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Interactions of human lacrimal and salivary cystatins with adenovirus endopeptidase

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Abstract

Over 100 serotypes of adenoviruses have been implicated in a variety of human and domesticated animal pathologies and some serotypes are widely used as gene transfer vectors. Aside from the limited use of vaccines for specific serotypes, little effort has been expended in the development of antivirals. The objective here was to study the effect of cystatins from human saliva (CS) and tears (CT), two points of viral entry, on adenain, the adenovirus type 2 encoded proteinase, which is absolutely required for infectivity. Two molecular weight species (13 and 14.5 kDa) were purified from both fluids at a yield of 5 mg/l. In vitro adenain activity was inhibited to 50% at a molar ratio of 5 CS:1 adenain and 3 CT:1 adenain. By comparison, papain was inhibited to 50% at a molar ratio of 2 CS:1 papain and 1.5 CT:1 papain. Adenain differed from papain in response to CS and chicken egg white (CEW) cystatin in being stimulated at low concentrations, and in being inhibited only at very high concentrations of cystatins. The presence of cleavage consensus sites specific to adenain in the human cystatins could drive the adenain–cystatin interaction predominantly in the substrate pathway direction. However, we found that the cystatins could only be digested after denaturation and by highly active fresh enzyme preparations. Our experiments designed to test the nature of the interaction between adenain and cystatins suggest a docking model for the adenain–human cystatin interaction, similar to that proposed for papain and CEW. At equilibrium the dissociation constant, K_d, between adenain and CT was 1.2 nM. The kinetic parameters determined here suggest a simple reversible mechanism for the inhibition of adenain by human cystatins. We conclude that the cystatins present in tears and saliva are unlikely to play a significant role in inhibiting adenovirus infections.

Keywords: Adenovirus type 2; Adenain; Papain; Cystatins

1. Introduction

Adenoviruses are implicated in a variety of human and domesticated animal pathologies and are currently under intensive investigation as gene transfer vectors (Horwitz, 1996; Qualikene et al.,

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2000; Wickham, 2000; Doronin et al., 2001). So far very little effort has been directed towards the development of antiviral strategies (De Clercq, 1993; Cornish et al., 1995; Sircar et al., 1996, 1998; Mentel et al., 1997). To partly fill this hiatus, the objective of the present investigation was to explore the potential inhibitory activity of the cystatins present in two biological fluids, saliva and tears which are at two common points of viral entry, towards the adenovirus endopeptidase, in comparison with the well characterized interaction between papain and chicken egg white (CEW) cystatin.

The human adenovirus 2 endopeptidase (adenain) is a 204 amino acid monomer of 24,838 Da and a member of the cysteine proteinase family (reviewed in Weber, 1999). Adenain complexed with its activating peptide has a K_m of 5 mM and its activity is optimal at pH 8 and 45 °C in the presence of 1 mM thiol compounds and positively charged polymers. The geometric disposition of the active site residues (H54, E/D71, C122) is identical to that of papain (H159, N175, C25) and so is the location of Q115 of adenain and Q19 of papain (Ding et al., 1996). In consequence some papain inhibitors also inhibit adenain (Sircar et al., 1998). The enzyme is specific for two consensus sites (M, I, L) XGG-X or (M, I, L) XGX-G, where X is any amino acid. A group of proteinases with similar substrate specificity and related structures includes the cysteine proteinases of African swine fever virus, vaccinia virus, fowlpox virus, and the UbI-specific proteinases of yeast and Chlamydia (Lopez-Otin et al., 1989; Li and Hochstrasser, 1999). Adenovirus maturation, infectivity and uncoating are dependent on proper adenain activity, suggesting that the viral proteinase is an appropriate target for the development of antivirals.

The endogenous cysteine proteinase inhibitors represent the final level at which cysteine proteinase activity can be regulated (Rawlings and Barrett, 1994; Lah and Kos, 1998). Various endogenous cysteine proteinase inhibitors are found in body fluids and tissues of animals and plant seeds. Animal cysteine proteinase inhibitors belong to the protein superfamily, cystatins (Brzin et al., 1984). Generally, cystatins do not form a covalent bond with cysteine proteinases, but instead cover the active site cleft blocking access to the active site. Physiological inhibitors that are specific for cysteine proteinases structurally related to papain all belong to the cystatin superfamily. Cystatins are tight binding, reversible inhibitors that may be subdivided in three main families of increasing structural complexity (Barrett, 1987; Barrett et al., 1986). Two low molecular weight cysteine proteinase inhibitors, cystatin A and cystatin B also called stefins, have been isolated from human skin, liver and granulocytes (Järvinen and Rinne, 1982; Ritonja et al., 1985). Two more low molecular weight inhibitors, cystatins S and SA, have been isolated from urine, blood serum (Brzin et al., 1984) and saliva (Isemura et al., 1984). Two high molecular weight cysteine proteinase inhibitors, also called kininogens, such as α_2-macroglobulin and kininogen, have been isolated from blood plasma and urine (Sueyoshi et al., 1985; Abrahamson, 1994).

The exact mechanism of inhibition which is related to a docking model is not yet fully understood, but there is evidence that several highly conserved fragments in the molecule are involved (Bode et al., 1988; Abrahamson, 1994). One or more of these conserved cystatin fragments behave in a substrate-like manner and rapidly interact with their target enzymes (Serveau et al., 1994; Turk et al., 1998). The crystal structure of papain complexed with the cystatin from egg white revealed three contact points (Machleidt et al., 1989). First, the N-terminal region which contains Gly, an absolutely conserved residue on position 9 in the case of CEW, is directed towards the opposite and narrower part of the active site cleft of the enzyme, which includes the substrate binding regions S1–S3. This segment does not interact with Cys25 of the papain active site but partially blocks it. Second and third, two β-hairpin loops have the appropriate shape and size to fill the more open part of the active site of papain which represent its putative S1′–S2′ subsites (Machleidt et al., 1989). The first β-hairpin contains the highly conserved sequence QXVXG (mainly QLSVG or QVVAG) and the second β-hairpin contains the absolutely conserved PW at position 103 and 104, respectively, in the case of CEW (Auerswald et al., 1992). A X-ray crystallographic analysis of human salivary cystatins was published by Ramasubbu et al. (1996) and is consis-

tent with the docking model proposed previously (Bode and Huber, 1992, 1994; Bode et al., 1990; Katunuma and Kominami, 1995).

2. Materials and methods

2.1. Enzymes and assays

Recombinant adenovirus type 2 proteinase (Adenain; EC3.4.22-) was purified by chromatography from an *Escherichia coli* expression system (pLPV) as described before (Keyvani-Amineh et al., 1995). Enzyme activity was measured with a fluorescent peptide substrate, R110, (rhodamine 110, bis-(L-leucyl-L-arginylglycylglycine amine) tetrachloride, purchased from Molecular probes, Inc. (4849 Pitchford Ave., Eugene, OR 97402), essentially as described before (Diouri et al., 1995). The reaction mixture contained TE buffer (10 mM Tris–HCl, pH 8, 1 mM ethylene diamine tetraacetic acid (EDTA)), 40 μM pVIc (oxidized enzyme activating peptide GVQSLKRRRCF), 3.3 μM R110, 1 μg of adenain in a total volume of 300 μl. Incubation was at 37 °C for 4 h and the fluorescence was measured.

Papain was purchased from Sigma and it was used at a concentration of 2.5 μg and the buffer/activator contained 340 mM sodium acetate, 60 mM acetic acid, 4 mM disodium EDTA, pH 5.5. On the day the buffer/activator was to be used, 8 mM cysteine was added. Incubation with papain was at 37 °C for 18 h.

In the case of protein substrates (cystatin from saliva, CS, cystatin from tears, CT, cystatin from CEW or high molecular weight kininogen (HMWK)) unless otherwise indicated, the reaction mixture contained 1 μg substrate which was boiled or not before reaction, 0.8 μg adenain in a total volume of 60 μl. The latter reactions were stopped by boiling in lysing solution.

Qualitative assays were done as described before with substrates of ts1 infected cell lysates labeled with ^{35}S-methionine at the non-permissive temperature (39 °C) at 24 h p.i. as a source of viral precursor proteins, particularly pVII (Keyvani-Amineh et al., 1995). Ts1 is defective for protease activity at 39 °C, consequently providing a ready source of viral precursor proteins. This substrate was boiled to inactivate any residual adenain. The adenain was preincubated with inhibitors (CS, CT, CEW or HMWK) before substrate addition. Enzyme activities were assessed from the conversion of viral precursor protein pVII to VII as visualized on autoradiograms of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separations.

2.2. Cystatins

CEW cystatin, HMWK and papain were purchased from Sigma. Cystatins from saliva (CS) were purified essentially as described by Isemura and Saitoh (1994), Isemura et al. (1991). Briefly, human whole saliva (10 ml per tube), in the absence of stimulation, was collected from three healthy female subjects into ice cooled test tubes containing 0.5 ml of a proteinase inhibitor solution (PIS; 500 μg pAPMSF, 25 μg phosphoramidon, 500 μg trasylol, 1 mg NaF, 10 mg EDTA · 2Na in 0.5 ml of 0.1 M Tris–HCl, pH 7.5). Four volumes of cold methanol were added to the 60 ml of pooled saliva and the precipitates formed were removed by filtration. Methanol was removed from the filtrate by evaporation under reduced pressure at 40 °C, and the residue was lyophilized. The lyophilized material was dissolved in bidistilled water and dialyzed against 20 mM Tris–HCl buffer (pH 7.5) containing 5% inhibitor solution. After removing insoluble materials by centrifugation, the supernatant was applied to a DE52 column equilibrated with the same buffer. After washing, the proteins were eluted with an incremental NaCl gradient (10; 50 mM; 0.1; 0.2; 0.5 M). Fractions were monitored by SDS-PAGE and the purest fractions were dialyzed against 0.02 M Tris–HCl buffer (pH 7.5) containing 5% inhibitor solution.

Tears from a 30-year-old healthy female donor, in the absence of stimulation, were collected using capillary tubes and processed essentially as described by Barka et al. (1991). The tears were flushed out from the tubes with sterile bidistilled water and then precipitated with methanol. The latter was removed by evaporation under reduced pressure at 40 °C. After lyophilization the material was resuspended in 100 μl of water. A mixture (400 μl) of methanol–acetone (50:50) was then added and the preparation was kept at −20 °C

overnight. The material was dialyzed against TE buffer (10 mM Tris–HCl, pH 8, 1 mM EDTA) using Spectrapor membrane tubing (m.w. cutoff 6000–8000).

Tears were also collected by the Schirmer paper strip method (absence of stimulation) from two healthy adult volunteers (Kitaoka et al., 1985). The whatman papers were dried at room temperature before extraction of the tear components with nanopure water. After lyophilization, the material was resuspended in 200 μl of water and dialyzed as described for the salivary cystatins. In order to protect cystatins against proteolytic breakdown, we have added an inhibitor cocktail PIS (5%) in the TE dialysis buffer. As blanks, extracts of the same size filter paper strips without tears were used. The purity of all the proteins and the protein concentration were determined by SDS-PAGE and the Bradford Assay, respectively, using bovine serum albumin (BSA) as standard.

2.3. Immunoprecipitation

Recombinant adenain (0.7 μg) and ^{125}I-labeled CS (iodogen method) or CT (1.5 μg) was incubated at 37 °C for 10 min, then 10 μl of anti-adenain antibodies or control non-immune serum were added in a total volume of 100 μl and incubated at 0 °C for 1 h. The immune complexes were precipitated with protein A bound to Sepharose CL-4B beads by rotation at 4 °C overnight. The material was pelleted by centrifugation and washed five times with equal volumes of phosphate buffer saline (PBS), the washes being combined with the initial supernatant. The radioactivity in the pellets and supernatants were counted in a LKB 1282 Compugamma. Anti-adenain was a polyclonal rabbit serum against the pLPV-produced purified recombinant enzyme.

2.4. Determination of equilibrium binding parameters

The binding of cystatins to adenain were studied under conditions of ligand excess, and were monitored by counting the γ-radiation in the immunoprecipitates.

The dissociation constant at equilibrium (K_d) was determined by means of a ligand (^{125}I-CT) saturation curve showing specific binding as a function of free ligand concentration by varying the ligand concentrations from 1 to 5 nmols (total volume of 100 μl). Adenain concentration was constant at 100 pmol. Non-specific binding was subtracted from total binding. An initial estimate of the dissociation constant of ^{125}I-CT was based on the equilibrium concentration of free ligand required to achieve 50% saturation of the binding sites. Scatchard analysis was performed to give a better estimate of the K_d. By plotting the ratio bound/free versus bound, the absolute value of the slope is an estimate of K_a or $1/K_d$ while the extrapolation of the Scatchard plot to the x-axis yields an estimate of maximal specific binding of 50% of adenain receptors. The K_d was determined in the presence and in the absence of adenain cofactor pVIc. k_{ass} is the association rate constant of the adenain–cystatin interaction. The dissociation rate constant, k_{diss}, was obtained by competition between radiolabeled cystatin from tears (CT) and excess unlabeled cystatins from saliva (CS). The dissociation of radiolabeled ^{125}I-CT from the complex adenain–^{125}I-CT, was studied by preincubation of adenain with ^{125}I-CT followed by addition of unlabeled competing CS. The cold competitor should have a chemical structure distinct from that of the labeled ^{125}I-CT to avoid displacement of non-specific as well as specific binding, therefore, we added excess cold CS (at least 100 times of ^{125}I-CT) because CS and CT are highly similar but with different p*I*s. The displacement of ^{125}I-CT by cold CS could occur if the binding is a simple reversible equilibrium. The dissociation constant of the complex between adenain and cystatins may be deduced from these displacement experiments when the displacing CS is in excess. The half-life corresponding to this dissociation constant was obtained for the adenain–cystatin complex.

The K_d is the dissociation constant at equilibrium and corresponds to k_{diss}/k_{ass}. The association rate constant at equilibrium or affinity constant of the binding reaction is $K_a = 1/K_d$. The kinetic calculations were as follows:

$$V_{\text{diss}} = -\frac{\text{d}[^{125}\text{I-CT–Adenain}]}{\text{d}t} = k[^{125}\text{I-CT–Adenain}] \Leftrightarrow -\frac{\text{d}[^{125}\text{I-CT–Adenain}]}{[^{125}\text{I-CT–Adenain}]} = k\ \text{d}t \quad (1)$$

Integration from $t = 0$ at time t gives

$$\ln[^{125}\text{I-CT–Adenain}]_t - \ln[^{125}\text{I-CT–Adenain}]_0 = -kt$$

$$\Leftrightarrow \frac{[\ln[^{125}\text{I-CT–Adenain}]_0 - \ln[^{125}\text{I-CT–Adenain}]_t]}{t} = k$$

3. Results and discussion

3.1. Cystatins from saliva and tears are weak inhibitors of adenain activity

The purified cystatins are shown in Fig. 1. The cystatins from tears (CT) resolved into two bands,

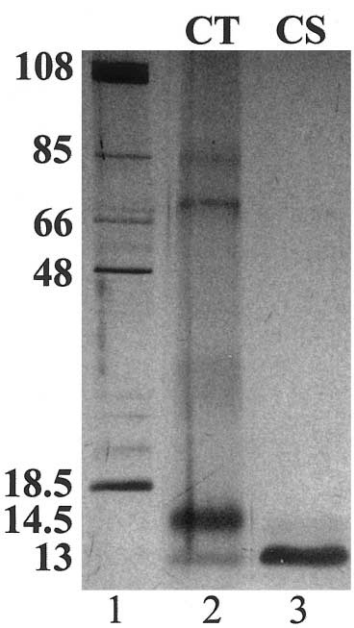

Fig. 1. Purification of human cystatins from saliva (CS) and tears (CT). SDS-PAGE (12.5%) of the purified proteins stained with silver nitrate. Samples were run in duplicate. Lane 1 contains Ad2 virus as molecular weight marker.

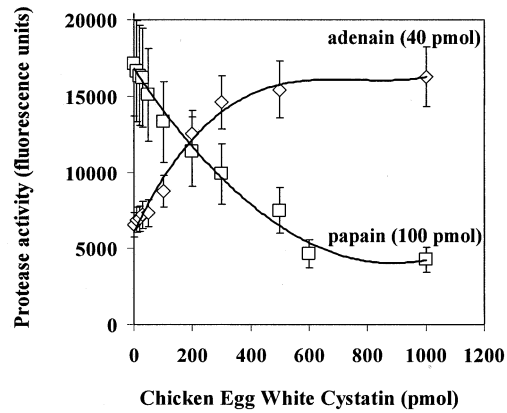

Fig. 2. The effect of egg white cystatin (CEW) on papain and adenain activity in vitro. The reaction mixtures contained 100 pmol of papain, 3.3 μM of R110 fluorescent peptide substrate and increasing concentrations of CEW (up to 1000 pmol, i.e. a molar ratio of papain to CEW of 1:10). For adenain, the reaction mixtures contained 40 pmol of adenain, 3.3 μM of R110 fluorescent peptide substrate and increasing concentrations of CEW (up to 1000 pmol, i.e. a molar ratio of adenain to CEW of 1:25). The fluorescence units of the cleaved substrate represent the mean of five experiments and experimental variation was less than 5%. The mean adenain and papain activity in the absence of CEW was 6334 and 17,150, respectively.

a main component of 14.5 kDa and a minor component of 13 kDa (Fig. 1, lanes 2–3). The purification from whole saliva also led to a mixture of two molecular species, the 13 kDa component being predominant (lanes 4–5). The yield of purified cystatins from both sources was around 5 mg/l. To study the effect of these cystatins on the activity of adenain we first compared the effect of CEW on adenain with the effect on papain, the latter being a well documented standard (Barrett, 1987; Lindahl et al., 1988). The reaction mixtures contained the enzymes and CEW prior to the addition of the fluorescent R110 peptide substrate. We observed a dose dependent decrease in papain activity as the ratio of CEW to papain was increased (Fig. 2). CEW did not show any inhibitory activity against adenain, but instead stimulated proteinase activity, possibly by stabilizing or buffering the enzyme. We next tested the effect of cystatins from saliva (CS) on papain and found that the enzyme was inhibited more efficiently than by CEW (Fig. 3). CS also inhibited adenain,

albeit with puzzling kinetics. The results with CT gave classical inhibition curves on both enzymes (Fig. 4). For a further comparison we also tested human kininogen, a high molecular weight cystatin. Kininogen was less efficient than either CT or CS on either enzyme (Fig. 5).

To facilitate comparison the results were expressed in terms of the molar ratio of cystatin to enzyme required to achieve 50% inhibition (Table 1). In comparison with papain, which is inhibited by all four cystatins tested, adenain was only efficiently inhibited by CT and CS. It should be noted that the cocktail of inhibitors used during the purification of CS and CT had no effect on adenain activity.

These experiments were repeated with adenain, using the ts1 precursor proteins as substrate. The results (not shown) were similar to the above, confirming that CEW does not inhibit adenain and that CT is more efficient than CS.

The proteins obtained from these two biological fluids, i.e. saliva and tears, are a mixture of several cystatins which may have different affinities for the active site of the proteinase. On SDS-PAGE, the predominant band of the CS mixture had an approximate molecular weight of 13 kDa while the

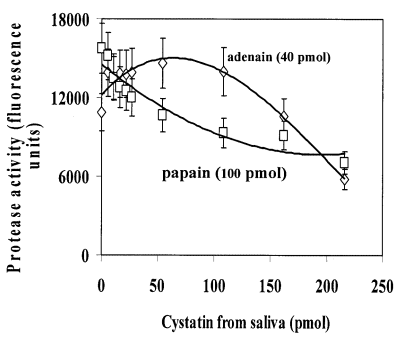

Fig. 3. The effect of salivary cystatins (CS) on papain and adenain activity in vitro. The reaction mixtures contained 100 pmol of papain, 3.3 μM of R110 fluorescent peptide substrate and increasing concentrations of CS (up to 216 pmol, i.e. a molar ratio papain of CS of 1:2.16). For adenain, the reaction mixtures contained 40 pmol of adenain, 3.3 μM of R110 fluorescent peptide substrate and increasing concentrations of CS (up to 216 pmol, i.e. a molar ratio of adenain to CS of 1:5). The fluorescence units of the cleaved substrate represent the mean of five experiments and experimental variation was less than 5%. The mean adenain and papain activity in the absence of CS was 10,582 and 15,740, respectively.

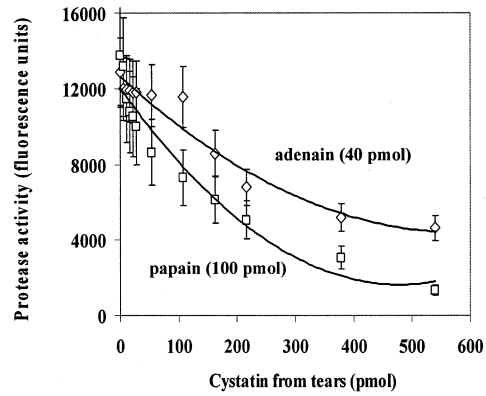

Fig. 4. The effect of cystatins from tears (CT) on papain and adenain activity in vitro. The reaction mixtures contained 100 pmol of papain, 3.3 μM of R110 fluorescent peptide substrate and increasing concentrations of CT (up to 540 pmol, i.e. a molar ratio of papain to CT of 1:5.4). For adenain, the reaction mixtures contained 40 pmol of adenain, 3.3 μM of R110 fluorescent peptide substrate and increasing concentrations of CT (up to 540 pmol, i.e. a molar ratio of adenain to CT of 1:16). The fluorescence units of the cleaved substrate represent the mean of five experiments and experimental variation was less than 5%. The mean adenain and papain activity in the absence of CT was 13,740 and 12,888, respectively.

predominant band of the CT mixture had an approximate molecular weight of 14.5 kDa (Fig. 1). CS and CT have been shown to contain the same cystatin variants (S, SA, SN, C, D) but in different ratios (Isemura and Saitoh, 1994; Isemura et al., 1991). We assume that the presence of the two molecular weight species in our purification is an indication of the presence of these different cystatins. Because CT was the more efficient inhibitor, the inhibition could be attributed to the higher molecular weight species, which are more abundant in CT. Another important fact to consider for the family 2 cystatins is the method of purification. These cystatins are frequently isolated in multiple isoforms of varying p*I* because of proteolytic cleavage of bonds in the N-terminal segment (Bobek et al., 1993). As noted above, we have attempted to minimize spurious proteolysis by the inclusion of a cocktail of inhibitors during the purification steps.

3.2. Denatured, but not native, cystatins are cleaved by adenain

The cystatins purified from saliva and tears

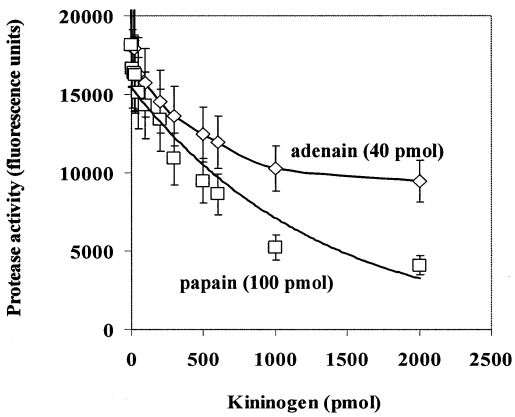

Fig. 5. The effect of HMWK on papain and adenain activity in vitro. The reaction mixtures contained 100 pmol of papain, 3.3 μM of R110 fluorescent peptide substrate and increasing concentrations of HMWK (up to 2000 pmol, i.e. a molar ratio of papain to HMWK of 1:20). For adenain, the reaction mixtures contained 40 pmol of rAVP, 3.3 μM of R110 fluorescent peptide substrate and increasing concentrations of CS (up to 2000 pmol, i.e. a molar ratio of adenain to HMWK of 1:50). The fluorescence units of the cleaved substrate represent the mean of five experiments and experimental variation was less than 5%. The mean adenain and papain activity in the absence of HMWK was 18,150 and 18,026, respectively.

contain adenain consensus cleavage sites while CEW and kininogen do not. CT and CS contain similar adenain consensus cleavage sites (IEGG for cystatin SA; IPGG for cystatins S and SN; LVGG for cystatin C; LAGG for cystatin D) in the N-terminal segment, which is thought to be involved in the inhibitory mechanism. Furthermore, the sequence of the first β-hairpin which is presumed to be involved in the inhibitory docking wedge contains a second adenain consensus cleavage site (IVGG) in the case of cystatins SA and D.

Table 1
Moles of cystatin required for 50% inhibition of one mole of proteinase

Cystatin	Papain	Adenain
CS (salivary)	2	5
CT (lacrimal)	1.5	3
HMWK	3.5	50
CEW	4	>50

These data were calculated from Figs. 2–5.

The first site is only about ten residues from the N-terminus, and would therefore be difficult to detect upon cleavage. The second site in the hairpin is located at a position in the molecule, which upon cleavage would easily be detected as new bands by SDS-PAGE. Even extensive incubation of native CT and CS with adenain failed to show hydrolysis. Heat denaturation by boiling resulted in partial hydrolysis of CT (Fig. 6A, lanes 3–4 and 6–7) and also CS (Fig. 6B, lanes 4 and 6).

In the case of the HMWK, the cleavage of the denatured protein was much more complex. HMWK was completely degraded when the protein was incubated overnight with adenain, pVIc and β-mercaptoethanol (Fig. 6C, lane 12). However, after only 2 h of incubation, we detected three main cleavage products. The size of these fragments corresponded approximately to cleavages at the two cystatin motifs QVVAG (Fig. 6C, lanes 10–11). Digestion occurred only with highly active enzyme and only after denaturation of the protein. The complete digestion of the protein in the presence of β-mercaptoethanol may be due to reduction of oxidation products formed by the presence of 19 cysteines. QVVAG is not an adenain consensus cleavage site. The observed cleavage adds a second unconventional site to that previously seen in the reactive site loop of a serpin, SCCA1 (Ruzindana-Umunyana et al., 2000). Denatured cystatins could, therefore, act as competitive inhibitors of adenain. But denatured cystatins (by boiling or treatment with 1% SDS) did not show any inhibitory activity towards adenain, possibly because of low affinity for this unconventional site (data not shown). It is interesting in this regard, that denatured CEW which also does not contain any consensus sites, was also marginally hydrolyzed, but not sufficiently to act as a competitive inhibitor (data not shown). We suggest that this light cleavage might occur in QLSVG within the first hairpin, which is thought to be involved in the inhibitory mechanism with papain.

These results are in agreement with theoretical studies and predictions reported recently (Tyndall and Fairlie, 1999; Fairlie et al., 2000). This group showed that aside from sequence/composition, the inhibitor/substrate conformation also influences proteinase recognition and subsequent cleavage.

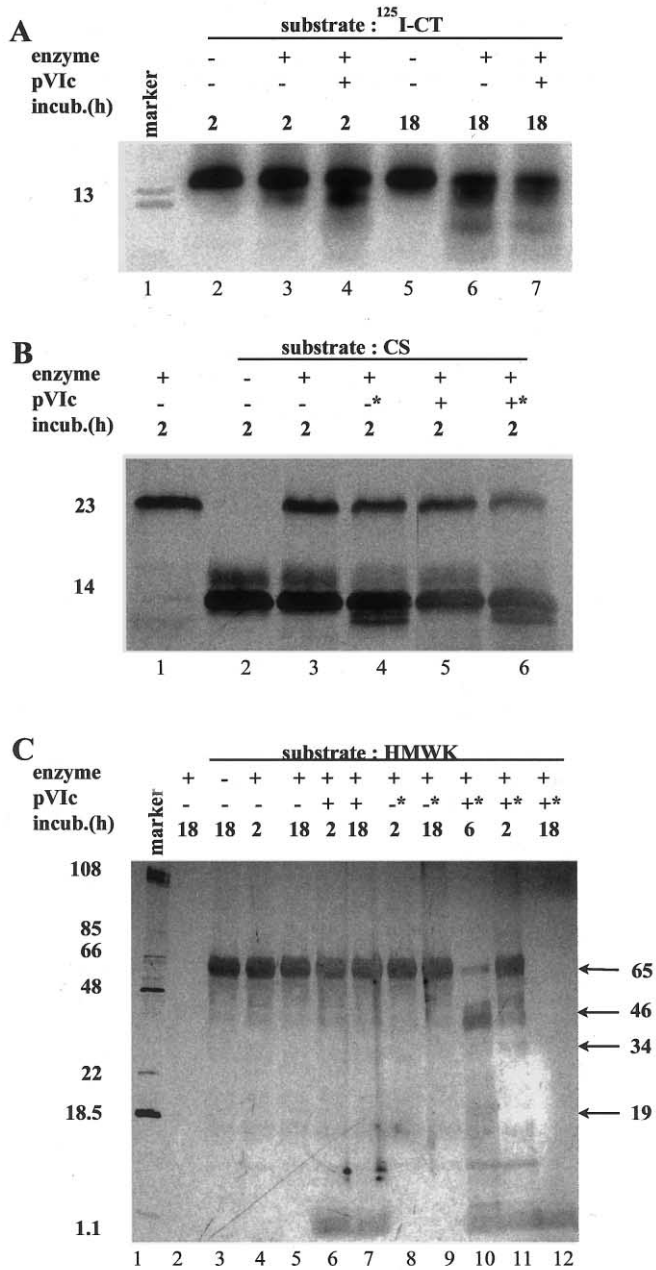

Fig. 6. The digestion of denatured cystatins by adenain. Panel A, ^{125}I-labelled cystatin from tears (CT) was incubated with 1 μg of adenain and activating peptide pVIc for the indicated times, then the reaction was stopped by adding lysing solution and boiling. Samples were separated on a 15% SDS-PAGE gel, dried and autoradiographed. Panel B, salivary cystatin (CS) was incubated with 2 μg of adenain, activating peptide pVIc and 1 mM β-mercaptoethanol (∗) for the indicated times, then the reaction was stopped by adding lysing solution and boiling. Samples were separated on a 15% SDS-PAGE gel and stained with silver nitrate.Panel C, HMWK was incubated with 0.15 μg of adenain, activating peptide pVIc and 1 mM β-mercaptoethanol (∗) for the indicated times, then the reaction was stopped by adding lysing solution and boiling. Samples were separated on a 12.5% SDS-PAGE gel and stained with silver nitrate. The arrows denote the cleavage products of approximately 46, 34, 31 and 19 kDa.

Indeed, the binding studies for human immunodeficiency virus (HIV)-1 proteinase inhibitors/substrates showed that when these inhibitors/substrates are preorganized in an extended conformation, they have significantly higher proteinase affinity.

3.3. Human cystatins bind to adenain and inhibit it by a simple bimolecular reversible mechanism

In order to characterize the cystatin–adenain interaction, we performed some preliminary studies of the kinetics of association and dissociation. The concentration dependence of specific binding provides direct information about the affinity of the cystatins for adenain binding sites. We observed that specific binding was saturable thereby suggesting a finite concentration of binding sites. In the simplest case described here, an initial estimate of the dissociation constant of ^{125}I-CT is provided by the equilibrium concentration of free ^{125}I-CT needed to give 50% occupancy of the binding sites. The K_d evaluated from this plot was slightly above 1×10^{-9} M (data not shown). A better estimate of this parameter can be obtained by a Scatchard plot of the ratio of bound/free versus bound ^{125}I-CT (Fig. 7A). Extrapolation of the Scatchard plot to the x-axis yields an estimate of the adenain concentration corresponding to the maximal specific binding, that is 0.24 nM. The slope provides an estimate of $-K_a$ or $-1/K_d$, i.e. $K_a = 0.8129$ and $K_d = 1.23$ nM in the absence of the adenain cofactor pVIc (Fig. 7A). Therefore, 1.23 nM is the free ^{125}I-CT concentration at 50% adenain saturation. In identical experiments, we also determined the dissociation constant at equilibrium in the presence of the adenain cofactor pVIc (Fig. 7B). The value obtained was 1.7 nM. The linearity of the kinetic profiles indicated that we are dealing with a homogeneous class of binding sites.

As shown by Eq. (1), the dissociation constant can be deduced from the displacement experiments (Fig. 8A and B). The k_{diss} is the absolute value of the inverse of the slope. The association constant k_{ass} is estimated by the ratio K_d/k_{diss}. The half-life of the complex when

$$[^{125}\text{I-CT}-\text{Adenain}]_t = \frac{1}{2}[^{125}\text{I-CT}-\text{Adenain}]_0$$

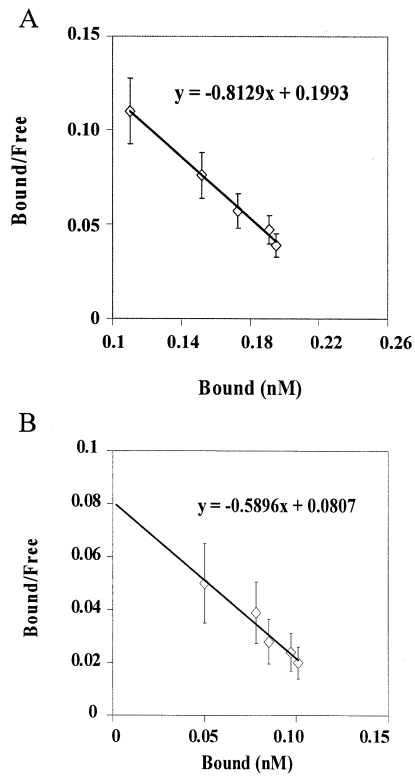

Fig. 7. Scatchard analysis of the binding of tear cystatins to adenain. The binding of 1–5 nmol of ^{125}I-CT to 100 pmol of adenain was assayed by immunoprecipitation (total volume of 100 μl). The assays are the means of three experiments, the bars indicating the S.E. y gives the equations of the linear regressions for the calculation of K_a and K_d. Panel A shows binding in the absence of pVIc and Panel B in the presence of pVIc.

is given by $\ln 2 = k\tau_{1/2} \Rightarrow \tau_{1/2} = \ln 2/k$.

Table 2 shows the kinetic parameters published for chicken cystatins and four cysteine proteinases (Lindahl et al., 1988; Björk et al., 1989, 1990) as compared with the kinetic parameters of lacrimal cystatin–adenain binding determined by the present studies. The inhibition constant (K_i) of the human cystatins towards adenain was not determined in the present studies, but the dissociation constant at equilibrium is a relevant estimate of the inhibitory efficiency of these cystatins. The K_a values provide a measure for the affinity of the cystatins to the different enzymes. The affinity between the different cysteine proteinases and cys-

tatins has been found to be highly variable (Table 2). These enzymes may use the same binding mechanism and the K_a merely reflects the differences in the nature of the interactions. Previous studies using inactivated forms of enzymes have shown different affinities for the cystatins (Björk et al., 1989). They were consistent with interactions of considerable strength occurring between the cystatins and domains of the enzymes at some distance from the reactive cysteine residue.

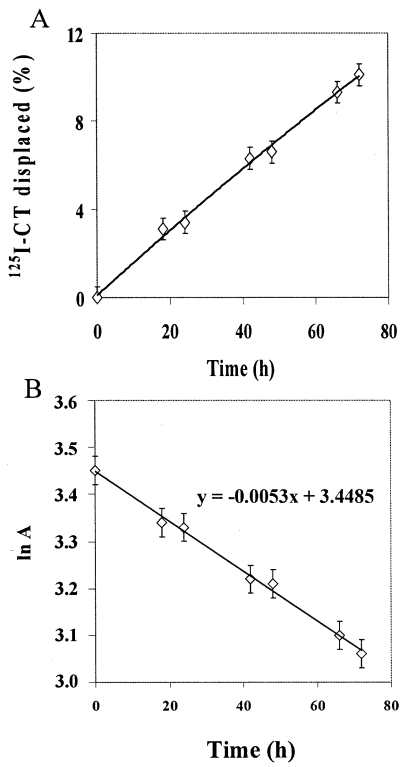

Fig. 8. Dissociation of the cystatin–adenain complex by competition with heterologous cystatin. Panel A, analysis by immunoprecipitation of the displacement of ^{125}I-CT from its complex with adenain by an excess of unlabeled CS. The complex between ^{125}I-CT and adenain was formed with equimolar amounts (100 pmol for both) for 15 min, and the unlabeled CS was added at a concentration of 10 nmol (total volume of 100 μl). The cystatin displaced from the complex by the competitor was corrected for dissociation in the absence of competitor and this value was plotted as a function of time. Each time point was repeated at least three times and the variation was less than 5%. Panel B, determination of k_{diss}/hour from the data in Panel A. Plot of ln A vs. time. 'A' is the percent of labeled cystatin complexed with adenain at $t = 0$ (in the absence of the competitor) up to $t = 72$ h after the addition of the competitor. Y is the linear regression equation of the plot for the calculation of k_{diss} and k_{ass}.

The present results suggest that the interaction between adenain and human cystatins is a non-covalent reversible interaction. However, because the complex dissociates slowly it appears irreversible. A pharmacologically effective proteinase inhibitor should fulfill the following conditions, the interaction between enzyme and inhibitor must be irreversible or if reversible, the dissociation constant of the enzyme–inhibitor complex must be less than 0.1 nM in order to maintain inhibitory activity for a longer period of time. As for adenain and human cystatins, this constant is 1.23 nM, therefore we consider these inhibitors as weak ones. Furthermore, the affinity constant deduced from the Scatchard analysis suggests that the presence of the adenain cofactor pVIc reduces the binding of cystatins to adenain (Fig. 7A and B; Table 2). The uncomplexed adenain seems to have a solvent-exposed active site that could accommodate inhibitor segments of cystatin loops more easily than the complexed adenain.

We conclude that the inhibitory mechanism of human cystatins on adenain is a tightly binding, reversible mechanism near the active site and appears to be competitive with the substrate although there was no detectable cleavage of the reactive domains of cystatins, which contain two adenain consensus cleavage sites. It is interesting to compare these natural inhibitors of cysteine proteinases to another natural inhibitor we tested in a previous study, the squamous cell carcinoma antigen (SCCA1; Ruzindana-Umunyana et al., 2000). This cross-class inhibitor, SCCA1, has no adenain consensus cleavage site but its native form was nevertheless cleaved by adenain in the reactive site loop. It inhibited adenain to 50% in a competitive and irreversible manner at a molar ratio of SCCA1 to adenain of 1.6:1. In the case of human cystatins, there was limited proteolysis by adenain and the 50% inhibition was not achieved until a molar ratio of CT to adenain of 3:1. These cystatins are therefore unlikely to play a significant role in inhibiting adenovirus infections.

Table 2
Equilibrium and kinetic constants of the interaction of cystatins with five cysteine proteinases

Enzyme	k_{ass} (M/s)	k_{diss} (per s)	K_d (M)	K_a (per M)	$\tau_{1/2}$
Papain[a]	9.9×10^{6}	5.7×10^{-7}	6×10^{-14}	1.7×10^{13}	14 days
Actinidin[a]	2.2×10^{6}	1.1×10^{-2}	5×10^{-9}	2×10^{8}	63 s
Chymopapain[a]	2.6×10^{6}	2.4×10^{-6}	9×10^{-13}	1.1×10^{12}	3 days
Ficin[a]	9.6×10^{6}	4.6×10^{-7}	5×10^{-14}	2×10^{13}	17 days
Adenain[b]	5.4×10^{4}	1.5×10^{-6}	1.2×10^{-9}	8.1×10^{8}	5 days
Adenain[b] + pVIc	ND	ND	1.7×10^{-9}	5.9×10^{8}	ND

k_{ass} is the association rate constant (on rate) of the enzyme–cystatin interaction; k_{diss} is the dissociation rate constant (off rate) of the enzyme–cystatin complex; K_d is the dissociation constant at equilibrium, $K_d = k_{diss}/k_{ass} = 1/K_a$; K_a is the association constant at equilibrium or affinity constant of the binding reaction (the inverse of the K_d); $\tau_{1/2}$ is the half-time of the complex dissociation reaction given by $\ln 2/K_d$.

[a] These constants are taken from Björk et al. (1989, 1990), Olsson et al. (1999), referring to CEW.

[b] The constants were determined with CT.

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References

Abrahamson, M., 1994. Cystatins. Methods Enzymol. 244, 685–700.

Auerswald, E.A., Genenger, G., Assfalg-Machleidt, I., Machleidt, W., Engh, R.A., Fritz, H., 1992. Recombinant chicken egg white variants of the QLSVG region. Eur. J. Biochem. 209, 837–845.

Barka, T., Asbell, P.A., van der Noen, H., Prasad, A., 1991. Cystatins in human tear fluid. Curr. Eye Res. 10 (1), 25–34.

Barrett, A.J., 1987. The cystatins: a new class of peptidase inhibitors. TIBS 12, 193–196.

Barrett, A.J., Rawlings, N.D., Davies, M.E., Machleidt, W., Salvesen, G., Turk, V., 1986. Cysteine proteinase inhibitors of the cystatin superfamily. In: Barrett, A.J., Salvesen, G. (Eds.), Proteinase Inhibitors. Elseveir, Amsterdam, pp. 515–569.

Björk, I., Alriksson, E., Ylinenjarvi, K., 1989. Kinetics of binding of chicken cystatin to papain. Biochemistry 28, 1568–1573.

Björk, I., Ylinenjarvi, K., Lindahl, P., 1990. Equilibrium and kinetic studies of the interaction of chicken cystatin with four cysteine proteinases. Biol. Chem. Hoppe-Seyler 371, 119–124.

Bobek, L.A., Wang, X., Levine, M.J., 1993. Efficient production of biologically active human salivary cystatins in *Escherichia coli*. Gene 123, 203–210.

Bode, W., Huber, R., 1992. Natural protein proteinase-inhibitors and their interaction with proteinases. Eur. J. Biochem. 204, 433–451.

Bode, W., Huber, R., 1994. Proteinase–protein inhibitor interactions. Fibrinolysis 8, 161–171.

Bode, W., Engh, E., Musil, D., Thiele, U., Huber, R., Karshikov, A., Brzin, J., Kos, J., Turk, V., 1988. The 2.0 Å X-ray crystal structure of chicken egg white cystatin and its possible mode of interaction with cysteine proteinases. EMBO J. 7 (8), 2593–2599.

Bode, W., Engh, E., Musil, D., Laber, B., Stubbs, M., Huber, R., Turk, V., 1990. Mechanism of interaction of cysteine proteinases and their protein inhibitors as compared to the serine proteinase-inhibitor interaction. Biol. Chem. Hoppe-Seyler 371 (Suppl.), 111–118.

Brzin, J., Popovic, T., Turk, V., Borchart, U., Machleidt, W., 1984. Human cystatin, a new protein inhibitor of cysteine proteinases. Biochem. Biophys. Res. Commun. 118 (1), 103–109.

Cornish, J.A., Murray, H., Kemp, G.D., Gani, D., 1995. Specific inhibitors of the adenovirus type 2 proteinase based on substrate-like tetrapeptide nitrites. Bioorg. Med. Chem. Lett. 5, 25–30.

De Clercq, E., 1993. Antiviral agents: characteristic activity spectrum depending on the molecular target with which they interact. In: Advances in Virus Research, vol. 42. Academic Press, New York.

Ding, J., McGrath, W.J., Sweet, R.M., Mangel, W.F., 1996. Crystal structure of the human adenovirus proteinase with its 11 amino acid cofactor. EMBO J. 15, 1778–1783.

Diouri, M., Geoghegan, K.F., Weber, J.M., 1995. Functional characterization of the adenovirus proteinase using fluorogenic substrates. Protein Pept. Lett. 6, 363–370.

Doronin, K., Kuppuswamy, M., Toth, K., Tollefson, A.E., Krajcsi, P., Krougliak, V., Wold, W.S.M., 2001. Tissue-specific, tumor-selective, replication-competent adenovirus vector for cancer gene therapy. J. Virol. 75 (7), 3314–3324.

Fairlie, D.P., Tyndall, J.D.A., Wong, A.K., Abbenante, G., Scanlon, M.J., March, D.R., Bergman, D.A., Chai, C.L.L., Burkett, B.A., 2000. Conformational selection of inhibitors and substrates by proteolytic enzymes: implications for drug design and polypeptide processing. J. Med. Chem. 43, 1271–1281.

Horwitz, M., 1996. Adenoviruses. In: Fields, B.N., et al. (Eds.), Virology, vol. 2. Lippincott-Raven, Philadelphia.

Isemura, S., Saitoh, E., 1994. Inhibitory activities of partially degraded salivary cystatins. Int. J. Biochem. 26 (6), 825–831.

Isemura, S., Saitoh, E., Ito, S., Isemura, M., Sanada, K., 1984. Cystatin S: a cysteine proteinase inhibitor of human saliva. J. Biochem. 96 (4), 1311–1314.

Isemura, S., Saitoh, E., Sanada, K., Minakata, K., 1991. Identification of full-sized forms of salivary (S-type) cystatins (cystatin SN, cystatin SA, cystatin S and two phosphorylated forms of cystatin S) in human whole saliva and determination of phosphorylation sites of cystatin S. J. Biochem. 110, 648–654.

Järvinen, M., Rinne, A., 1982. Human spleen cysteine proteinase inhibitor. Purification, fractionation into isoelectric variants and some properties of the variants. Biochim. Biophys. Acta 708 (2), 210–217.

Katunuma, N., Kominami, E., 1995. Structure, mechanisms and assays of cysteine proteinase inhibitors: cystatins and E-64 derivatives. Methods Enzymol. 251, 382–397.

Keyvani-Amineh, H., Labrecque, P., Cai, F., Carstens, E.B., Weber, J.M., 1995. Adenovirus protease expressed in insect cells cleaves adenovirus proteins, ovalbumin and baculovirus protease in the absence of activating peptide. Virus Res. 37, 87–97.

Kitaoka, M., Nakazawa, M., Hayasaka, S., 1985. Lysosomal enzymes in human tear fluid collected by filter paper strips. Exp. Eye Res. 41, 259–265.

Lah, T.T., Kos, J., 1998. Cysteine proteinases in cancer progression and their clinical relevance for prognosis. Biol. Chem. 379, 125–130.

Li, S., Hochstrasser, M., 1999. A new protease required for cell-cycle progression in yeast. Nature 398, 246–251.

Lindahl, P., Alriksson, E., Jörnvall, H., Björk, I., 1988. Interaction of the cysteine proteinase inhibitor chicken cystatin with papain. Biochemistry 27, 5074–5082.

Lopez-Otin, C., Simon-Mateo, C., Martinez, L., Vinuela, E., 1989. Gly-Gly-X, a novel consensus sequence for the proteolytic processing of viral and cellular proteins. J. Biol. Chem. 264, 9107–9110.

Machleidt, W., Thiele, U., Laber, B., Assfalg-Machleidt, I., Etserl, A., Wiegand, G., Kos, J., Turk, V., Bode, W., 1989. Mechanism of inhibition of papain by chicken egg white cystatin. FEBS Lett. 243 (2), 234–238.

Mentel, R., Kinder, M., Wegner, U., von Janta-Lipinski, M., Matthes, E., 1997. Inhibitory activity of 3′-fluoro-2′ deoxythymidine and related nucleoside analogues against adenoviruses in vitro. Antiviral Res. 34, 113–119.

Olsson, S.L., Ek, B., Björk, I., 1999. The affinity and kinetics of inhibition of cysteine proteinases by intact recombinant bovine cystatin C. Biochim. Biophys. Acta 1432, 73–81.

Qualikene, W., Lamoureux, L., Weber, J.M., Massie, B., 2000. Protease-deleted adenovirus vectors and complementing cell lines: potential applications of single-round replication mutants for vaccination and gene therapy. Hum. Gene Ther. 11, 1341–1353.

Ramasubbu, N., Weaver, T., Tseng, C.C., Bobek, L.A., Levine, M.J., 1996. Preliminary X-ray crystallographic analysis of human salivary cystatins. Acta Crystallogr. D52, 869–870.

Rawlings, N.D., Barrett, A.J., 1994. Families of cysteine peptidases. Methods Enzymol. 244, 461–486.

Ritonja, A., Machleidt, W., Barrett, A.J., 1985. Amino acid sequence of the intracellular cysteine proteinase inhibitor cystatin B from human liver. Biochem. Biophys. Res. Commun. 131 (3), 1187–1192.

Ruzindana-Umunyana, A., Sircar, S., Schick, C., Silverman, G.A., Weber, J.M., 2000. Adenovirus endopeptidase hydrolyses human squamous cell carcinoma antigens in vitro but not ex vivo. Virology 268, 141–146.

Serveau, C., Juliano, L., Bernard, P., Moreau, T., Mayer, R., Gauthier, F., 1994. New substrates of papain, based on the conserved sequence of natural inhibitors of the cystatin family. Biochimie 76, 153–158.

Sircar, S., Keyvani-Amineh, H., Weber, J.M., 1996. Inhibition of adenovirus infection with protease inhibitors. Antiviral Res. 30, 147–153.

Sircar, S., Ruzindana-Umunyana, A., Neugebauer, W., Weber, J.M., 1998. Adenovirus endopeptidase and papain are inhibited by same agents. Antiviral Res. 40, 45–51.

Sueyoshi, T., Enjyoji, K., Shimada, T., Kato, H., Iwanaga, S., Bando, Y., Kominami, E., Katunuma, N., 1985. A new function of kininogens as thiol-proteinase inhibitors: inhibition of papain and cathepsins B, H and L by bovine, rat and human plasma kininogens. FEBS 182 (1), 193–195.

Turk, D., Guncar, G., Podobnik, M., Turk, B., 1998. Revised definition of substrate binding sites of papain-like cysteine proteases. Biol. Chem. 379, 137–147.

Tyndall, J.D.A., Fairlie, D.P., 1999. Conformational homogeneity in molecular recognition by proteolytic enzymes. J. Mol. Recognit. 12, 363–370.

Weber, J.M., 1999. Role of endopeptidase in adenovirus infection. In: Seth, P. (Ed.), Adenoviruses: From Basic Research to Gene Therapy Application. R.G. Landes Company.

Wickham, T.J., 2000. Targeting adenovirus. Gene Ther. 7, 110–114.