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Covalent immobilization of tobacco-etch-virus NIa protease: a useful tool for cleavage of the histidine tag of recombinant proteins

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Addition of tags [such as His (histidine) tags] is extremely helpful for the affinity purification of recombinant proteins. In several cases, these tags must be removed before performing functional and structural studies. The enzyme most frequently used to cleave tags of recombinant proteins is the TEV-protease (tobacco-etch-virus NIa protease). The continuous production of this enzyme in soluble form is quite an expensive process and not easily accessible to many laboratories. Thus an interesting alternative is the use of TEV-protease in an immobilized form, which may be reutilized several times. The main objective of the present study was to obtain a TEV-protease in an immobilized form, by covalent immobilization on to solid supports through selective use of different amino acid residues, lysine or cysteine. High protein immobilization yields (75–97%) were obtained with both strategies. The TEV-protease immobilized through its exposed cysteine thiol groups maintained its ability for cleaving a 20 kDa substrate. While the activity of the immobilized TEV-protease maintained only 30% of the activity of the enzyme in soluble form, its stability at 4°C was improved three times. Moreover, this enzyme could be reutilized in at least five cycles of cleavage without loss of performance. The present results indicate that the use of a TEV-protease in an immobilized form is a potentially useful tool for the cleavage of His tags of recombinant proteins and may be useful for reducing the cost of the total process of cleavage.

Introduction

The use of affinity tags has facilitated the purification of a high number of proteins for applications in biochemical, therapeutic and structural studies [1–3]. The most common affinity tags include the His (histidine) tag [4], the *Schistosoma japonicum* GST (glutathione transferase) [5], the *Escherichia coli* MBD (maltose-binding domain) [6] and the *E. coli* TRX (thioredoxin) [7]. In some cases, the use of MBD or TRX increased the solubility of the target protein [6,8,9]. However, the presence of a tag may disturb the protein conformation, as reported for the DNA-binding protein AreA from *Aspergillus nidulans* [10]. The relative position of the His tag (N-terminus or C-terminus) seems also to be important. For example, positioning a His tag in the C-terminus of the tumour-associated single-chain Fv construct covered the antigen-binding site and adversely affected its binding properties [11]. In the case of the trimeric cytokine TNFα (tumour necrosis factor α), the biological activity was recovered to the expected normal values only when the His tag was removed [12]. Furthermore, the cleavage of the His tag from the SH3 domain (Src homology 3 domain) from chicken Src tyrosine kinase (Src SH3) was essential for the crystallization of both wild-type (WT) and mutant Src SH3 [13]. All these lines of evidence emphasize the importance of characterizing, at a functional level, recombinant proteins expressed with purification tags before performing biochemical and structural studies.

A large repertoire of proteases with different specificities and varying length of recognition sequence are available to remove the tags, such as Factor Xa [14], thrombin [15], enterokinase [16] and TEV-protease (tobacco-etch-virus NIa protease) [17]. High specificity and good activity under a wide range of conditions make TEV-protease a popular choice for cleaving tags of fusion proteins. TEV-protease

Key words: covalent immobilization, histidine tag, recombinant protein, thiolsulfinate–agarose, tobacco etch virus NIa protease (TEV-protease).

Abbreviations used: DTT, dithiothreitol; G–agarose, glutaraldehyde–agarose; His tag, histidine tag; IPTG, isopropyl β-D-thiogalactoside; LB, Luria–Bertani; MBD, maltose-binding domain; SH3 domain, Src homology 3 domain; TEV-protease, tobacco-etch virus NIa protease; TRX, thioredoxin; TSI–agarose, thiolsulfinate–agarose; WT, wild-type.

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recognizes an extended seven-amino-acid consensus sequence (EXXYXQ$^{*S}/_{G}$) (where X is any residue and $*$ indicates the cleavage site) [18,19] improbably present in non-viral sequences. TEV-protease cleaves tags efficiently even at low temperature and in the presence of various protease inhibitors [20]. The production of TEV-protease in *E. coli* has been hampered by auto-inactivation, codon bias and low solubility of the protein. The problem with codon bias can be solved by tRNA supplementation and mutations [21]. Auto-inactivation has been largely eliminated by substitutions on residue 219 [22,23]. The low solubility has been improved by three strategies. In one case, the solubility of the TEV-protease was enhanced by introducing three amino acid substitutions into the protein (protein mutant named TEV_{SH}-protease), resulting in a 5-fold increase in the yield of purified protease [24]. In another case, 80% of TEV-protease was expressed in the soluble fraction (65 mg/l) by chaperone co-expression and low temperature [25]. Based on these works, Blommel and Fox [26] combined a new construct of TEV-protease and the use of fermenters, resulting in 400 mg/l of culture. When the TEV-protease is used in soluble form, it should be separated from the tag-cleaved protein. This separation is normally achieved by one step of size-exclusion chromatography, which allows also the separation from the His tag and in some cases from the non-cleaved protein. However, when the molecular mass of the cleaved protein is similar to that of the TEV-protease, additional purification steps are needed, increasing the steps of the total process. Moreover, when a great number of proteins are studied, as in structural genomic projects, for protein crystallization, the production of the soluble TEV-protease should be performed continuously in the laboratory, increasing the total cost of the purification process.

As not many laboratories have the possibility of a continuous production of soluble TEV-protease, an interesting alternative is the use of TEV-protease covalently immobilized onto a solid support. This would allow the separation of the TEV-protease by a simple filtration or centrifugation process as well as its reuse, reducing the cost of the process. The properties of the immobilized protein can be manipulated depending on several factors such as immobilization chemistry, the amino acid residues involved in the interaction, the number of bonds between the protein and the support and the environment surrounding the immobilized molecule [27–31]. The objective of the present study was to obtain a TEV-protease immobilized by covalent bonds to an insoluble support through the selective use of different residues of its surface (lysine or cysteine). Two immobilization chemistries were analysed in order to obtain an active and stable biocatalyst that can be reused in several processes of cleavage of His tags of recombinant proteins.

Methods

Protein expression and purification

The plasmid pTH24–TEV_{SH} encoding TEV_{SH}-protease was provided by the group of Helena Berglund (Karolinska Institutet, Stockholm, Sweden). Competent *E. coli* BL21(DE3)-pLysS cells were transformed with pTH24-TEV_{SH} to produce recombinant His-tagged proteins. Colonies were selected on LB (Luria–Bertani) agar plates containing 100 μg/ml ampicillin and 50 μg/ml chloramphenicol. Precultures of 10 ml of LB supplemented with the same antibiotics were grown overnight at 37 °C and then diluted 200-fold into the same medium and incubated with agitation (140 rev./min) until the attenuance at 600 nm (D_{600}) reached 0.6. Then, cultures were incubated at 20 °C, and, after equilibration at this temperature, protein expression was induced with 1 mM IPTG (isopropyl β-D-thiogalactoside) for 20 h. Cells were harvested by centrifugation at 2500 ***g*** for 15 min. The cell pellet from 1 litre of culture was resuspended in 50 ml of cold lysis buffer (20 mM Tris/HCl, pH 8.0, 500 mM NaCl, 10% glycerol and 10 mM imidazole) containing a protease inhibitor cocktail (Amersham Biosciences) and lysed by sonication (Fisher Scientific model 60 sonic dismembrator) in iced water. Cell debris was removed by centrifugation at 8500 ***g*** for 1 h at 4 °C and the His tag TEV_{SH} protein was purified by metal-affinity chromatography, from the supernatant, using Chelating Sepharose Fast Flow (GE Healthcare, Uppsala, Sweden) previously loaded with 0.1 M $CuSO_4$. The supernatant was incubated for 1 h at 4 °C under agitation with 6 ml of the resin equilibrated in lysis buffer, following the manufacturer's instructions. The unbound protein was then washed with 5 bed volumes of buffer A (20 mM Tris/HCl, pH 8.0, 500 mM NaCl, 10% glycerol, 20 mM imidazole and 1 mM 2-mercaptoethanol) and with buffer B (buffer A with 30 mM imidazole). The recombinant proteins bound to the resin were eluted in five steps with 2 bed volumes of buffer C (buffer A with 300 mM imidazole). Fractions were analysed by SDS/PAGE and visualized by staining with Coomassie Brilliant Blue, and the fractions containing purified recombinant protein were pooled and dialysed at 4 °C in four steps of 2 h against buffer D [25 mM Tris/HCl, pH 8.0, 200 mM NaCl, 2 mM EDTA, 20% glycerol and 2 mM DTT (dithiothreitol)] to gradually decrease the concentration of imidazole to avoid protein precipitation (150, 75, 35 and 0 mM imidazole).

The plasmid encoding TEV_{WT}-protease was provided by the group of Pedro Alzari (Institute Pasteur, Paris, France). TEV_{WT}-protease was expressed by induction with 0.5 mM IPTG for 3 h and purified using denaturant conditions. For this, cells from 1 litre of culture were resuspended in 50 ml of buffer E (10 mM Tris/HCl, pH 8.0, 100 mM NaH_2PO_4 and 6 M guanidinium chloride) and incubated for 30 min at 65 °C and lysed by sonication. Cell debris was removed by

centrifugation at 8500 ***g*** for 1 h at 4 °C and the supernatant was incubated with 4 ml of Cu^{2+}-chelating Sepharose. The unbound protein was then washed in four steps with 6 bed volumes of buffer F (100 mM NaH_2PO_4, pH 8.0, 8 M urea and 10 mM Tris/HCl) and with buffer F at pH 6.0. The recombinant proteins bound to the resin were eluted in four steps with 2 bed volumes of buffer F at pH 4.0. The fractions containing purified recombinant protein were pooled, adjusted to pH 8.5 and dialysed at 4 °C in two steps of 2 h against buffer G (100 mM Tris/HCl, pH 8.5, 500 mM NaCl, 0.5 mM EDTA, 20% glycerol and 5 mM DTT) and two steps of 2 h against the buffer G containing 40% glycerol.

The plasmid pDEST17 encoding a phosphotyrosine phosphatase (Tc00.1047053503471.10) from *Trypanosoma cruzi* was obtained from the laboratory of Dr Samuel Goldenberg (Instituto de Biologia Molecular do Paraná, Curitiba, Paraná, Brazil). This 20 kDa TEV-substrate was expressed and purified as described for the TEV_{SH}-protease, but with some modifications: protein expression was carried out at 15 °C; cells from 1 litre of culture were resuspended in 30 ml of lysis buffer; after sonication, the supernatant was incubated with 3 ml of Cu^{2+}-chelating Sepharose; the unbound protein was washed with buffer A and buffer B containing 0.5% Triton X-100; the dialysis was performed in buffer H (20 mM Tris/HCl, pH 8.0, 0.5 mM NaCl, 5 mM DTT and 10% glycerol) containing decreasing imidazole concentrations.

Any precipitated material appearing during the dialysis of TEV_{SH} and TEV_{WT} proteases and 20 kDa TEV-substrate was removed by centrifugation and protein concentration was determined by the Bradford assay (Bio-Rad) using BSA as the standard [32]. To purify these proteins and to separate monomeric proteins from possible aggregates present in the samples, a further step of purification by size-exclusion chromatography was performed in an AKTA Purifier System (AKTA Basic; General Electrics), using the Superdex 200 16/60 prep. grade column (GE Healthcare) previously washed and equilibrated in the last dialysis buffer, following the manufacturer's instructions. Samples were eluted with 2 bed column volumes of the equilibration buffer and fractions containing the recombinant protein (TEV_{SH} and TEV_{WT} proteases, or 20 kDa TEV-substrate) were pooled and concentrated in 10 kDa cut-off concentrators (Millipore Amicon Ultra-15 membrane) to a final concentration of 2 or 3 mg/ml and stored at −20 °C.

TSI–agarose (thiolsulfinate–agarose) synthesis

TSI–agarose was prepared and titrated, essentially as described by Batista-Viera et al. [33]. In this method, the agarose beads (Sepharose-4B) are first made to react with epichlorohydrin in an alkaline medium. The oxirane groups thus formed are then converted with sodium thiolsulfate into support-bound thiosulfate groups (Bunte salt), which are finally reduced with DTT to thiol groups. Then the thiol groups are first oxidized with potassium ferricyanide to disulfide structures, and in a second step, controlled oxidation of the agarose disulfide groups to form TSI moieties is performed with the oxidizing agent magnesium monoperoxyphthalate (see scheme in Figure 2B). The titration of the TSI structures is performed by back titration of the remaining GSH free in solution, after its incubation with TSI supports. The free GSH is made to react with 2,2′-dipyridyldisulfide, and the 2-thiopyridone released is measured at 343 nm. The TSI–agarose used contained 16 μmol of reactive structures per gram of suction-dried support.

G–agarose (glutaraldehyde–agarose) synthesis

G–agarose was prepared and titrated as described previously by Guisán et al. [34]. In this method, the agarose beads (Sepharose-4B) are first made to react with glycidol in an alkaline medium in the presence of sodium borohydride. The glyceryl-agarose thus formed is then oxidized to glyoxyl-agarose with sodium periodate. The titration of the aldehyde groups obtained is performed by back titration of the non-consumed sodium periodate with potassium iodide. Then the glyoxyl-agarose is made to react with ethylenediamine at pH 10 to form a Schiff base, which is reduced with sodium borohydride to a stable alkyl-amine bond (Amine–agarose). The last step of the support synthesis is the reaction of the bound amine groups with glutaraldehyde to form G–agarose (see scheme in Figure 2A). Two G–agarose supports were used, one prepared using agarose with 75 μmol of amine groups/g of suction-dried support (G75–agarose) and the other prepared with agarose containing 15 μmol of amine groups/g of suction-dried support (G15–agarose).

TEV-protease immobilization

For the immobilization on to TSI–agarose, aliquots of 0.1 g of suction-dried TSI–agarose were incubated with 1 ml of TEV-protease solution (1 mg/ml) in 0.1 M sodium phosphate (pH 8.0). The mixtures were gently agitated for 16 h at 4 °C and then washed with 0.1 M sodium phosphate buffer (pH 7.0). To completely block the remaining TSI groups and to wash out the non-specifically bound ligands, the TEV-protease–TSI–agarose was washed in three steps with 1 ml of buffer I (0.1 M sodium phosphate, pH 7.0, and 2.5 mM of GSH), washed in three steps with 1 ml of buffer J (0.1 M sodium phosphate, pH 7.0, and 0.5 M NaCl), washed in three steps with 1 ml of buffer I and incubated with buffer I for 30 min at 25 °C. After this, the support was washed again as described previously with buffer J, buffer I and once more with buffer J. Finally, it was washed with activity buffer (50 mM Tris/HCl, pH 7.0, 0.5 mM EDTA and 1 mM DTT) and immediately used or stored at 4 °C. To avoid the release of TEV-protease from the support during the activity tests,

the DTT concentration of the activity buffer was previously defined.

For the TEV-protease immobilization on to G–agarose, aliquots of 0.1 g of suction-dried G–agarose was incubated with 1 ml of TEV_{SH}-protease solution (1 mg/ml) in 0.2 M sodium phosphate buffer (pH 7.0). The mixtures were gently agitated for 16 h at 4 °C, and then washed with 0.1 M sodium phosphate buffer (pH 7.0) and equilibrated in 40 mM sodium carbonate buffer (pH 10). To reduce the excess of active groups and Schiff bases the TEV_{SH}-protease–G–agarose was suspended in 1 ml of the same buffer containing 1 mg/ml sodium borohydride and gently stirred for 30 min at 25 °C. Then it was washed with activity buffer and immediately used or stored at 4 °C.

The amount of protein immobilized on to TSI–agarose and G–agarose was determined as the difference between the amount of protein applied to the support and the protein recovered in the supernatant and washing fractions and expressed as a percentage of the applied protein.

TEV-protease activity determination

The TEV_{SH}-protease activity was assayed using as substrate a 20 kDa protein phosphatase from *T. cruzi*, which contains the His tag (MSYYHHHHHHLESTSLYKKAGS) and the TEV-protease cleavage site (ENLYFQG). For both soluble and immobilized TEV-proteases, the molar enzyme/substrate ratio used was 1:10. For the soluble TEV-protease assay, 7.6×10^{-5} μmol of TEV_{SH}-protease was incubated with 7.6×10^{-4} μmol of 20 kDa TEV-substrate in a final volume of 65.5 μl of activity buffer (50 mM Tris/HCl, pH 7.0, 0.5 mM EDTA and 1 mM DTT). For the immobilized TEV-protease assay, 6.3×10^{-4} μmol of TEV_{SH}-protease was incubated with 6.25×10^{-3} μmol of 20 kDa TEV-substrate in 625 μl of activity buffer. In both cases, the mixture was agitated gently at 25 °C. For the soluble enzyme, aliquots of 12.5 μl were taken at regular intervals, 4 μl of sample buffer [5×; 0.5 M Tris/HCl, pH 8.2, 10% glycerol, 10% (w/v) SDS, 250 mM DTT and 0.001% Bromophenol Blue] was added and samples were boiled during 5 min to stop the enzymatic reaction. In the case of the immobilized enzyme, the mixture was centrifuged at regular intervals; aliquots of 12.5 μl were taken from the supernatant and treated in the same way as for the soluble enzyme. SDS/PAGE (12 or 15% gel) was carried on, and the amount of cleaved TEV-substrate was quantified using Scion Image analysis (Scion). In each line of the gel the optical density of the protein bands representing the cleaved and non-cleaved form of the substrate was determined. The sum of both values of each line on the gel was used as the 100% to determine the percentage of cleavage. In order to evaluate the enzyme activity, the amount of cleaved TEV-substrate/μg of TEV-protease was plotted against time and the μg of cleaved TEV-substrate/h per μg of TEV-protease was determined using the linear part of the curve.

Soluble and immobilized enzyme storage stability at 4 °C

A solution in activity buffer of the soluble enzyme and a suspension of the immobilized enzyme, containing 0.76 μg of TEV-protease/μl, were stored in a refrigerator at 4 °C for several days. At different periods, aliquots were taken and the remaining enzymatic activity was measured as described above. The residual activities represent the activity at each time of incubation expressed as the percentage of the initial enzyme activity. The SF (stabilization factor) was calculated as the ratio of the half-life of the immobilized enzyme to that of the corresponding soluble enzyme.

Reuse of the TEV-protease–TSI–agarose

0.1 g of immobilized TEV-protease containing 5 mg of protein/g of support was incubated with 100 μg of TEV-substrate in a final volume of 250 μl of activity buffer during 24 h at 25 °C. The TEV-protease–TSI–agarose was filtered, ending the enzymatic reaction. An aliquot of 12 μl was taken from the supernatant, 4 μl of sample buffer was added and it was boiled during 5 min. SDS/PAGE was carried out and the percentage of cleaved TEV-substrate was determined. The TEV-protease–TSI–agarose was washed with activity buffer and stored for 1 day at 4 °C until the next reuse.

Results

Purification of recombinant proteins

The proteases TEV_{WT} and TEV_{SH} and the TEV-substrate were initially purified by affinity chromatography and then by size-exclusion chromatography. Figure 1(A) shows the chromatographic profile of both proteases and the substrate. SDS/PAGE of each major peak (inset of Figure 1A) showed bands at the expected molecular masses, approx. 30 kDa for TEV_{WT} and TEV_{SH} proteases and 20 kDa for TEV-substrate. Possibly due to differences in the purification protocols, more contaminants were observed associated with the TEV_{WT}-protease than with the TEV_{SH}-protease. The yield of purified proteins from 1 litre of bacterial culture was 20 mg for both TEV_{WT} and TEV_{SH} proteases and 5 mg for TEV-substrate. As shown in Figure 1(B), both TEV_{WT} and TEV_{SH} purified proteases were active on the TEV-substrate. Cleavage of the 20 kDa TEV-substrate produced a 16.5 kDa protein and a 3.5 kDa His tag.

TEV-protease immobilization and ability for substrate cleavage

The knowledge of the sequence and structure of TEV-protease allowed us to predict that in solution 12 of 14 lysine

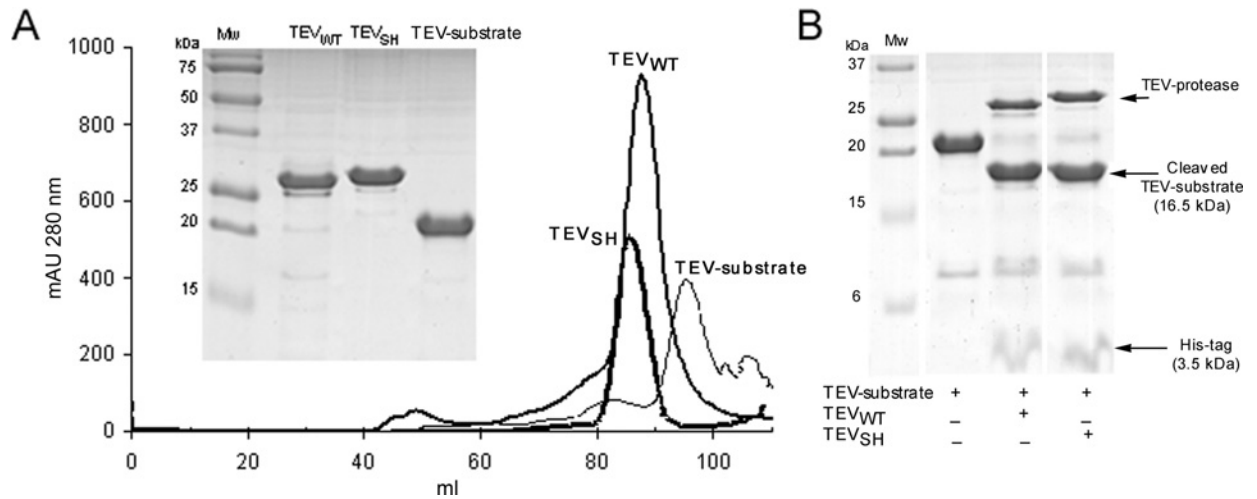

Figure 1 Protein purification and TEV-protease activity

(**A**) Chromatographic profiles of purified TEV_{WT} and TEV_{SH} proteases, and TEV-substrate (20 kDa His tag recombinant protein containing the TEV cleavage site). Following affinity purification, proteins were dialysed, concentrated and injected on a Superdex 200 16/60 prep. grade column (see the Methods section for details). Peaks corresponding to TEV_{WT} and TEV_{SH} proteases, and TEV-substrate, were collected, concentrated and analysed by SDS/PAGE (12% gel) (inset). (**B**) SDS/PAGE (15% gel) of TEV-substrate digested by TEV_{WT} and TEV_{SH} proteases. The molar enzyme/substrate ratio was 1:5, and the reaction mixture was incubated for 24 h at 25 °C. Mw, molecular mass markers. Gels were stained with Coomassie Brilliant Blue R-250.

residues are exposed and distributed all over the protein surface, and only two of four cysteine residues are exposed (Cys-130 and Cys-110) and both are on the same side of the protein surface (see Supplementary Figure S1 at http://www.babonline.org/bab/53/bab530165add.htm). Moreover, these two cysteine residues are distant from the substrate-binding site of the TEV-protease, and are not the catalytic cysteine (Cys-151), which is located inside the protein. On the contrary, several lysine residues are located near the binding site of the TEV-substrate (Lys^{215}, Lys^{220}, Lys^{141} and Lys^{147}).

Considering this information two covalent immobilization strategies were used. One used agarose activated with glutaraldehyde (G15–agarose or G75–agarose) for protein coupling through the ε-amino groups of lysine residues (Figure 2A). The other was based on the formation of disulfide bonds between the TSI groups of the activated agarose support (TSI–agarose) and the thiol groups of cysteine residues (Figure 2B).

After immobilization on supports, the proteins recovered in the supernatant and in the washing fractions were re-collected and subjected to SDS/PAGE, together with the amount of protease offered to the support (Figure 3). Attenuance of the bands corresponding to the TEV-protease was measured. The amount of immobilized protein was calculated as the difference between the protein applied to the support (Figures 3A–3C, line 1) and the protein recovered in the supernatant and washing fractions (Figures 3A–3C, lines 2 and 3), and expressed as a percentage of the applied protein. High protein immobilization yields were obtained with both supports. In the case of TSI–agarose, 75 and 96% of the offered protein were immobilized for TEV_{WT} and TEV_{SH} proteases respectively. For the TEV_{SH}-protease, the immobilization yield on to the G15–agarose and G75–agarose support was 87 and 97% respectively.

Incubation of the supports in the SDS-sample buffer (containing DTT) promoted the release of the TEV-protease immobilized on to TSI–agarose (Figures 3A and 3B, step 4) but not of the TEV-protease immobilized on to the G–agarose (Figure 3C, line 4). This result was as expected; in fact, while both immobilization strategies are covalent, the attachment of proteins on to the TSI–agarose is reversible, whereas the other is not (see Figure 2).

In order to evaluate whether the activity of the TEV-protease after immobilization on supports was maintained, their ability to cleave a 20 kDa TEV-substrate was tested (Figure 4). The supernatant of the cleavage reaction was subjected to SDS/PAGE. In the case of the TEV-protease immobilized on to TSI–agarose, only one band corresponding to the cleaved substrate (16.5 kDa) appeared on the gel, indicating a high efficiency of cleavage (Figure 4A, line 2). On the contrary, under the same assay conditions, the TEV-protease immobilized on to G–agarose was less or not efficient. In the case of using G15–agarose as support, two bands corresponding to the cleaved and the non-cleaved form of the substrate appeared on the gel (Figure 4B, line 2), whereas no cleaved form appeared after immobilization on G75 support (results not shown). Thus we detected a loss

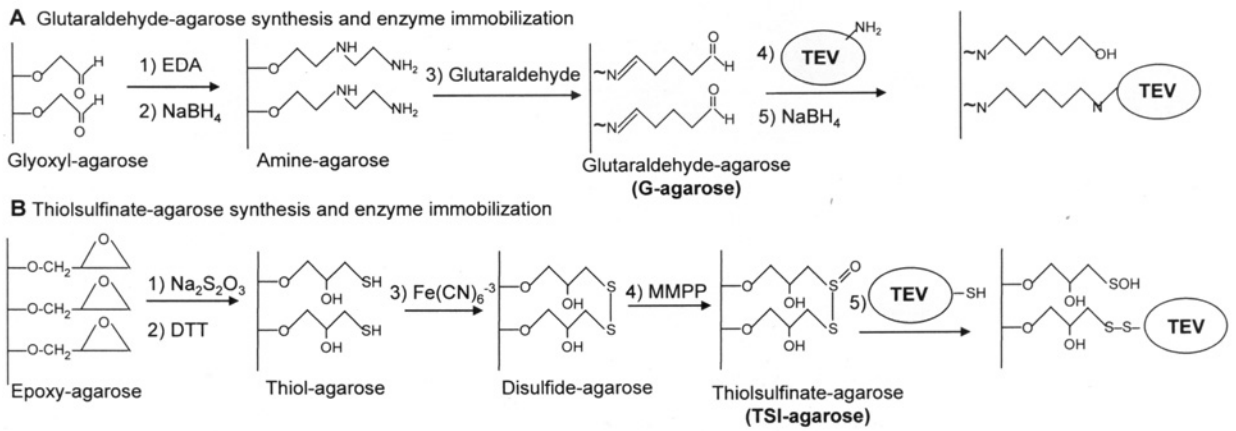

Figure 2 Support synthesis and enzyme immobilization strategies

(**A**) G–agarose synthesis and immobilization of the TEV-protease. Step 1, glyoxyl-agarose is made to react with ethylenediamine (EDA) at pH 10 to form a Schiff base; step 2, reduction of Schiff base with sodium borohydride ($NaBH_4$) to a stable alkyl-amine bond (Amine–agarose); step 3, reaction of the bound amino groups with glutaraldehyde to form G–agarose; step 4, immobilization of TEV-protease on to G–agarose by amino groups forming Schiff base; step 5, reduction of Schiff base with $NaBH_4$. Two G–agarose supports were synthesized, one using amino-agarose containing 75 μmol of amine groups/g of suction-dried support (G75–agarose) and the other containing 15 μmol of amine groups/g of suction-dried support (G15–agarose). (**B**) TSI–agarose synthesis and immobilization of the TEV-protease. Step 1, oxirane groups of epoxy-agarose are converted with sodium thiosulfate ($Na_2S_2O_3$) into support-bound thiosulfate groups; step 2, thiolsulfate groups are reduced with DTT to thiol groups; step 3, the thiol groups are oxidized with potassium ferricyanide [$Fe(CN)_6^{-3}$] to disulfide structures; step 4, agarose disulfide groups are converted into thiolsulfinate moieties with the oxidizing agent magnesium monoperoxyphthalate (MMPP); lane 5, immobilization of TEV-protease on to TSI–agarose by thiol groups.

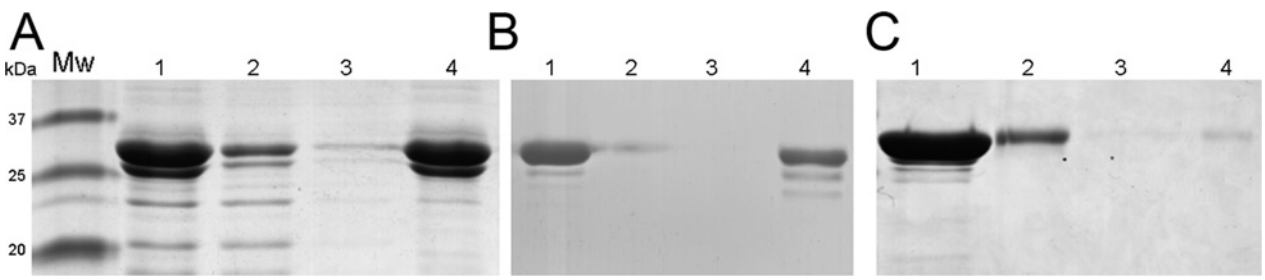

Figure 3 SDS/PAGE analysis of the immobilization of TEV-protease on to TSI–agarose and G15–agarose

(**A**) Immobilization of TEV_{WT}-protease on to TSI–agarose. (**B**) Immobilization of TEV_{SH}-protease on to TSI–agarose. (**C**) Immobilization of TEV_{SH}-protease on to G15–agarose. Lane 1, amount of TEV-protease applied to the support; lane 2, protein recovered in the supernatant; lane 3, protein recovered in the washing fraction; lane 4, TEV-protease released from the support after incubation in sample buffer containing DTT. Mw, molecular mass markers.

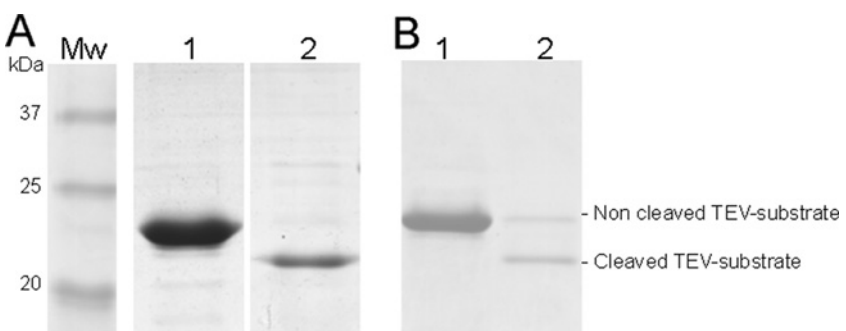

Figure 4 SDS/PAGE analysis of the activity of TEV_{SH}-protease immobilized on to TSI–agarose and G–agarose

(**A**) SDS/PAGE (12% gel) of TEV-substrate digested by TEV_{SH}-protease-TSI–agarose. (**B**) SDS/PAGE (12% gel) of TEV-substrate digested by TEV_{SH}-protease–G–agarose. Lane 1, amount of TEV-substrate initially present in the reaction mixture. Lane 2, amount of TEV-substrate (cleaved and not cleaved) collected from the supernatant after the activity assay. Reactions were performed using a molar ratio of 1:5 (immobilized enzyme/substrate) in the activity buffer at 25 °C for 24 h. Mw, molecular mass markers. Gels were stained with Coomassie Brilliant Blue R-250.

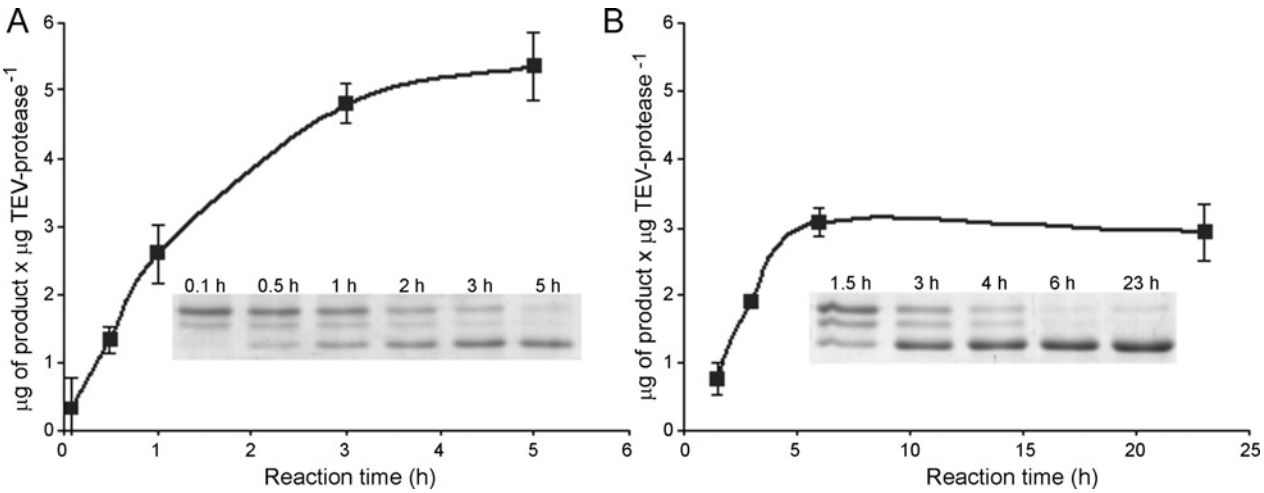

Figure 5 Activity of soluble and immobilized TEV-proteases

(**A**) Kinetic profile of soluble TEV-protease. (**B**) Kinetic profile of TEV-protease immobilized on to TSI–agarose. Reactions were performed using a molar ratio of 1:10 (enzyme/substrate) in the activity buffer at 25 °C. Samples were collected at different stages during the process of the cleavage of the TEV-substrate and analysed by SDS/PAGE. In some cases, before the cleaved assay the purified TEV-substrate was shown as two bands in SDS gels. The presence of a modification in the tag of the substrate may explain this difference, because after tag cleavage with the TEV-protease only one band of the substrate was detected. Values are the means ± S.E.M. for two independent experiments. Inset: SDS/PAGE showing an example of a kinetic profile.

of activity when the TEV-protease is immobilized on to a G–agarose support. This may be attributed to a multipoint interaction of the TEV-protease with the support, or to the immobilization conditions (buffer composition, time of incubation, temperature and blocking treatments). The latter is unlikely, as control assays performed with the soluble TEV-protease under the same conditions showed no detrimental effects on its activity.

Comparison of the intensity of the band representing the total amount of substrate offered to the protease (Figure 4, line 1) with that of the bands appearing after incubation with the immobilized protease (Figure 4, line 2) clearly indicates a great loss of the total amount of substrate (see also Figure 1B for the TEV-soluble activity). This suggests that under these conditions (absence of precipitate protein), part of the substrate directly interacts with the support. In fact, control experiments showed that even in the absence of the TEV-protease, part of the substrate was covalently bound and retained by the matrix. A partial loss of the substrate was also detected when the support was treated with an excess of glutathione or 2-mercaptoethanol to block all remaining TSI active groups. This evidence suggests that the substrate used in the present study, through some of its five cysteine residues, can compete with the disulfide bridges formed between the blocking agent (glutathione) and the TSI groups of the support and that this interaction may occur because of the long periods of incubation used for the activity assay (24 h). However, this effect was minimized by an increase in the steps of blocking with glutathione as described in the Methods section. Under these conditions, the substrate loss represented only 3% of the protein used in the activity assay.

Due to the better ability of the TEV-protease–TSI–agarose to cleave the TEV-substrate, as compared with the low performance of the TEV-protease–G–agarose, the first one was utilized for additional studies. As the immobilized TEV_{SH} and TEV_{WT} proteases presented a similar behaviour and the protocol for purification of the TEV_{SH}-protease is easier (no denaturant conditions) than the TEV_{WT}-protease (see the Methods section), further experiments were performed using only the TEV_{SH}-protease.

Assessment of soluble and immobilized TEV-protease kinetic activity

The kinetics of the TEV-protease activity, soluble and immobilized on to TSI–agarose, were determined using an enzyme/substrate molar ratio of 1:10. Figure 5 shows a plot representing the amount (μg) of protein substrate cleaved per μg of TEV-protease as a function of the reaction time. A linear relationship was observed at 1 and 3 h for the soluble and immobilized TEV-protease respectively. The activity calculated in the linear region of the curve was 2.48 and 0.76 μg of cleaved TEV-substrate/μg of TEV-protease per hour, for the soluble and the immobilized enzyme respectively. Thus the immobilization process caused an inactivation of approx. 70% of the protease. The reduction of activity cannot be attributed to the experimental conditions used for the immobilization process. In fact, as mentioned above, the activity of the soluble TEV-protease remained at normal value when incubated under the same

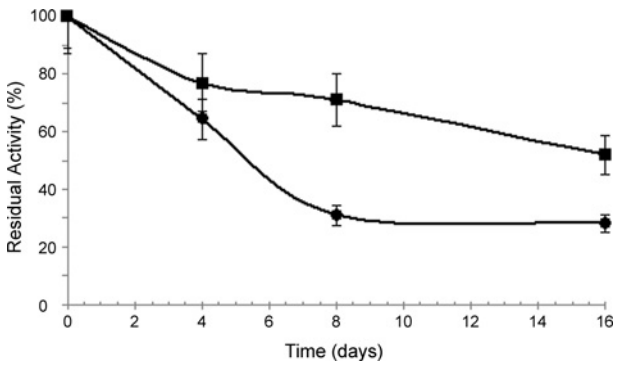

Figure 6 Stability at 4 °C of the soluble and immobilized TEV-proteases

Soluble TEV-protease (●) and TEV-protease immobilized on to TSI–agarose support (■). A dilution of the soluble enzyme and a suspension of the immobilized enzyme, containing 0.76 μg of TEV-protease/μl, were stored in a refrigerator at 4 °C for several days. At different intervals, aliquots were taken and the remaining enzymatic activity was measured using a molar ratio of 1:10 (enzyme/substrate) in the activity buffer at 25 °C.

conditions (buffer, pH, time and glutathione concentration) used for the immobilization protocols (results not shown). Also diffusional problems are unlikely as doubling the amount of substrate when performing the activity assay resulted in no increase in the activity of the immobilized enzyme.

Storage stability and operational reuse of TEV_{SH}-protease-TSI–agarose

The storage stability at 4 °C of the TEV-protease immobilized on to TSI–agarose was analysed and compared with the soluble enzyme. Samples of both enzymes were taken and the residual activity was assayed at 0, 4, 8 and 16 days of storage (Figure 6). Between 0 and 8 days of storage at 4 °C, the stability of the soluble enzyme was reduced, showing rapid inactivation kinetics with a residual activity of approx. 50% after 5 days of storage. Between 8 and 16 days, a slow inactivation kinetics was observed, with a residual activity of approx. 30%. The TEV-protease immobilized on to the TSI–agarose was three times more stable than the soluble enzyme, showing a reduction in the activity of approx. 50% only after 16 days of storage. The operational reuse of TEV-protease-TSI–agarose was assayed in five cycles of reactions at 25 °C, of 24 h each. In all the cycles tested, the immobilized enzyme cleaved 100% of the TEV-substrate (results not shown).

Discussion

In the present study, we used different strategies to immobilize the TEV-protease on to agarose supports. The coupling chemistries selected were based on the reaction of the thiol groups of the cysteine residues with the TSI support-bound groups (TSI–agarose), and on the reaction of the ε-amino groups of lysine residues with glutaraldehyde support-bound groups (G–agarose). The ability of both immobilized derivatives to cleave the His tag of a 20 kDa TEV-substrate was evaluated. High protein immobilization yields were achieved with both the strategies. However, the TEV-protease immobilized on to TSI–agarose showed a better cleaving ability than the enzyme immobilized on to G–agarose. A possible reason for this difference arises from the analysis of the TEV-protease structural data [21]. In fact, the crystallized TEV-protease contains only two cysteine residues exposed on its surface and both are distant from the substrate-binding site, whereas 12 out of 14 lysine residues are solvent-exposed and distributed all over the protein crystal surface, and several near the active site. Thus, when cysteine residues were used for the protease immobilization, possibly one or two points of attachment to the support were obtained, leaving the active site available to the binding of the substrate. On the contrary, it is possible that, when the immobilization was obtained through lysine residue ε-amino-groups, a multiple attachment to the matrix occurred [35]. This may have determined a distortion in the three-dimensional structure of the TEV-protease [35], explaining the lower ability of the protease immobilized on to highly activated G–agarose (G75–agarose) to cleave the substrate, as detected in the present study. In addition, as several of the exposed lysine residues are located near the active site, it is possible that the immobilization of the protease may have also partially blocked access of the macromolecular substrate to the binding site, lowering the cleaving ability of the TEV-protease–G–agarose. This may explain the partial activity detected when the enzyme was immobilized on to G–agarose containing a low amount of reactive aldehyde groups (G15–agarose), which allows immobilization by a lower number of lysine residues. A similar type of inactivation has also been reported in other studies [36,37].

In the present study, we report for the first time the immobilization of the TEV-protease on to an insoluble support. The immobilization of another enzyme belonging to the same family of the virus that codifies the TEV-protease, the coxsackie virus protease 3C, has been previously reported [38]. In this case, the protease was covalently immobilized on to a commercial *N*-hydroxysuccinimide-activated Hitrap column, and the ability for cleaving fusion proteins and reusability were tested. However, a possible loss of activity due to the immobilization process was not studied. In our work, kinetic experiments performed to compare the activity of the soluble TEV-protease with that of the TEV-protease immobilized on to TSI–agarose showed that the latter maintained only 30% of the soluble enzyme activity. This partial loss of activity was unexpected considering the structural data mentioned above. In fact, the cysteine residues used for the immobilization are exposed and distant from the active site, and in theory should

allow at best two direct interactions with the support, and should not create tension in the structure of the enzyme that may generate such an inactivation. Moreover, the reduction in activity cannot be attributed to the experimental conditions of the immobilization process as demonstrated by the performed controls. In some cases, the lower activity of immobilized enzymes compared with soluble ones can be attributed to diffusional effects [27,39]. However, experiments of activity performed with TEV-protease–TSI–agarose using twice the normal amount of substrate suggested the absence of diffusional interference. Another possible reason for the partial inactivation detected is that the TEV-protease in solution may adopt a different conformation than that in the crystal structure, exposing to the media other cysteine residues. It is known that the thiol group is one of the most reactive nucleophilic groups found in proteins, which react at a relatively high rate even at neutral pH [40] forming reversible covalent bonds. Thus, if in the TEV-protease in solution the catalytic cysteine (Cys^{151}) is exposed, it could covalently bind to the TSI–agarose support causing inactivation of the enzyme. Experiments where the TEV-protease is immobilized in the presence of a peptide substrate to protect the active site would help in analysing this hypothesis.

The use of the TEV immobilized on the TSI–agarose may be very useful for laboratories that cannot afford a continuous production of soluble TEV or that only need to process a small amount of substrate. The decreased specific activity of the immobilized TEV makes necessary a three times-longer incubation or -higher amount of immobilized enzyme than the soluble one to obtain the same yield of cleavage. This is not inconvenient as usually the reaction is performed overnight (1 mg of immobilized enzyme can cleave 12 mg of substrate in 16 h) and does not assume an increase in the total cost of the process as the immobilized enzyme can be reused several times without loss of its cleavage capacity. Also the soluble TEV can be repurified following the cleavage reaction. However, this process is a multistep work: trap the soluble protein by affinity chromatography, elute from the support, dialyse, concentrate and evaluate the residual activity before use. On the contrary, the TEV-protease immobilized on to TSI–agarose can be easily separated from the cleaved protein by a simple step of filtration or centrifugation allowing its immediate reuse in a new cleavage cycle.

The stability of biocatalysts is another key parameter that limits their industrial application, and enzyme stabilization is a central issue in biotechnology [41]. Therefore, in order to be suitable for different applications, an enzyme needs to be stable or needs to be stabilized. Immobilization on to solid carriers is perhaps the most used strategy to improve the operational stability of biocatalysts [42,43]. The TEV-protease–TSI–agarose used in the present study was shown to be three times more stable than the soluble enzyme. A possible interpretation for this behaviour is that the protease was attached to the support through a 'critical area' for enzyme stability [30]. It has been reported that denaturation of a protein begins in a defined region of the protein called 'critical area'. So when enzymes are immobilized through this area, their stability will be improved [44].

In summary, our observations indicate that the loss of activity detected during immobilization on to TSI–agarose is compensated by the higher stability and by the possibility of a high number of reuses, which implies concomitant economical benefits. These results confirm that immobilization of this kind of protease on to solid supports represents an interesting alternative to the use of the soluble enzyme in the removal of tags from recombinant proteins.

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online data

SUPPLEMENTARY ONLINE DATA

Covalent immobilization of tobacco-etch-virus NIa protease: a useful tool for cleavage of the histidine tag of recombinant proteins

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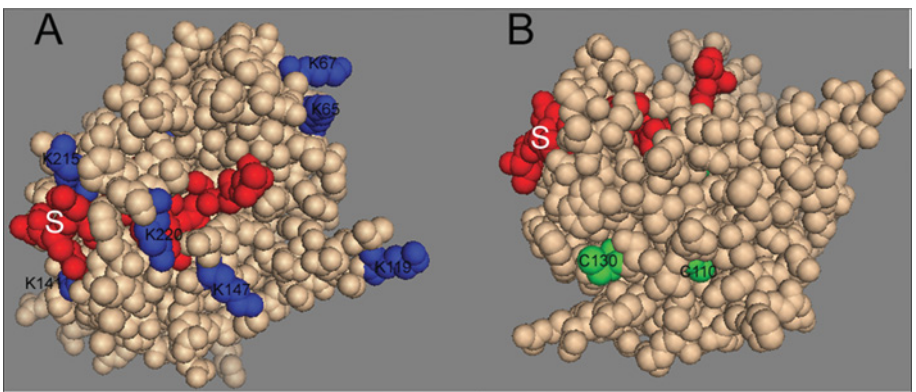

1-GESLF**K**GPRD YNPISSTI**C**H LTNESDGHTT SLYGIGFGPF IITN**K**HLFRR
NNGTLLVQSL HGVF**K**V**K**NTT TLQQHLIDGR DMIIIRMP**K**D FPPFPQ**K**L**K**F
REPQREERI**C** LVTTNFQT**K**S MSSMVSDTS**C** TFPSSDGIFW **K**HWIQT**K**DGQ
CGSPLVSTRD GFIVGIHSAS NFTNTNNYFT SVP**K**NFMELL TNQEAQQWVS
GWRLNADSVL WGGH**K**VFMS**K** PEEPFQPV**K**E ATQLMN-236

Figure S1 TEV-protease structure and sequence analysis

Representation of the crystallographic structure of the TEV-protease showing some of the solvent-exposed lysine residues (**A**) and cysteine residues (**B**). Note the proximity of the lysine residues (Lys-215, Lys-220, Lys-141 and Lys-147) to the binding site of the TEV-substrate (S). The protein sequence (bottom) shows the 14 lysine (underlined and boldface) and the 4 cysteine (boldface) residues of TEV-protease. The number of lysine and cysteine residues is the same for both TEV variants used. If the tag sequence is considered, TEV_{SH} has two lysine residues more than TEV_{WT}. TEV-protease structure analysis was performed using data for the catalytically active (PDB code 1LVM) and inactive (PDB code 1LVB_B) TEV-proteases. Images were created using PyMOL (DeLane Scientific; http://pymol.sourceforge.net/) and PDB code 1LVB_B of TEV-protease in complex with the peptide substrate (TENLYFQSGT) [1].

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