was achieved when SPI::CP was used (Fig. 7, lanes 10 and 11). SPI2 fused to CP protected from hydrolysis by proteinase K not only its fusion partner domain (CP) but also added BSA (Fig. 7, lane 10). In contrast, AEBSF used at a concentration equal to the concentration of SPI::CP was unable to prevent proteolysis (Fig. 7, lanes 12 and 13*).*

DISCUSSION

The aim of our study was to determine whether selected small proteinase inhibitors fused to a target protein could be used as tools protecting the target proteins from proteolysis. To this end, SPI2 from *G. mellonella* silk (Nirmala *et al.*, 2001) and CMTI I from *C. maxima* (Bolewska *et al.*, 1995) were selected as proteinase inhibitors, and the CP of PVY and *E. coli* GUS as target proteins. Both SPI2 and CMTI I are small inhibitors with a

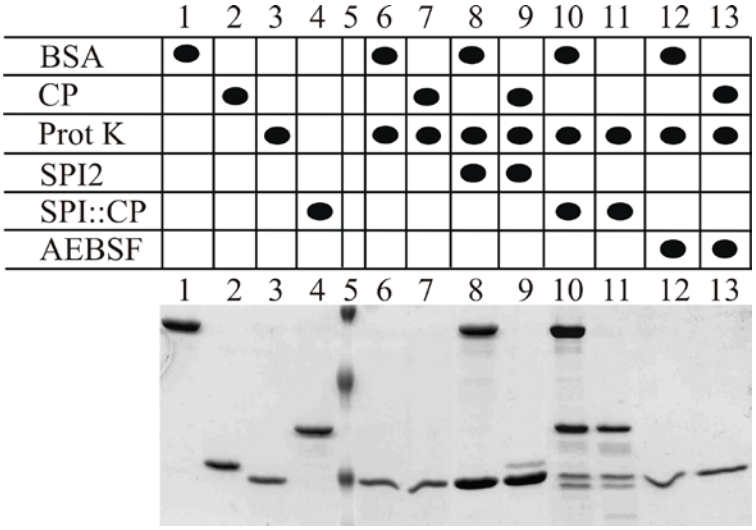

Figure 7. SDS/PAGE analysis of recombinant CP and SPI::CP after proteolytic digestion with proteinase K in the absence or presence of inhibitors.
SDS/PAGE was performed for recombinant CP, SPI::CP and BSA (used as internal control) after incubation without (lanes 1, 2 and 4) or with (lanes 6–13) proteinase K. The inhibitors were present in the reactions electrophoresed in lanes 8–13 (Materials and Methods). Protein ladder: lane 5, bands are: 90, 48, 36 kDa, respectively. The components of the digestion mixtures are marked by black ovals in the diagram above the gel.

well-known rigid structure stabilized by conserved disulfide bridges. Both are serine proteinase inhibitors but belong to two different families. They are competitive inhibitors and share the same canonical-like mechanism of inhibition. They inhibit proteolysis by fitting their reactive loop with an exposed reactive site residue P1 into a cavity of the active site of the proteinase (Laskowski *et al.*, 2000). Considering the composition of the fusion proteins it is worth noting that the P1 residues of SPI2 and CMTI I are situated in the N-terminal regions of each sequence. Moreover, the C-terminus of CMTI I makes only 7 of 138 contacts with trypsin (Otlewski & Krowarsch, 1996). Similarly, the C-terminus of SPI2 seems to be spatially distant from the active centre of the inhibitor and its P1 residue (I. Zhukov, unpublished results). The above data strongly suggest that fusion of the target protein to the C-terminus of SPI2 or CMTI I should be neutral with respect to the functions of the two inhibitors. Selection of the target proteins was performed to allow easy testing of two different polypeptide functions: the capacity of the CP to self-organize and the enzymatic activity of GUS. Both their genes and the target proteins selected are genetically and biochemically very well characterized. Native PVY CP possesses the capacity to form well-defined superstructures — VLPs, with the N- and C-terminal regions exposed on the surface of the VLPs (Shukla & Ward, 1989). Moreover, surface exposition of the long and highly immunogenic N-terminus prevails over exposition of the C-terminus (Shukla *et al.*, 1988) suggesting that fusing an additional polypeptide to the N-terminus of the CP should not affect its self-organization capacity. The tolerance of the N-terminal fusion has previously been demonstrated for other potyviruses (Jagadish *et al.*, 1996; Fernandez-Fernandez *et al.*, 1998). GUS was chosen as a second target since it allows easy testing of the enzymatic properties of its projected fusion proteins, and since it tolerates large amino-terminal fusions without loss of enzymatic activity (Elmayan & Tepfer, 1994; Vilaine *et al.*, 1998; Huang *et al.*, 2003). For these reasons, the C-terminus of each inhibitor was fused to the N-terminus of the CP or GUS.

The expression of the recombinant proteins with a His-tag at their C-terminus was quite efficient, especially for CP, SPI::CP and GUS (Fig. 2B, D); in the case of CMTI::CP and both SPI::GUS and CMTI::GUS, the expression level was lower. Although the expression level of the fusion proteins was lower than that of the target proteins (CP and GUS) expressed alone, sufficient amounts of product could be obtained; even with CMTI::GUS, the recombinant protein with the lowest expression level, it was possible to purify about 15 mg of protein from one litre of culture. The growth of cells producing GUS and its fusions was not correlated with protein expression level, since neither high expression of GUS alone nor low production of GUS fused to the proteinase inhibitors affected cell growth (Fig. 2C, D). In contrast, expression of CP and its fusions was deleterious to cell growth (Fig. 2A, B). In this case the accumulation of proteins and their oligomerization into VLPs could be an additional obstacle for bacterial metabolism (Jagadish *et al.*, 1993; Edwards *et al.*, 1994; Kurland & Dong, 1996; Hammond *et al.*, 1998).

Despite numerous similarities between the two proteinase inhibitors, when fused to the target proteins they affected the function of the targets in quite different manners. Whereas fusion with SPI2 did not influence the self-assembly of CP and only slightly decreased the enzymatic activity of GUS, fusion with CMTI I clearly hindered VLP formation and significantly reduced GUS activity (Figs. 3 and 4A; Table 2). Since literature data suggest that both target proteins should easily tolerate an additional polypeptide at their N-terminus (Elmayan & Tepfer, 1994; Jagadish *et al.*, 1996; Fernandez-Fernandez *et al.*, 1998; Vilaine *et al.*, 1998; Huang *et al.*, 2003), the differences observed may result from the impact exerted on the target protein by the inhibitory domain. CMTI I is slightly shorter (by 5 amino acids) than SPI2 and its structure is stabilized by three disulfide bridges. It has been reported that mutation of even one amino acid can considerably affect the stability and folding process of CMTI I (Zhukov *et al.*, 2000). Therefore, it is probable that the fusion of the CMTI I C-terminus with the target protein could have an impact on the proper folding of the inhibitory domain and consequently might structurally destabilize the target domain. In contrast, SPI2 is 5 amino acids longer than CMTI I and its structure is stabilized by only two disulfide bridges. Nuclear magnetic resonance spectroscopy suggests that the structure and folding processes of SPI2 are very stable (I. Zhukov, unpublished data). Therefore, the additional domain fused to the C-terminus of SPI2 should be well tolerated and proper folding of both fusion domains would be achieved. Additionally, according to a BLAST query, domains with homology to SPI2 seem to be present in various multidomain proteins (not shown). On the contrary, homologues of CMTI I were not detected in multidomain proteins. These observations could reflect the evolutionary tolerance of functional fusions of SPI2-like domains with numerous polypeptides. This is in agreement with our observation that CMTI I in the fusion proteins could lead to destabilization of the target polypeptide domains whereas fusion with SPI2 seems to be rather neutral to the function of the targets analysed. Such incompatibility of partner domains also seems to be responsible

for the loss of inhibitory properties of the CMTI I domain in the CMTI::GUS fusion (Table 4).

The IC_{50} values obtained and the results of proteolysis experiments showed that both SPI2 and CMTI I used alone or as fusions were much stronger inhibitors of proteinase K and trypsin than AEBSF, a commercial inhibitor recommended for irreversible inactivation of serine proteinases (Fig. 7; Tables 3 and 4). Interestingly, *in vitro* proteolysis experiments demonstrated that SPI fused to the target protein protected its fusion partner domain against proteolysis as well other proteins present in the digestion mixture (Fig. 4B and 7).

Fusion of SPI2 to the target proteins preserved the structural features of the CP as well as the enzymatic activity of GUS and displayed excellent inhibitory activity against proteinase K, indicating proper folding of the SPI2 domain in these fusion products. Preservation of the inhibitor and the fused protein's activities in the model systems presented as well as the observed evolutionary tendency of SPI2-type inhibitors to be a component of multidomain proteins strongly suggest potential biotechnological applications of SPI::target protein fusions. Overexpression of some proteins can be difficult to achieve. Not rarely these difficulties result from proteolytic activities inside the host cells or, as in the case of *Bacillus* expression systems, are due to a large number of extracellular proteinases. In such cases the use of inhibitor::target protein fusions could be preferable to addition of a protease inhibitor separately, or to the coexpression of two independent constructs, one containing the inhibitor and the other the target protein. In the first case, when added separately the inhibitor could meet the barrier of the cell wall and would not be able to protect the target proteins inside the host cells. In the second case, on the contrary to the coexpression of two proteins overproduction of the inhibitor::target protein would guarantee identical expression levels of both the inhibitor and target domains and better protection of such target domains. In *Bacillus* expression systems the problems with degradation of foreign, secreted proteins have been overcome by using extracellular proteinase-deficient mutants. Nevertheless, reports have suggested that growth of such mutants is limited to some extent in protein-rich industrial media (Schallmey *et al.*, 2004). Considering the strong inhibition of bacterial proteinases (especially subtilisin from *B. subtilis*) by SPI2, the use of SPI::target protein in *Bacillus* expression systems could be advantageous. Moreover, during the purification steps the inhibitor fused to the target protein would always copurify with the protein of interest, even during fractionation that normally would lead to loss of protection for separately expressed components. It is worth noting that both SPI2 and CMTI I used alone appeared to be much stronger proteinase inhibitors than a commercially available inhibitor of this class of proteinases. Therefore, the practical application of these inhibitors as replacements or supplements of commercially available inhibitors or inhibitor cocktails should be considered. Finally, molecular engineering of the active centre of the inhibitor (including the P1 residue) may enlarge the present range of target proteinases (Laskowski & Kato, 1980; Otlewski & Krowarsch, 1996; Grzesiak *et al.*, 2000).

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