Comparison of complexes formed by a crustacean and a vertebrate trypsin with bovine pancreatic trypsin inhibitor – the key to achieving extreme stability?

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This paper provides evidence for the extremely high resistance of a complex of crayfish trypsin (CFT) and bovine pancreatic trypsin inhibitor (BPTI) against heating and chemical denaturing agents such as sodium dodecyl sulfate (SDS) and urea. To dissociate this complex, 15 min boiling in SDS was necessary, compared to a complex of bovine trypsin (BT) (EC 3.4.21.4) and BPTI, which dissociates in SDS without boiling. The CFT–BPTI complex remained stable even in 9 M urea, while the BT–BPTI complex started to dissociate at concentrations of approximately 4 M urea. The melting temperatures of the BT–BPTI and CFT–BPTI complexes, as determined by differential scanning calorimetry, were found to be 79.6 and 100.1 °C, respectively. The behaviour of the apo-enzymes - CFT was found to have a less stable structure compared to BT - did not provide a definite indication regarding the differential effects on their stabilities. To explore the structural features responsible for this extreme stability, we crystallized CFT in complex with BPTI, and identified extended contacts compared to the BT-BPTI complex. Comparison of the B-factors of similar trypsin–trypsin inhibitor complexes suggests that molecular flexibility of the components is also required for the strong protein–protein interaction. Although the structural reason for the extreme stability of the CFT–BPTI complex is not yet fully understood, our study may be a starting point for the development of new protein complexes with enhanced stability.

Structured digital abstract

- **BT and BPTI bind** by comigration in sds page (View interaction)
- **BPTI and BT bind** by comigration in gel electrophoresis (View interaction)
- **CFT and BPTI bind** by molecular sieving (View interaction)
- **CFT and BPTI bind** by x-ray crystallography (View interaction)
- **BPTI and CFT bind** by comigration in gel electrophoresis (View interaction)
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- **CFT and BPTI bind** by comigration in sds page (View interaction)
- **BT and BPTI bind** by molecular sieving (View interaction)

Abbreviations

BPTI, bovine pancreatic trypsin inhibitor; BT, bovine trypsin; CFT, crayfish trypsin; ME, β-mercaptoethanol; SEC, size-exclusion chromatography; SGTI, Schistocerca gregaria trypsin inhibitor; TUG, transverse urea gradient.
Introduction

Protein stability is the net balance of forces that derives from equilibrium of the native folded conformation and the denatured state(s) of a protein. The word is used in different ways, but it normally refers to the physical (thermodynamic) stability. Another important property of proteins is their chemical stability against processes that cause loss of integrity, due to bond cleavage (deamidation, hydrolysis of peptide bonds, oxidation of methionine, etc.) or generation of new chemical entities (e.g. thiol-catalysed disulfide interchange) [1,2]. At yet another level, biological stability may refer to the differences in the natural turnover of proteins [3–5] in the cellular environment, where proteins are continuously exposed to a whole range of post-translational modifications controlling their effective life time. Exploring the structural features and mechanisms responsible for protein stability is important for understanding the basic thermodynamics of the process of folding. The ability to enhance protein stability may result in multi-billion dollar profits for biotechnological, food and drug industries.

The classic target and model in protein stability research is subtilisin [6]. In the 1980s, many experiments were performed to increase the stability of subtilisin against heat, oxidation and denaturing agents by using protein engineering [7–9]. These efforts, mostly undertaken in the labs of Genentech Inc. (San Francisco, CA, USA), were successful, and genetically engineered mutants of subtilisin are now used worldwide as a component of laundry and dishwashing detergents.

Since the discovery of trypsin more than 130 years ago [10], serine proteases (EC 3.4.21) complexed with their natural protein inhibitors have been the most common model for studying protein–protein interactions [11–14]. A highlight of our own research in this area was elucidation of the X-ray structure of a complex [15,16] formed between a taxon-specific protease inhibitor from the desert locust Schistocerca gregaria (SGTI) [17] and trypsin from narrow-fingered crayfish (Pontastacus leptodactylus). The conclusion of this study was that a series of extended electrostatic interactions account for the strong and highly specific interaction between these two proteins [15]. More recently, based on an ultra-high-resolution X-ray structure of another serine protease–protease inhibitor complex formed between bovine trypsin (BT) and a mutant of SGTI selected by phage-display [18], we proposed a refined mechanism of trypsin action [19].

Hepatopancreas trypsin from P. leptodactylus (UniProt ID Q52V24) is a member of the S1 family of serine endopeptidases. It was firstly characterized by Zwilling et al. [20] as a link between vertebrate and bacterial trypsins. It is a 25 kDa serine protease with characteristics of the crustacean trypsins: it has an acidic pI, a decreased number of cysteines (compared to vertebrate trypsins), sequence insertions, and is irreversibly inactivated at low pH.

In the present study, we demonstrate the extremely high resistance of a non-covalent complex of crayfish trypsin (CFT) with bovine pancreatic trypsin inhibitor (BPTI) to various denaturing agents and heating. Similar findings were reported by Radisky and Koshland [21] for analysis of the complex formed between subtilisin (EC 3.4.21.62) and a chymotrypsin inhibitor. Our experimental results with CFT–BPTI complex, derived from structural and biophysical comparisons, confirmed that both the thermodynamic and chemical stability of this complex are much greater than those of the structurally highly homologous complex formed between BT and BPTI. The results of these observations may contribute to development of resistance of proteins under various denaturing conditions (e.g. heat or chemical denaturants) by protein engineering methods.

Results

Comparative electrophoretic assays of crayfish and BTs and their complexes with BPTI

Our unpublished studies showed that BPTI inhibits trypsin isolated from the arthropod narrow-fingered crayfish (P. leptodactylus). The present study on the stability of this complex was initiated by the unexpected SDS/PAGE pattern obtained for a mixture of CFT and BPTI (Fig. 1A). While two protein bands in this pattern correspond to the enzyme and the inhibitor, the identity of the third one, with higher electrophoretic mobility than that of CFT, was not evident at first. However, boiling a mixture of these proteins in the SDS sample buffer progressively dissociated the third protein band into two components, CFT and BPTI (Fig. 1B). Interestingly, this dissociation process was not significantly affected by β-mercaptoethanol (ME). The pattern shown in Fig. 1B suggests that the disulfide bridges in the intact complex are either not accessible to ME, or do not significantly contribute to the strength of interaction between these proteins. In contrast to the high stability of the CFT–BPTI complex in SDS/ME, a structurally homologous complex formed between BT and
BPTI is not resistant to either SDS alone or SDS/ME (Fig. 1C). We compared the results of SDS/ME treatment of free CFT and its complex with BPTI, and found that the free enzyme underwent a dramatic structural change upon 1 min boiling in the SDS/ME-containing sample buffer (Fig. 1D). However, its stability in SDS is much lower than that of its complex with BPTI.

Comparison of thermal stabilities by differential scanning calorimetry

Surprisingly, the interaction between CFT and BPTI was significantly more stable than that of BT complexed with its own inhibitor. According to calorimetric measurements (Fig. 2), the melting temperatures \( T_m \) of CFT and BT without BPTI were approximately 54 and 67 °C, respectively. The different melting profiles indicated the lower structural stability of CFT. This was also reported by Fodor et al. [15]. BT exhibited two denaturation peaks. We know from our experience (unpublished results) that the smaller peak at lower temperature is related to unfolding of the autolytic product of BT, and its amplitude depends on the level of autolysis.

Addition of BPTI to BT increased the \( T_m \) value to 79.6 °C, suggesting that the stability of BT is increased by the interaction. However, the presence of BPTI caused significantly higher increase in the thermal stability of CFT, with a \( T_m \) value of 100.1 °C, indicating stronger enzyme–inhibitor interaction. A similarly dramatic but lower increase was observed previously on addition of SGTI to CFT \( (T_m = 91 \, ^\circ \text{C}) \) [15]. Generally, the unfolding transitions of trypsins and their complexes were irreversible. However, the observed differences in melting temperatures were dramatic and did not depend significantly on the experimental conditions (e.g. heating rate, concentration), suggesting real thermodynamic stability differences (data not shown).

Comparative studies by transverse urea gradient (TUG) PAGE

We used TUG gels to analyse the chemical stability of the two trypsins and to investigate the effect of BPTI

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**Fig. 1.** SDS/PAGE assays. (A) Reduced SDS/PAGE (sample buffer contains 5% v/v ME) for CFT, BPTI and a 1 : 4 molar ratio mixture of the trypsin and the inhibitor. (B) Reduced and non-reduced SDS/PAGE (absence of ME) of a mixture of CFT and BPTI in a 1 : 4 molar ratio. Before loading onto the gel, samples were boiled for 1–30 min in SDS-containing sample buffer. (C) Reduced and non-reduced SDS/PAGE of a 1 : 4 molar mixture of BT and BPTI. The samples were boiled in sample buffer as in Fig. 1B. (D) Comparison of the resistance to SDS of CFT and a mixture of CFT and BPTI (in a 1 : 3 molar ratio) by reducing SDS/PAGE.
binding on their stability. The unfolded protein occupies a larger hydrodynamic volume than the folded one, and thus migrates more slowly [22]. Therefore, the structural alterations and urea concentrations at which urea unfolding transitions occur may be studied by this method. Remarkable differences were observed between the two trypsins in the absence of BPTI, as shown in Fig. 3. CFT shows one transition between 2 and 4 m urea concentration, but unexpected bands appeared above 2 m urea (Fig. 3A). One transition was detected for BT at 3 m urea, but the unfolding of the protein did not produce additional bands (Fig. 3B). Thus the unfolding of BT in urea appears to be a one-step process. The complex of CFT and BPTI showed no unfolding transition, and its electrophoretic mobility was not changed at all up to 9 m urea (Fig. 3C). Although increased stability was detected for BT in the presence of BPTI, the complex was partially dissociated above 4 m urea (Fig. 3D).

Fluorescence measurements in urea

Detecting the emission maxima of the intrinsic fluorescence of the protein at various urea concentrations is a highly sensitive way to monitor urea-induced conformational changes [23–26]. CFT showed one steep transition centred at 6.2 m urea (in contrast to the transition detected by TUG gel electrophoresis) (Fig. 4A), whereas its complex with BPTI exhibited no transition and no shift in the emission maximum (Fig. 4B). In the absence of BPTI, BT showed one
transition near 3 M urea (which may correspond to the hydrodynamic volume change, detected by TUG) (Fig. 4A), whereas the BT–BPTI complex showed an increasing emission wavelength maximum without a transition (Fig. 4B).

Size-exclusion chromatography (SEC)

SEC gives us highly reliable and accurate data on the hydrodynamic dimensions of a given protein under native and denaturing conditions. Thus, protein conformers formed during denaturation may be separated physically [27]. Figure 5 shows the SEC chromatograms of CFT and BT in the absence (Fig. 5A) and presence of BPTI (Fig. 5B). In agreement with the TUG gel experiments, the increase in urea concentration not only caused a shift in the retention volume of CFT, but also led to the appearance of new peaks in the chromatogram. The BT chromatogram showed a gradual decrease in the retention volume. Binding of BPTI decreases the effect of urea on both trypsins. However, the BT–BPTI complex becomes partially dissociated in 4 M urea while the CFT–BPTI complex is still stable in 8 M urea.
Structural differences between CFT–BPTI and BT–BPTI complexes

The amino acid sequence of trypsin from *P. lepto-ductylus* was compared to that of BT (Fig. S1) and other vertebrate and non-vertebrate trypsins by Fodor *et al.* [15]. Their results suggest that the arthropod crustacean trypsins represent an evolutionary pathway that is different from that of other non-crustacean arthropod trypsins and vertebrate trypsins. The overall structure of CFT corresponds to the general chymotrypsin-like fold [28–30], but its disulfide bond pattern and extended loop regions suggest that it is more flexible than BT, and it has a lower structural stability.

Here we compared the X-ray structure of the CFT–BPTI complex with that of the BT–BPTI complex to determine whether certain structural features are responsible for the extreme physical and chemical stability. The natural crayfish trypsin in complex with BPTI was crystallized and the crystals diffracted to 1.99 Å resolution (PDB ID 4BNR). Data collection and refinement statistics are shown in Table 1. The overall fold of CFT is similar to that of BT, thus most of the structures are superimposable (Fig. 6).

Figure 7A,B shows the conformation of the P3–P2′ regions of BPTI in complex with CFT (Fig. 7A) and BT (Fig. 7B) (designation of the residues of the inhibitor follows the Schechter–Berger convention [31]). The only difference is the conformation of the P2′ (Arg17) residue. As described by Fodor *et al.* [15], there are remarkable differences in five loops of the CFT: Loop37, Loop60, Loop145, Loop173 and Loop202.

### Table 1. Crystallographic data and refinement.

<table>
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<tr>
<td>Rmerge (%)</td>
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<tr>
<td>Completeness (%)</td>
<td>98.1 (83.2)</td>
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<tr>
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<tr>
<td>Refinement</td>
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<tr>
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<tr>
<td>Number of unique reflections</td>
<td>45 201</td>
</tr>
<tr>
<td>Rwork/Rfree (%)</td>
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<td>Number of non-hydrogen atoms</td>
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*a* Values for the highest-resolution shell are shown in parentheses. 
*b* $R_{merge} = \Sigma l_o/\langle \Sigma l_o \rangle$, where $l_o$ observed intensity of a reflection, and $\langle \Sigma l_o \rangle$ the average intensity from multiple reflections. 
*c* $R$-factor = $\Sigma |F_o| - |F_c| / \Sigma |F_o|$. 
*d* The percentage of peptide bonds in the most favoured and additional region of the Ramachandran plot (determined by MolProbity [52]).

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Fig. 6. (A) Crystal structure of the CFT–BPTI complex (PDB ID 4BNR). The inhibitor is presented as its backbone structure, and shown in green. The color codes for trypsin are as described in (B). (B) Comparison of the overall conformation of CFT and BT. Structures are derived from the CFT–BPTI and BT–BPTI complexes (PDB ID 4BNR and 3OTJ, respectively). Conserved structural elements are coloured grey, loop regions are coloured red (for CFT) and blue (for BT). The catalytic triads (His57, Asp102 and Ser195) of the enzymes are shown in magenta. The figures were generated using PyMOL (Schrödinger, Portland, OR, USA).
Two of these loops are important parts of the inhibitor-binding region. Loop37 of CFT has five inserted residues, including three phenylalanine residues and one isoleucine residue, that are oriented towards the inhibitor and form a hydrophobic cluster with the P3′ and P4′ isoleucine residues of BPTI (Fig. 8A). The shorter Loop37 of BT (Fig. 8B) is less hydrophobic, as it contains only one phenylalanine (Phe41). Two further differences may explain the increased stability of the CFT–BPTI complex compared with BT–BPTI. First, the Gln175 residue of BT (Fig. 8D) is substituted by Glu175 in CFT (Fig. 8C). The negatively charged residue is oriented towards the inhibitor, and is close enough to form a salt bridge with the Arg39 residue of BPTI. Second, the Tyr96 residue is also oriented towards the inhibitor (Fig. 8C) and may form a hydrogen bond with the amino group of Arg39. Due to its shorter side chain, Ser96 of BT (Fig. 8D) cannot make contact with the inhibitor.

We also examined the flexibility of the CFT–BPTI complex based on the B-factors determined by X-ray crystallography, and compared them to previously studied trypsin–trypsin inhibitor complexes containing CFT, BPTI, BT, SGTI and SGPI-1-PO2 (the phage-selected mutant of SGTI [18]). The results shown in Fig. 9A are intriguing, as the B-factors do not correlate well in all regions. In the region between residues 150 and 180, the B-factors for CFT in the more stable CFT–BPTI complex are much higher than those of the other complexes. If ‘equivalent’ atoms of BT and CFT (see Experimental procedures) are compared in complex with BPTI, as shown in Fig. 9B, the same local region displays a slightly increased B-factor in CFT. As shown in Fig. 9C, this property is probably associated with the BPTI–CFT pair. When atoms of BT are compared in complexes with BPTI and SGPI-1-PO2, the B-factors in the BPTI complex do not show the same type of increase as seen in atoms of CFT in its CFT–BPTI complex compared to CFT–SGTI.

**Discussion**

The present study was initiated by our previous investigations on the protease specificities of two protease inhibitors isolated from the haemolymph of a desert locust, *Schistocerca gregaria* [17,33]. One of these inhibitors, SGTI, shows structural features of a trypsin inhibitor. However, it does not inhibit BT at all. An interesting finding was that CFT is a potent target enzyme of SGTI [15]. Based on studies of the X-ray structure and stability of the crayfish trypsin–SGTI complex [15,16], we examined the inhibitory effect of BPTI on CFT. Although the CFT–SGTI and CFT–BPTI complexes do not have any biological relevance (as no natural inhibitors of crustacean decapod trypsins have been identified), studying such highly stable but physiologically irrelevant protein–protein complexes is important to obtain insight into the molecular mechanism of protein–protein interactions. The practical purpose of these studies is to use this new knowledge to alter the strength of interactions between particular proteins, including those that are used in the pharmaceutical or other industries.

First, SDS/PAGE was used to compare the effects of SDS on the two proteases and their complexes with BPTI (Fig. 1). We observed several anomalies regarding the electrophoretic mobility of CFT in the absence
and presence of BPTI. A possible explanation for these observations may be that CFT is at least partially SDS-resistant due to its highly compact structure and predominantly negative charges on its surface. The free enzyme unfolds only under more severe SDS treatment conditions (boiling). The most remarkable finding of these experiments was that CFT, unlike BT, forms an extremely tight complex with BPTI that fully dissociates only upon 15 min boiling in the SDS sample buffer. SDS resistance has been described previously, but only for individual proteins (so-called ‘kinetically stable proteins’) [34–36], but in our case the extreme stability is the result of the complex formation of two different proteins.

Although the chemical mechanisms of denaturation of a protein by SDS and urea treatment are quite different [37–39], the extent of resistance of the CFT–BPTI complex to urea was similar to that for SDS/boiling.

Fig. 8. Superposition of the CFT–BPTI and bovine trypsin–BPTI complexes. The highlighted structural differences between CFT (A and C) and BT (B and D) may be responsible for the higher stability of the CFT–BPTI complex. BPTI is coloured cyan, conserved structural motives of trypsins are shown in grey, N and O atoms are shown in blue and red, respectively. The carbon atoms of the differing residues of trypsin (chymotrypsin numbering) are coloured deep blue (for BT) and green (for CFT). Hydrogen bonds and ionic interactions are indicated by dark grey and yellow dashed lines, respectively. (A, B) Insertion in Loop37. CFT has five inserted residues, including three phenylalanine residues and one isoleucine residue, in Loop37 (A), and may therefore form an extended hydrophobic region with the P3′ and P4′ isoleucine residues of BPTI. The shorter, less hydrophobic Loop37 of BT (B) has only one phenylalanine (Phe41) and one aromatic residue (Tyr39). (C, D) A glutamine/glutamic acid and a serine/tyrosine substitution. The Glu175 residue of CFT stabilizes the Arg39 residue of the BPTI through formation of a salt bridge (D). The Tyr96 residue of CFT also interacts with and stabilizes Arg39 of BPTI through a hydrogen bond (C). The distance between the OH group of Tyr96 and the α-amino group of the Arg39 is 3.1 Å. The Ser96 residue of BT has shorter side chain (D), and therefore is too far from the Arg39 to interact with it. The figures were generated using PyMOL (Schrödinger).
The results obtained by three methods – transverse urea gradient gel electrophoresis (Fig. 3), tryptophan fluorescence measurements (Fig. 4) and size-exclusion chromatography (Fig. 5) – are consistent, with a few exceptions. Each approach (except tryptophan fluorescence of CFT) shows that both CFT and BT undergo structural changes at approximately 2–4 M urea. A unique property of CFT is that, during the course of its transition, new molecular forms of the enzyme with decreasing mobilities are formed (Figs 3A and 5A). N-terminal sequencing of these components (data not shown) showed that they are autolysis products of the enzyme. Although native CFT is resistant to self-digestion [20], the partially unfolded forms are cleavable by the active enzyme population. The cleaved products may be separated by electrophoresis and SEC, but the fluorescence measurements showed only one steep transition at approximately 6 M urea concentration. A possible explanation for this phenomenon is that cleavage and urea concentration do not significantly affect the environment of the Trp side chains, i.e. they are still buried in a hydrophobic structure. Above 6 M urea, this structure unfolds. It is worth noting that, in the urea gel, enzyme activity is detected up to 6 M urea. Above this concentration, no further autolysis occurs, indicating complete unfolding of the enzyme.
enzyme molecules, i.e. loss of the globular structure. This is consistent with the transition above 6 m urea revealed by tryptophan fluorescence. Therefore, the major conclusion of these experiments is that BPTI stabilizes CFT against both heating and urea treatment. It is also clear from our data that these stabilizing effects cannot be simply explained by the behaviour of the free enzymes. The heat stability of the CFT–BPTI complex (Fig. 2) is remarkably high, and raises the question of what kind of structural interactions make this complex so much more stable to various denaturing effects compared to the BT–BPTI complex.

Comparison of the X-ray structures of the two complexes may answer this question (Figs 6–8). We found extended contacts in CFT–BPTI compared to BT–BPTI, including hydrophobic interactions, salt bridges and hydrogen bonds, but it is unclear whether these contacts are sufficient in themselves to account for the stability differences of the two complexes. Cloning and expression of CFT, and mutation of the recombinant enzyme at the various binding sites, are required to further study this phenomenon. Furthermore, obtaining the X-ray structure of apo-CFT would also be useful to study the possible flexibility differences between the free and complexed forms. Our hypothesis is that the structural dynamics of at least one unbound component may affect the stability of the resulting complexes. The results of the B-factor comparisons of the trypsin–trypsin inhibitor complexes are consistent with this hypothesis. We identified a region between residues 150 and 180 of CFT in complex with BPTI that has higher flexibility compared with other homologous complexes. However, it should be borne in mind that the structures were derived from different crystal forms and with very different resolution of diffraction data, and also very different modeling protocols were used. Despite these considerations, the approximated isotropic B-factors for equivalent atoms correlate strikingly well for most of the structure even though the absolute values of the atomic B-factors are very different due to the different resolutions of the data [varying from 0.95 Å (PDB ID 2XTT) to 1.99 Å (PDB ID 4NBR)]. Thus, in complex with BPTI, unlike the complex of SGTI, the structure of CFT became locally disordered. BT in complex with SGPI-1-PO2 or with BPTI also show lower flexibility than CFT complexes with similar or identical inhibitors.

On the basis of these comparisons, we believe that flexibility of one partner (e.g. CFT) in a protein–protein complex may be required for an extremely strong interaction. However, how flexibility increases stability is not yet clear.

Experimental procedures

Bovine pancreatic trypsin and bovine pancreatic trypsin inhibitor were purchased from Sigma (St Louis, MO, USA). Crayfish trypsin was isolated from Pontastacus leptodactylus as described below.

Isolation of crayfish trypsin (CFT)

Narrow-fingered crayfish (P. leptodactylus) trypsin was isolated and purified as described by Zwilling et al. [20] and modified by Fodor et al. [15]. Crayfish cardia fluid was collected using a teflon capillary connected to a syringe. The collected cardia fluid was diluted threefold with distilled water (pH 8.0) and filtered through a 0.22 μm MILLEX®-GP syringe driven filter (Merck Millipore, Billerica, MA, USA). The filtered cardia fluid was loaded onto a CNBr-Sepharose 4B soybean trypsin inhibitor column. The column was washed with three column volumes of distilled water (pH 8.0), and then eluted with dilute NH₃ solution (pH 11.0). Fractions containing CFT were pooled and loaded onto a MONO Q (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) ion-exchange column equilibrated with 10 mM MES buffer (pH 6.0), and eluted using a linear gradient of 0–3 m NaCl. Four isoforms of CFT were collected, and checked by SDS/PAGE and activity measurements. All isoenzymes were found to be identical with regard to their enzymatic activity and sensitivity to inhibition. For further studies, the most abundant form was chosen, and was concentrated by ultrafiltration using a Vivaspin® 20 centrifugal concentrator (Sartorius Stedim Biotech GmbH, Goettingen, Germany).

Electrophoretic assays

SDS/PAGE

SDS/PAGE was performed as described by Laemmli [40] using a 15% w/v polyacrylamide gel in a vertical slab gel apparatus (Bio-Rad, Hercules, CA, USA). The proteins were prepared as reduced and non-reduced forms. A four-fold molar excess of BPTI was added to the trypsin samples in order to study the enzyme–inhibitor complexes. Reduced samples were treated with reducing sample buffer containing 55 v/v β-mercaptoethanol; non-reduced samples were treated with sample buffer lacking β-mercaptoethanol. After vortexing, samples were boiled for 5 min (except for the time-dependent measurements).

Transverse urea gradient PAGE

Discontinuous TUG gels were prepared as described by Gentile et al. [41]. Slab gels were designed with a 0–9 m urea gradient perpendicular to the direction of electrophoresis. The increased viscosity caused by the increasing con-
centration of urea was compensated for by an inverse acryl-
amide gradient. The gradient was produced using two solu-
tions corresponding to the ends of the desired gradient: solu-
tion A contained 15.0% w/v acrylamide and no urea; solu-
tion B contained 11.6% w/v acrylamide and 9 m urea. Elec-

fluorescence measurements. Denaturation transition curves were
obtained from the wavelength of the tryptophan fluores-
cence maximum determined using an FLS-920 spectrotelo-

**Preparation and crystallization of the CFT–BPTI complex**

A fourfold molar excess of BPTI was added to CFT and in,
putated for 15 min at room temperature. The complex was
loaded onto a Superdex™ 75 HR 10/30 gel filtration column (GE Healthcare Bio-Sciences AB) and eluted using
150 mM NH₄HCO₃ (pH 8.0). The excess inhibitor was

**Structural analysis**

The diffraction data were collected at beamline ID14-1 of the
European Synchrotron Radiation Facility (λ = 0.9334 Å, 6.6 × 10¹¹ photons per second, 1° oscillation, 5 s
exposure per image, 180 images in total). The diffraction
data were integrated, scaled and merged using the XDS
package [44] (Table 1). Molecular replacement by the pro-
gram Phaser [45] identified two tightly packed heterodimers
in the asymmetric unit using trypsin subunit of PDB ID
2F91 [16] as the search model. Once the trypsin moiety was
identified, the BPTI subunit from PDB ID 1BZX [46] was
added following a superposition defined by the trypsin
subunits. Using the combined phase information from the
CFT and BPTI subunits, automated iterative model build-
ing was performed using the program Arp/wArp [47].
Model improvement was continued by iterative manual
rebuilding using the program Coot [48] and refinement by
phenix.refine of the PHENIX package [49]. Improvement
of the structure was continuously monitored by Molprobity
[50]. In the last rounds of refinement, the TLS model [51]
for B-factors (14 TLS groups in the asymmetric unit as
identified by the PHENIX package) was introduced, which
lead to an improvement of 2.4% in R̄free.

In the B-factor comparisons, atoms were defined as
equivalent if they shared the same residue number, inser-
tion code and atom name, and, if alternative conformations
exist, if the atom belonged to group A. It is important to
note that this procedure does not necessarily imply chemi-
cal or positional equivalency, but may be applied to substi-
tuted residues, and, to some extent, defines the distance of side-chain atoms from the main chain.

**Ethical statement**

All animals were cared for and treated according to the local ethical rules, which conform with the guidelines of the European Communities Council Directive 24 November 1986 (86/609/EEC). All efforts were made to minimize pain and suffering and to reduce the number of animals used.

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**References**


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**Supporting information**

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Fig. S1. Amino acid sequence alignment of CFT and BT.