Glutathione S-transferase can be used as a C-terminal, enzymatically active dimerization module for a recombinant protease inhibitor, and functionally secreted into the periplasm of *Escherichia coli*

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Abstract

Glutathione S-transferase (GST) from *Schistosoma japonicum*, which is widely used for the production of fusion proteins in the cytoplasm of *Escherichia coli*, was employed as a functional fusion module that effects dimer formation of a recombinant protein and confers enzymatic reporter activity at the same time. For this purpose GST was linked via a flexible spacer to the C-terminus of the thiol–protease inhibitor cystatin, whose binding properties for papain were to be studied. The fusion protein was secreted into the bacterial periplasm by means of the OmpA signal peptide to ensure formation of the two disulfide bonds in cystatin. The formation of wrong crosslinks in the oxidizing milieu was prevented by replacing three of the four exposed cysteine residues in GST. Using the tetracycline promoter for tightly controlled gene expression the soluble fusion protein could be isolated from the periplasmic protein fraction. Purification to homogeneity was achieved in one step by means of an affinity column with glutathione agarose. Alternatively, the protein was isolated via streptavidin affinity chromatography after the *Strep*-tag had been appended to its C-terminus. The GST moiety of the fusion protein was enzymatically active and the kinetic parameters were determined using glutathione and 1-chloro-2,4-dinitrobenzene as substrates. Furthermore, strong binding activity for papain was detected in an ELISA. The signal with the cystatin–GST fusion protein was much higher than with cystatin itself, demonstrating an avidity effect due to the dimer formation of GST. The quaternary structure was further confirmed by chemical crosslinking, which resulted in a specific reaction product with twice the molecular size. Thus, engineered GST is suitable as a moderately sized, secretion-competent fusion partner that can confer bivalency to a protein of interest and promote detection of binding interactions even in cases of low affinity.

Keywords: avidity; bivalency; cystatin; fusion protein; GST; papain; secretion

Fusion partners that provoke homo-dimerization of certain ligand-binding proteins and provide moreover enzymatic reporter activity may be useful for a number of applications. First, catalysis of a chromogenic reaction permits the facile detection and quantification of the fusion protein. Second, the display of two identical ligand binding sites within the same bio-molecule should give rise to an avidity effect that can be exploited for the detection of binding events, especially in cases of low ligand affinity (Pack & Plückthun, 1992).

The avidity effect was first discovered for antibody molecules, which carry two separate but identical antigen-binding sites. Due to an entropy-based mechanism this bivalency leads to an increase in the apparent strength of binding, called avidity, if an oligomeric or surface-immobilized antigen is encountered (Crothers & Metzger,

1972). It has been well recognized today that this effect is not restricted to immunoglobulins but can, in principle, happen whenever a ligand-binding protein is oligovalent. Therefore, in the case of univalent ligand-binding proteins it would be desirable to have access to a stably folded dimeric enzyme that confers bivalency when employed in a recombinant fusion protein.

Unfortunately, examples of enzymes that appear suitable in this respect are rare, especially if secretion is needed as, for example, when structural disulfide bonds must be formed in the protein of interest. Bacterial alkaline phosphatase was utilized as a dimeric fusion enzyme for recombinant antibody fragments (Ducancel et al., 1993). However, the yield of correctly folded fusion protein was low and a generic purification method, which would be helpful in most applications, was not described. The limited folding efficiency may result from the large size of this enzyme (450 amino acids per subunit) and its complex folding mechanism, due to the presence of disulfide bonds and of metal ions that are important for its stability (Walker & Gilbert, 1994).

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In the search for a homo-dimeric enzyme with useful properties our attention was attracted towards glutathione S-transferase (GST) from *S. japonicum*, which has gained widespread application for the bacterial production of polypeptides that were fused to its C-terminus (Smith & Johnson, 1988). The main advantages of this system have been the high level of soluble protein that can be obtained in the cytoplasm of *E. coli* and the facile affinity purification on glutathione agarose before GST is then usually cleaved off. Considering, however, the use of GST as a permanent instead of a transient fusion partner, it must be kept in mind that many proteins of interest need an intact N-terminus for their biochemical activity.

One example is the thiol-protease inhibitor cystatin from chicken egg white, a protein of 116 amino acid residues (Bode et al., 1988). The N-terminal peptide segment as well as two internal loops are involved in the complex formation with its cognate protease papain. The same region was chosen as an epitope when cystatin was employed in a protein design study with an antibody (Schiweck & Skerra, 1997). To make GST amenable as a dimerization module for cystatin it had thus to be tested whether GST is still a functional enzyme when another protein gets fused to its N-terminal end.

Furthermore, the fact that cystatin possesses two structural disulfide bonds required secretion of the recombinant polypeptide into the periplasm of *E. coli* to obtain the correctly folded protein (Auerswald et al., 1991). GST, in contrast, is a typical cytoplasmic enzyme with altogether eight free cysteine residues on its surface. Apart from the deleterious effect that had to be expected from exposure of this large number of thiol groups to an oxidizing environment, it was not known whether GST exhibits stop-transfer sequences that might prevent membrane translocation at all. In a previous attempt to direct, for example, the human placental GST P1-1 into the periplasm of *E. coli* secretion and processing of the heterologous protein appeared to be hampered (Battistoni et al., 1995).

In the present study we demonstrate that GST can be successfully used as a secretory fusion enzyme in spite of these caveats, and we describe its functional characteristics when attached to cystatin as an exemplary fusion partner.

Results

Construction of an expression vector for the bacterial secretion of a cystatin-GST fusion protein

The hybrid polypeptide was designed such that a truncated form of chicken egg white cystatin constituted the N-terminal domain, which was linked via a flexible spacer of five glycine residues to GST as the C-terminal dimerization module (Fig. 1). The corresponding structural gene was constructed in several steps, using a synthetic gene for the inhibitor (Auerswald et al., 1989) and the cloned cDNA for *S. japonicum* GST (Smith & Johnson, 1988). The coding region was placed under transcriptional control of the tightly regulated *tet* promoter (Skerra, 1994), yielding the expression plasmid pTTX6 (Fig. 2). For secretion of the polypeptide into the bacterial periplasm the gene sequence that coded for the mature part of cystatin, starting with residue Gly 9, was precisely fused to the OmpA signal sequence (Fig. 2B). Gly 9 was chosen as the first residue of cystatin because this isoform constitutes a major secondary processing product that can be isolated from chicken egg white. In addition, the crystal structure of this truncated protein had been solved (Bode et al., 1988).

Fig. 1. Molecular model for the dimerized fusion protein between cystatin and GST. One monomer is shown in dark gray and the other in light gray. A bound GSH molecule is depicted in each GST active site (ball and stick, opposite gray shade). The ribbon representation was generated using InsightII modeling software (BIOSYM/MSI) and corresponds to the mature polypeptide sequence as shown in Figure 2. The crystal coordinates of the Gly 9 version of chicken egg white cystatin (Bode et al., 1988; PDB entry 1CEW) and of *S. japonicum* GST (Lim et al., 1994; PDB entry 1GNE) were connected via a segment with the sequence Gly₅-Met that was modeled in an extended conformation. The gp41 peptide epitope that had been present at the C-terminus in the GST crystal structure (cf. Lim et al., 1994) was deleted.

From the recently described crystal structure of the *S. japonicum* GST (Lim et al., 1994) it became clear that the four Cys residues in each subunit are located close to the protein surface. Especially the Cys residues 85, 138, and 178 appeared to be highly solvent exposed. To prevent unwanted reactions of these side chains upon transfer to an oxidizing environment the coding sequence for GST was changed to incorporate Ser residues instead (cf. Fig. 2B). Cys 169, in contrast, was found to be partially buried by neighboring hydrophobic side chains and, therefore, not replaced. The entire GST coding sequence, beginning with a Met residue and ending with its native C-terminus, was placed downstream of the five Gly residues following Gln 116 of cystatin (Fig. 2B).

In a variant of the fusion protein the *Strep*-tag was appended to the C-terminus of GST. This nine amino acid sequence had previously been engineered for conferring binding activity towards streptavidin (Schmidt & Skerra, 1993, 1994). The corresponding expression plasmid, pTTX7, was otherwise identical with pTTX6. Finally, the vector pTTX9 was constructed from pTTX7 for general use by deleting the cystatin coding region and replacing it with a multiple cloning site for the restriction enzymes *Stu*I, *Xho*I, and *Not*I. Thus, it is possible to insert different structural genes in front of GST. Because the reading frame was not interrupted between the OmpA signal peptide and GST, pTTX9 was also suited for secreting the plain enzyme, yet fused with the *Strep*-tag, into the bacterial periplasmic space.

Bacterial production and purification of the cystatin-GST fusion

After induction of recombinant gene expression with anhydrotetracycline the secreted cystatin-GST fusion protein encoded on pTTX6 was liberated from the periplasm of *E. coli* in a soluble form by a standard cell fractionation protocol. As with other types of GST fusion proteins, purification was achieved in a single step by means of a glutathione (GSH) affinity column. Upon applica-

Fig. 2. Genetic construct for the secretion of the recombinant fusion protein between the Gly 9 version of chicken egg white cystatin and the engineered *S. japonicum* GST. **A:** Expression vector pTTX6. The structural gene for the cystatin–GST fusion was equipped with the OmpA signal sequence and placed under transcriptional control of the tightly regulated tetracycline promoter/operator (*tet^{p/o}*). The hybrid gene is flanked by the *Xba*I and *Hind*III restriction sites. Outside this region the plasmid is identical with the generic secretion vector pASK75 (Skerra, 1994). *tetR*, *tet* repressor gene; *t_{lpp}*, lipoprotein terminator; *ori*, origin of replication; *f1-IG*, intergenic region of filamentous phage *f1*; *bla*, β -lactamase gene. **B:** Sequence of the coding region for the cystatin–GST fusion protein on pTTX6. The amino acid sequence is given below the nucleotide sequence, whereby the reading frames for the OmpA signal peptide, cystatin, and GST are labeled. The truncated cystatin sequence starts with the amino acid Gly 9 and ends with Gln 116. The four Cys residues in cystatin that give rise to the disulfide bonds Cys 71–Cys 81 and Cys 95–Cys 115 are underlined. Following a spacer of five Gly residues the sequence of the GST moiety starts with Met 1. The three Cys residues at the positions 85, 138, and 178 of GST were replaced by Ser as indicated. Cys 169 was retained for reasons given in the text. The sequence of the fusion protein encoded on pTTX6 ends with the original C-terminal residue of the *S. japonicum* GST, Lys 218. In the case of pTTX7 the *Strep*-tag sequence was appended (cf. Skerra, 1994) and the *Xba*I site within the cystatin gene (nt. 289–294) was removed. On pTTX9 the whole cystatin gene was furthermore replaced by a sequence encoding “Ser-Ser-Ala-Ala-Ala” and including restriction sites for *Stu*I, *Xho*I, and *Nco*I (cf. Materials and methods).

tion of a 5-mM GSH solution the fusion protein was eluted in a sharp peak (Fig. 3), yielding routinely 0.35 mg homogeneous protein per 1 L of bacterial culture. The correct removal of the signal peptide was confirmed by N-terminal amino acid sequencing of the isolated protein. The sequence “Gly-Ala-Pro-Val” was unambiguously detected so that the bacterial signal peptidase had directly processed the precursor of the fusion protein to the corresponding cystatin Gly 9 form.

As shown below, both parts of the affinity-purified fusion protein were apparently active, at least when the GSH that had been used for elution was quickly removed by dialysis. To protect the disulfide bridges in cystatin from any possible reduction and cleavage by the free thiol compound a 5-mM solution of oxidized

glutathione (GSSG) was alternatively utilized in the affinity chromatography. Indeed, the fusion protein was purified with the same efficiency (not shown). Yet, due to the intrinsic UV absorption of GSSG the elution could not be directly monitored by measuring the A_{280} .

For comparison, the cystatin–GST fusion protein that had the four Cys residues in the enzyme still present was produced instead of the Ser 85, Ser 138, Ser 178 version using the same experimental conditions. Although a band of similar intensity was detected in the SDS PAGE of a total cell lysate after three hours of induction (not shown) no soluble protein could be isolated in this case. In addition, the cells tended to lyse during the preparation of the periplasmic protein fraction.

Fig. 3. Bacterial production of the cystatin-GST fusion protein. **A:** Purification of the protein expressed from pTTX6 via GSH agarose. After applying the periplasmic protein fraction and washing with chromatography buffer the pure protein was eluted with 5 mM GSH dissolved in the same buffer. **B:** Purification of the protein expressed from pTTX7 on a column with immobilized streptavidin. The bound protein was eluted with 2.5 mM desthiobiotin. **C:** SDS PAGE, followed by staining with Coomassie brilliant blue. Lanes: M, molecular mass standard (kDa); 1, cystatin-GST (pTTX6) purified on GSH agarose; 2, cystatin-GST/Strep-tag (pTTX7) purified by streptavidin affinity chromatography; 3, GST/Strep-tag (pTTX9) purified on GSH agarose.

The variant of the engineered cystatin-GST fusion protein that was encoded on pTTX7 and carried the C-terminal *Strep*-tag could also be purified by means of a streptavidin affinity column, apart from the GSH chromatography (Fig. 3B). Specific elution of the bound protein was achieved under gentle buffer conditions by the application of desthiobiotin in this case (Voss & Skerra, 1997). The protein preparations from either chromatography were indistinguishable in terms of yield and quality. In contrast with the streptavidin matrix, however, the GSH affinity column lost its activity after ca. four uses. Finally, the plain GST enzyme carrying

the *Strep*-tag, which was encoded on pTTX9, was also successfully purified by both methods from the periplasmic protein fraction of *E. coli* (Fig. 3C).

Functional characterization of the cystatin-GST fusion protein

The enzymatic activity of the fusion protein was measured with the two standard substrates for glutathione S-transferases, 1-chloro-2,4-dinitrobenzene (CDNB), and GSH (Habig et al., 1974). The

Table 1. Enzymatic properties of the cystatin–GST fusion protein

Specific activity ^a ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	2.08 $\pm$ 0.06
Varied [GSH] ^b	
V_{max} ($\mu\text{M min}^{-1}$)	26.8 $\pm$ 2.3
$K_{\text{M}}^{\text{GSH}}$ (mM)	0.456 $\pm$ 0.117
$k_{\text{cat}}/K_{\text{M}}$ ($\text{mM}^{-1} \text{s}^{-1}$)	18.5
Varied [CDNB] ^c	
V_{max} ($\mu\text{M min}^{-1}$)	19.5 $\pm$ 0.8
$K_{\text{M}}^{\text{CDNB}}$ (mM)	0.864 $\pm$ 0.087
$k_{\text{cat}}/K_{\text{M}}$ ($\text{mM}^{-1} \text{s}^{-1}$)	7.12

^aSpecific activity was measured at 30 °C with 1 mM CDBN and 1 mM GSH.

^bApparent V_{max} and $K_{\text{M}}^{\text{GSH}}$ values in the presence of 2.5 mM CDBN measured with 4 $\mu\text{g/mL}$ of the fusion protein by varying GSH from 0.1 to 2 mM.

^cApparent V_{max} and $K_{\text{M}}^{\text{CDNB}}$ values in the presence of 2.5 mM GSH measured with 4 $\mu\text{g/mL}$ of the fusion protein by varying CDBN from 0.1 to 2 mM.

kinetic parameters are listed in Table 1. The specific activity and the K_{M} values were almost identical with the numbers that had been reported for recombinant wild-type *S. japonicum* GST (Walker et al., 1993), demonstrating that the enzyme is fully active as part of the fusion protein.

The quaternary structure was investigated in a chemical cross-linking experiment. When the purified cystatin–GST fusion protein was incubated in the presence of disuccinimidyl suberate and subsequently analyzed by SDS PAGE, a band of approximately double the molecular size became apparent (Fig. 4). Neither monomeric protein nor higher oligomers were visible. The same effect was observed with the plain secreted GST, encoded on pTTX9. This dimeric status is in agreement with previous biochemical data from gel filtration experiments (Walker et al., 1993) and with the crystal structure of the enzyme (Lim et al., 1994; McTigue et al., 1995). Thus, the cystatin–GST fusion protein exists as a defined and stable homodimer.

The functionality of the cystatin moiety in the fusion protein was determined in an ELISA, resting on its binding activity for papain (Fig. 5). Two different methods were employed for the detection of the fusion protein, which became bound to the immobilized carboxymethylated thiol–protease. First, a commercially available antiserum against GST, followed by an antibody–alkaline phosphatase conjugate, was used. Second, the variant of the cystatin–GST fusion protein that carried the C-terminal *Strep*-tag was directly detected with a streptavidin–alkaline phosphatase conjugate. In both cases a strong, concentration-dependent signal was obtained (Figs. 5A, B). The binding for papain was specific because the background was very low in its absence and no papain binding signal could be detected when the secreted GST/*Strep*-tag enzyme was used for comparison (not shown).

Therefore, the cystatin domain appeared to be fully functional and correctly folded as part of the fusion protein and its N-terminus was obviously accessible. Remarkably, the binding activity that was observed with the cystatin–GST fusion protein was much higher compared with the plain monomeric cystatin/*Strep*-tag protein, which had been applied under the same conditions (Fig. 5B). This phenomenon was in agreement with the expected avidity effect for the fusion protein because the affinity of the Gly 9 version

Fig. 4. Analysis of the chemically crosslinked fusion protein by SDS PAGE. Lanes: 1, crosslinked cystatin–GST fusion protein; 2, crosslinked GST/*Strep*-tag enzyme. Staining was performed with Coomassie brilliant blue. Molecular sizes are indicated. The calculated molecular masses were 37,872 Da for the monomer and 75,744 Da for the dimer of the mature cystatin–GST fusion protein expressed from pTTX6 and 27,119 Da and 54,238 Da, respectively, for the GST/*Strep*-tag enzyme.

of cystatin for carboxymethylated papain is low, with a published K_{d} value of 1.15 μM (Lindahl et al., 1992). This number is already in the problematic range of low thermodynamic affinities, where reproducible ELISA signals are difficult to obtain due to rapid equilibration and leakage during the washing steps (cf. Pack & Plückthun, 1992).

Discussion

The GST from *S. japonicum* has been successfully employed as a secretable dimerization and effector module for a disulfide bond-containing recombinant protein. Usually, the immunoglobulins are considered as the paradigm for a type of universal adapter molecules that can with one end specifically recognize a target and with the other end (the effector or F_{c} part) trigger downstream signals. In addition, each immunoglobulin molecule possesses at least two identical antigen-binding sites (located in the F_{ab} parts), which results in an increased apparent binding strength, called avidity, when a multimeric or surface-bound antigen is met. In the present study we attempted to introduce these two general features, i.e., bivalency and an (enzymatic) effector activity, for investigating the binding function of the protease inhibitor cystatin by employing glutathione S-transferase as a fusion partner.

Fig. 5. Detection of papain-binding activity in an ELISA. Rows of an ELISA plate were coated with carboxymethylated papain. After blocking with BSA and washing, varying concentrations of the purified fusion protein were applied. **A:** Detection of the cystatin-GST fusion protein (circles) with anti-GST immunoglobulins from goat and an anti-goat IgG/alkaline phosphatase conjugate. Wells that had not been coated with the papain but just blocked were used as a control (triangles). **B:** Detection of the cystatin-GST/Strep-tag fusion protein (circles) or of cystatin/Strep-tag (squares) with streptavidin-alkaline phosphatase conjugate. In the case of the fusion protein a control was performed as above (triangles). Alkaline phosphatase activity was detected with *p*-nitrophenyl phosphate by measuring the change in A_{410} per time. Note the differing abscissa scales. The concentration values for half-maximal binding of the fusion protein were 10 nM in both cases.

GST, a dimeric cytosolic enzyme from *S. japonicum*, is ordinarily used as an N-terminal fusion domain for the production of recombinant proteins in the cytoplasm of *E. coli* and proteolytically removed thereafter. Our system is different in several aspects. First, the N-terminus of cystatin plays an essential role for the binding to its cognate protease papain as well as to other possible targets (Schiweck & Skerra, 1997). Therefore, GST was placed at the C-terminus of the inhibitor, after introduction of a flexible spacer. Second, cystatin carries two structural disulfide bonds. Thus, the whole fusion protein was secreted into the periplasm of *E. coli* where proper folding was permitted. For this purpose three of the

four unpaired cysteine residues in each GST subunit were replaced to prevent mis-folding due to the formation of non-natural disulfide bonds. Fortunately, it turned out that GST did not contain a stop-transfer sequence that could have interfered with its secretion.

Thus, we obtained a soluble fusion protein between cystatin and GST, which associated to a stable non-covalent dimer in vivo. This protein was conveniently purified from the periplasmic protein fraction of *E. coli*, either on GSH agarose or via the *Strep*-tag, in a single step. Both the cystatin and the GST part were shown to be fully functional and the dimeric structure gave rise to a strong avidity effect. The described GST fusion protein can now be used as a tool for studying binding interactions with possibly weak affinities. The proposed strategy should not be limited to cystatin but also be applicable to other ligand-binding proteins of interest, including F_v and F_{ab} fragments of antibodies.

Materials and methods

Vector constructions

pTTX6 was constructed in several steps according to standard methodology (Sambrook et al., 1989). The generic expression plasmid pASK75, carrying the *tet* promoter/operator (Skerra, 1994), was used as the cloning vehicle, i.e., the structural gene for the GST fusion protein described here (Fig. 2B) replaced the cassette between the *Xba*I and *Hind*III restriction sites. The DNA sequence encoding the fusion between the OmpA signal peptide and an N-terminally truncated cystatin was derived from pASK95 (Skerra, unpubl.), which carried a synthetic gene for the protease inhibitor (Auerswald et al., 1989). The GST gene was PCR-amplified from the pGEX-3X plasmid (Pharmacia, Uppsala, Sweden), a derivative of pGEX-1 with a factor X_a cleavage/recognition sequence (Smith & Johnson, 1988), using the primers 5'-AAA AGT CCC CTA TAC TAG GTT ATT GG and 5'-GCG ACC ATC CTC CAA AAT AAG CTT TGA TCG. Introduction of the Gly₅ linker sequence and replacement of the three Cys residues 85, 138, and 178 in GST was performed by site-directed mutagenesis (Geisselsoder et al., 1987) with the single-stranded plasmid DNA after assembly of the different parts. The final nucleotide sequence of the whole coding region on pTTX6 (see Fig. 2) was confirmed by DNA sequencing using the T7 sequencing kit (Pharmacia).

For the production of the cystatin-GST fusion protein with the *Strep*-tag (Schmidt & Skerra, 1993) attached to its C-terminus the plasmid pTTX7 was constructed from pTTX6 in two steps. First, an *Eco*47III restriction site was introduced after the codon for Lys 218 in GST by site-directed mutagenesis with the oligodeoxynucleotide 5'-CAC AGG TCA AGC TTA AGC GCT TGG AGG ATG GTC CG, and an internal *Xba*I restriction site in the synthetic cystatin gene was eliminated at the same time with the oligodeoxynucleotide 5'-CAC ACG AAC AAC ACG AGA CGA GTA TTT G. The whole structural gene was then excised from the resulting plasmid via *Xba*I and *Eco*47III and inserted into the correspondingly cut pASK75, resulting in a precise fusion with the coding sequence for the *Strep*-tag on this vector (cf. Skerra, 1994).

The generic expression vector for the secretion of GST fusion proteins, pTTX9, was obtained from pTTX7 by deleting the cystatin gene via site-directed mutagenesis with the oligodeoxynucleotide 5'-CAT ACC ACC GCC TCC ACC TGC GGC CGC ACT CGA GGC CTG CGC TAC GGT AGC, whereby singular cloning sites for *Stu*I, *Xho*I, and *Not*I were introduced. The construction of pTTX7 and pTTX9 was checked by DNA sequencing as above.

Recombinant protein production

E. coli K-12 strain JM83 (Yanisch-Perron et al., 1985), transformed with pTTX6, pTTX7, or pTTX9, was grown at 37 °C (200 rpm) in 2-L LB medium containing 100 mg/L ampicillin. Expression was induced at an $A_{550} = 0.5$ by the addition of 0.2 mg/L anhydrotetracycline, aTc (Acros Chimica, Belgium), and shaking was continued for three hours. Cells were harvested by centrifugation, resuspended in 20-mL ice-cold 0.5 M sucrose, 1 mM EDTA, 100 mM Tris/HCl pH 8.0, and kept on ice for 30 min, whereby the periplasmic content was released. The plasmolyzed cells were removed by centrifugation and the supernatant was recovered as the periplasmic protein fraction, dialyzed over night against the chromatography buffer (1 mM EDTA, 150 mM NaCl, 50 mM Tris/HCl pH 8.0), and filter sterilized. Monomeric cystatin (Gly 9 variant with the *Strep*-tag fused to its C-terminus) was expressed from pCys-Strep as described (Schmidt & Skerra, 1994).

Affinity chromatography

For GSH affinity chromatography (Smith & Johnson, 1988) 170 mg freeze-dried GSH agarose (Sigma, St. Louis, MO, USA) was swollen in 35 mL water for two hours and packed into a column with 2 mL bed volume. The column was washed with water and equilibrated with chromatography buffer (see above). The periplasmic protein solution (ca. 20 mL, from a 2-L *E. coli* culture) was applied (flow rate 20 mL/h) and the column was washed with chromatography buffer until the A_{280} of the flow through fell to the baseline. The fusion protein was then eluted with 5 mM reduced glutathione, GSH (Sigma), dissolved in the chromatography buffer. Alternatively, 5 mM oxidized glutathione, GSSG (Sigma), was used. The eluted protein fraction was dialyzed against PBS (4 mM KH_2PO_4 , 16 mM Na_2HPO_4 , 115 mM NaCl) containing 1 mM EDTA to remove the thiol compound.

Proteins that carried the *Strep*-tag (expressed from pTTX7, pTTX9, or pCys-Strep) were also purified by streptavidin affinity chromatography (Schmidt & Skerra, 1994). A streptavidin mutant with improved binding strength was employed for this purpose and a solution of 2.5 mM desthiobiotin in the chromatography buffer was used for competitive elution (Voss & Skerra, 1997). For SDS PAGE the samples were treated with loading buffer containing β -mercaptoethanol and analyzed on a discontinuous 0.1% (w/v) SDS, 15% (w/v) polyacrylamide gel (Fling & Gregerson, 1986). N-terminal amino acid sequencing was performed with an Applied Biosystems Procise Sequencer after electro-transfer of the purified protein onto an Immobilon P membrane (Millipore, Bedford, MA, USA).

Detection of papain-binding activity in an ELISA

For carboxymethylation of papain (Anastasi et al., 1982) 6 mg of the protein (Boehringer Mannheim, Germany) was dissolved in 5 mL 1 mM DTT, 1 mM EDTA, 0.1 M Na phosphate pH 7.5, and activated for 10 min at 20 °C. One milliliter of a 100 mM solution of iodoacetic acid (Fluka, Switzerland) in 0.1 M Na phosphate pH 7.5 was added and incubation was continued for 10 min. The solution was desalted on a PD-10 column with Sephadex G-25 M (Pharmacia) that had been equilibrated with 0.1 M NaHCO_3 pH 8.0. The protein concentration was determined by A_{280} measurement with an absorption coefficient of $2.39 \text{ mL mg}^{-1} \text{ cm}^{-1}$ (Brocklehurst et al., 1973).

The ELISA was performed at ambient temperature in 96-well microtiter plates (Becton Dickinson, Germany). Ten wells in a row

were coated with 100 μL of the carboxymethylated papain at a concentration of 100 $\mu\text{g/mL}$ for two hours, followed by blocking with 2% (w/v) BSA, 0.5% (v/v) Tween in PBS for 2 h. The plates were washed three times with PBS containing 0.1% (v/v) Tween.

Fifty microliters of a dilution series of the purified cystatin-GST fusion protein (produced with pTTX6) in PBS/Tween was applied and incubated for 1 h. After washing three times with PBS/Tween the bound fusion protein was detected in two steps, using anti-GST immunoglobulins from goat (Pharmacia), diluted 1:100 in PBS/Tween, and an anti-goat IgG/alkaline phosphatase conjugate (Sigma), diluted 1:1000 in PBS/Tween. The signal was developed with 0.5 mg/mL *p*-nitrophenyl phosphate in the presence of 0.1 M Tris/HCl pH 8.8, 0.1 M NaCl, 5 mM MgCl_2 and measured as the change in absorption at 410 nm per time with a SpectraMax 250 instrument (Molecular Devices, Sunnyvale, CA, USA). The background signal from a well in the same row where the fusion protein had been omitted was subtracted.

Alternatively, the purified cystatin-GST/*Strep*-tag fusion protein (produced with pTTX7) and the monomeric cystatin/*Strep*-tag (from pCys-Strep) were used in the ELISA. In this case the bound fusion protein was detected with a streptavidin-alkaline phosphatase conjugate (Amersham, Buckinghamshire, UK), diluted 1:1000 in PBS/Tween (Schmidt & Skerra, 1993). The ELISA was developed by reaction with *p*-nitrophenyl phosphate as above.

Enzyme kinetics

The enzymatic activity of the fusion protein was determined in 0.1 M K phosphate pH 6.5 using GSH (Sigma) and CDNB (Sigma) as substrates and monitoring the appearance of the product at 340 nm in an Ultrospec 2000 instrument (Pharmacia) with $\Delta\epsilon = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$ (Habig et al., 1974). The reaction was carried out in a thermostatted quartz cuvette with 1-cm path length at 30 °C. GSH was directly dissolved in the assay buffer, whereas appropriate stock solutions in ethanol were prepared for CDNB such that the concentration of the organic solvent in the assay mixture was always 4% (v/v). Measurements were made with the concentration of one substrate varied in the presence of a constant concentration of the other substrate. The reaction was initiated by the addition of the fusion protein, which had been dialyzed against the assay buffer, at a final concentration of 4 $\mu\text{g/mL}$. The protein concentration was determined by A_{280} measurement with a calculated absorption coefficient of $1.40 \text{ mL mg}^{-1} \text{ cm}^{-1}$ (Gill & von Hippel, 1989) and a molecular mass of 75,744 Da for the dimer. The initial velocity was measured and the rate of product formation in the absence of the enzyme, determined in a separate experiment, was subtracted. The data were then fitted by non-linear least-squares regression according to the Michaelis-Menten formula. The specific activity was determined as the mean from a concentration row of the enzyme between 0.5 and 5 $\mu\text{g/mL}$.

Crosslinking experiments

The oligomeric structure of the fusion protein was elucidated by chemical crosslinking (Hill et al., 1979). One hundred microliters of a solution of either the purified cystatin-GST fusion protein (159 $\mu\text{g/mL}$) or the purified GST/*Strep*-tag enzyme (163 $\mu\text{g/mL}$) was adjusted to 0.2 M triethanolamine/HCl pH 8.5, 0.5 M NaCl using a Bio-Spin chromatography column (Bio-Rad). One microliter of a 10 mg/mL solution of disuccinimidyl suberate (Sigma) in dimethyl formamide was added and the reaction mixture was in-

cubated under gentle agitation for 1 h at room temperature. The crosslinking products were analyzed in a 12% SDS PAGE without the addition of β -mercaptoethanol.

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