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# Kinetics of inhibition of bovine cathepsin S by bovine stefin B

Boris Turk[a,*], Adrijana Čolić[a,b], Veronika Stoka[a], Vito Turk[a]

[a]*Department of Biochemistry and Molecular Biology, J. Stefan Institute, Jamova 39, 61111 Ljubljana, Slovenia*
[b]*Center for Genetic Engineering and Biotechnology, Filipa Kljajića 12, Sarajevo, Bosnia and Herzegovina*

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**Abstract**

The kinetics of the complex formation between bovine cathepsin S and bovine stefin B was studied by conventional and stopped-flow techniques. The inhibition at low inhibitor concentrations was tight and reversible ($k_{ass} = 5.8 \times 10^7$ $M^{-1} \cdot s^{-1}$, $k_{diss} = 4.9 \times 10^{-4}$ $s^{-1}$ at pH 6.0 and 25°C), whereas at higher inhibitor concentrations it was pseudo-irreversible ($k_{ass} = 6.14 \times 10^7$ $M^{-1} \cdot s^{-1}$). The complex was formed directly lacking the fast pre-equilibrium step with the dissociation equilibrium constant of ~ 8 pM. The competitive nature of inhibition was confirmed. The $k_{ass}$ was found to be pH-independent between pH 6.0 and 7.5 and decreased at lower or higher pH values in a way that strongly suggests involvement of two ionizable groups in the interaction ($pK_1 = 5.2$, $pK_2 = 8.3$). The enzyme–substrate interaction seems to be influenced by different ionizable groups ($pK_1 = 4.4$, $pK_2 = 7.8$).

*Key words:* Stefin B; Cathepsin S; Cystatin; Cysteine proteinase; Stopped-flow; Kinetics

## 1. Introduction

Stefin B is a protein inhibitor of cysteine proteinases with $M_r$ of about 11,000 [1,2]. It was first isolated from rat liver [3] and later also from human [4–6] and bovine origin [7]. The latter was found to be the first mammalian member of the stefin family, where highly conserved QVVAG region was replaced with the QLVAG sequence [8] without any effect on its inhibitory activity [7,9]. The inhibitor forms a tight, equimolar complex with cysteine proteinases [10] like other cystatins [11]. Among them cathepsin B is inhibited with the lowest affinity [5,9]. The crystal structure of recombinant human stefin B in the complex with carboxymethylated papain [10] confirmed the proposed mechanism of interaction of cysteine proteinases and their inhibitors from the cystatin superfamily [12], which differs from the classical one. Recently, stefin B was found to be able to inhibit cathepsin L at neutral pH, demonstrating its potential physiological role also at neutral conditions [13].

Cathepsin S is a lysosomal cysteine proteinase from papain family [14]. Like other lysosomal cathepsins, this enzyme is believed to be involved in intracellular protein turnover [15]. It has been isolated as a single chain protein with $M_r$ around 24,000 from various mammalian tissues [16–19]. For a long time it was confused with cathepsin L, although both enzymes significantly differ in their pH stability [13,20]. Finally, the amino acid sequence of both enzymes from bovine origin clearly showed that these are two different enzymes [21,22]. The activity of papain-like cysteine proteinases is generally believed to be controlled by their protein inhibitors from the cystatin superfamily [1,2]. Some inhibitory kinetics of cathepsin S by stefin B has already been reported [23].

Although interactions between plant cysteine proteinases and cystatins have been extensively studied [24,25], no such data are available for mammalian papain-like cysteine proteinases. In this study we have investigated in more details the interaction between two mammalian proteins, bovine cathepsin S and bovine stefin B, with the aim to characterize the mechanism of their interaction, and to evaluate the potential physiological role of stefin B as an inhibitor of cathepsin S.

## 2. Materials and methods

### 2.1. Materials

Z-Phe-Arg-AMC and dithiothreitol were purchased from Serva (Heidelberg, Germany), dimethylsulfoxide was from Merck (Darmstadt, Germany). Stock solution of the substrate was made in dimethylsulfoxide. Ep-475 was from Peptide Research Institute (Osaka, Japan); EDTA and papain (EC 3.4.22.2; 2× crystallized) were from Sigma (St. Louis, USA). Bovine cathepsin S [19] and bovine stefin B [7] were purified using published procedures. Cathepsin S (EC 3.4.22.27) and additionally purified papain [26] were active-site titrated with Ep-475 as described previously [9], whereas stefin B was titrated with active-site titrated papain. Protein concentrations were determined using the method of Lowry et al. [27]. The papain concentration was determined

* Corresponding author. Fax: (386) (61) 27-3594.

*Abbreviations:* CPI, cysteine proteinase inhibitor; Ep-475, L-3-carboxy-*trans*-2,3-epoxypropyl-leucylamido-(3-guanidino)butane; -AMC-4-methyl-7-coumarylamide; Z-, benzyloxycarbonyl-.


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using an absorption coefficient of 2.39 $l \cdot g^{-1} \cdot cm^{-1}$ [28] and $M_r$ 23,400 [29]. All other chemicals were of analytical grade. Unless otherwise stated, the kinetic experiments were done in phosphate buffer (100 mM $Na_2HPO_4/KH_2PO_4$, 50 mM NaCl, 1 mM EDTA) pH 6.0 and at 25°C. The following buffers were also used, when the pH was varied: 100 mM citrate buffer at pH 3.5 and 4.0, 100 mM acetate buffer at pH 4.5–5.5, 100 mM phosphate buffer at pH 6.0–7.5, 100 mM Hepes at pH 8.0 and 100 mM Tris at pH 8.5. All the buffers contained also 50 mM NaCl and 1 mM EDTA. The activating buffer for all the experiments was 3 mM EDTA, containing 8 mM dithiothreitol, pH 6.0. Dimethylsulfoxide concentration was less than 3% throughout.

*2.2. Kinetics of the inhibition of cathepsin S by stefin B*

The inhibition of cathepsin S by stefin B in the presence of Z-Phe-Arg-AMC was followed at excitation and emission wavelengths 370 and 460 nm, respectively, using a Perkin Elmer LS3 spectrofluorimeter (USA), as described previously [9]. Fluorimeter was on-line connected with an IBM XT computer, and Flusys program [30] was used for fluorimeter control.

Faster reactions were monitored with a DX 17MV stopped-flow apparatus (Applied Photophysics, UK). The emission of released product was observed at an excitation wavelength of 360 nm through the cut-off filter with ~ 50% transmission at 400 nm. One syringe was filled with buffer and stefin B + substrate, whereas the second was filled with preactivated cathepsin S (5 min in the activation buffer). A 10:1 volume ratio of mixing was used in all the experiments. Stopped-flow traces were digitized and stored in an Archimedes computer. The stored progress curves were composed of 400 data pairs (fluorescence, time). All kinetic constants obtained are averages of 6–10 individual runs.

All the experiments were done under pseudo-first-order conditions with a 10-fold molar excess of stefin B over cathepsin S, and with less than 3% of substrate hydrolysis.

*2.3. Determination of* $k_{cat}$ *and* $K_m$

The pH dependence of kinetic parameters for cathepsin S-catalysed Z-Phe-Arg-AMC hydrolysis was studied in the pH range 3.5–8.0. Substrate (various concentration) was dissolved in 1.95 ml of appropriate buffer. The reaction was started by the addition of 50 ml of preactivated cathepsin S (0.05–0.5 nM final concentration, depending on pH), which was found to be stable during the experiment. Initial velocities of the cathepsin S-catalysed substrate hydrolysis were then measured in a Perkin Elmer LS3 spectrofluorimeter (USA) at excitation and emission wavelengths 370 and 460 nm, respectively. Seven to ten different substrate concentrations were used at each pH value. Double reciprocal plots showed that the kinetics adhered to the Michaelis-Menten model and also provided initial estimates of $k_{cat}$ and $K_m$. The refined kinetic parameters $k_{cat}$ and $K_m$ were then determined by nonlinear regression analysis.

## 3. Results

### *3.1. Inhibition kinetics*

The tight-binding inhibition of cathepsin S by stefin B was studied in the presence of substrate. For their interaction we assumed a simple, competitive mechanism of inhibition, which is shown in Scheme I:

$$\begin{array}{l} E + S \rightleftharpoons ES \longrightarrow E + P \\ + \\ I \\ k_{ass} \Big\downarrow\Big\uparrow k_{diss} \\ EI \end{array}$$

where $k_{ass}$ is the second-order rate constant of complex formation and $k_{diss}$ is the rate constant of its decomposition. The release of product was recorded after mixing of enzyme with inhibitor + substrate in order to determine the kinetic constants. Progress curves obtained at low inhibitor concentrations (0.1–1.5 nM) were biphasic and could be best fitted to the following integrated equation [31]:

$$[P] = v_s t + (v_z - v_s)(1 - e^{-kt})/k \quad (1)$$

where [P] presents the product concentration, and $v_z$ and $v_s$ are the initial and steady-state velocities, respectively. In this equation $k$ is the apparent pseudo-first-order rate constant for the approach to steady state and is given by the following relationships [31]:

$$k = k_{ass}[I_o]/(1 + [S_o]/K_m) + k_{diss} \quad (2)$$

The pseudo-first-order rate constant $k$ was found to increase linearly with increasing concentration of stefin B and a $k_{ass}$ value of $(5.8 \pm 1.0) \times 10^7\ M^{-1} \cdot s^{-1}$ was calculated from the slope of the plot (data not shown). The dissociation rate constant $k_{diss}$ was calculated using the following equation [31]:

$$k_{diss} = k \times v_s/v_z \quad (3)$$

All $k_{diss}$ values obtained in this way from individual measurements were very close, giving an average value of $(4.9 \pm 0.5) \times 10^{-4}\ s^{-1}$.

It is possible that the stable EI complex is not formed directly (Scheme I) but through the fast pre-equilibrium as it is illustrated on Scheme II:

$$\begin{array}{l} E + S \rightleftharpoons ES \longrightarrow E + P \\ + \\ I \\ k_{ass} \Big\downarrow\Big\uparrow k_{diss} \\ \\ \quad\quad k_{+1} \\ EI \rightleftharpoons EI^* \\ \quad\quad k_{-1} \end{array}$$

The apparent first-order rate constant $k$ of Eqn. 1 is here given by the following equation [31]:

$$k = k_{+1}[I_o]/([I_o] + K_i(1 + [S_o]/K_m)) + k_{-1} \quad (4)$$

In an attempt to detect the intermediate (EI) the inhibition of cathepsin S by stefin B was also studied at high inhibitor concentrations ($[I_o] = 0.2–8.0\ \mu M$) at 20 $\mu$M Z-Phe-Arg-AMC with a stopped-flow apparatus. Progress curves obtained were analysed by nonlinear regression analysis and could be best fitted to single exponentials at all concentrations of stefin B investigated (Fig. 1), indicating that the interaction is pseudo-irreversible ($k_{ass}[I_o] >> k_{diss}$). The fluorescence amplitude corresponding to the amount of product formed was constant at all inhibitor concentrations, indicating that the equilibrium between E and ES is established rapidly in comparison to the equilibrium between E and EI. Similarly like at low inhibitor concentrations, pseudo-first-order

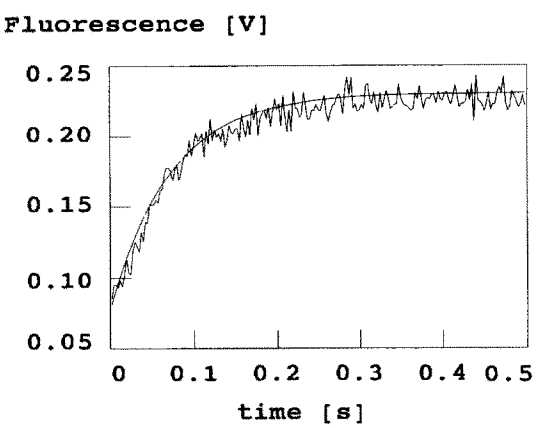


Fig. 1. Progress curve for the inhibition of cathepsin S by stefin B at 25°C and pH 6.0. The reaction medium contained 500 nM stefin B, 50 nM cathepsin S and 20 $\mu$M Z-Phe-Arg-AMC. The solid line crossing the stopped-flow trace is the theoretical curve generated using the best estimates of $k$ and the intercept.

rate constant $k$, increased linearly with increasing concentration of stefin B up to the highest concentration investigated (Fig. 2), thus indicating that the proposed mechanism (Scheme I) is obeyed. Scheme II predicts a hyperbolic dependence of $k$ upon $[I_o]$. The slope of the regression line gave an association rate constant of $(6.14 \pm 0.11) \times 10^7$ $M^{-1} \cdot s^{-1}$, while the intercept of the line on the ordinate was indistinguishable from zero. This value is in good agreement with that determined at low reactant concentration, namely $5.8 \times 10^7$ $M^{-1} \cdot s^{-1}$. From both association and dissociation rate constants the equilibrium constant $K_i$ ($= k_{diss}/k_{ass}$) of $(8.0 \pm 0.9)$ pM was calculated for the interaction at pH 6.0 and 25°C. The interaction was also investigated at different substrate concentration with $[I_o] = 500$ nM and $[E_o] = 50$ nM, and $k$ was plotted as a function of $1/(1 + [S_o]/K_m)$ as can be seen in inset of Fig. 2. The linearity of the plot is in agreement with other experiments thus indicating lack of the fast pre-equilibrium (Scheme I). Similarly as for the dependence on inhibitor concentration Scheme II also predicts a hyperbolic dependence of $k$ upon $(1 + [S_o]/K_m)$ (see Eqn. 4). The decrease of $k$ with $[S_o]$ also provides evidence for the assumed competitive nature of the inhibition (Scheme I). The $k_{ass}$ value calculated from the slope of this line ($6.6 \times 10^7$ $M^{-1} \cdot s^{-1}$) was also in good agreement with those obtained at low and high inhibitor concentrations, whereas the intercept on the ordinate was indistinguishable from zero, thus precluding the determination of $k_{diss}$ (i.e. $k_{ass}[I_o] >> k_{diss}$).

### 3.2. pH-dependence of inhibition

The pH-dependence of the association rate of stefin B with cathepsin S was studied with the stopped-flow apparatus at 25°C under pseudo-first-order-conditions (Fig. 3A). All the curves except at pH 3.5 reached a plateau, indicating that the effect of the complex dissociation under the conditions used in the experiments is negligible, whereas at pH 3.5 practically no inhibition was observed. The association rate constant increased steeply from pH 4.0–6.0 and remained practically constant up to pH 7.5. Above this value a steep decrease was observed. The profile of the pH-dependence of $k_{ass}$ suggests, that at least two ionizable groups are involved in the interaction. The data were fitted to the following equation:

$$k_{ass} = k_{ass(lim)} / (1 + [H^+]/K_1 + K_2/[H^+]) \quad (5)$$

where $k_{ass(lim)}$ presents the limiting value of $k_{ass}$. The two groups were compatible with involvement of an unprotonated acid group with $pK_a$ $5.2 \pm 0.1$ and of a protonated basic group with $pK_a$ $8.3 \pm 0.1$. Also the pH-dependence of $k_{cat}/K_m$, the second-order rate constant for the reaction between the enzyme and the substrate was bell-shaped, indicating the influence of two ionizable groups on the substrate hydrolysis (Fig. 3*b*). When the data were fitted to the following equation:

$$k_{cat}/K_m = (k_{cat}/k_m)_{(lim)}/(1 + [H^+]K_1 + K_2/[H^+]) \quad (6)$$

with $(k_{cat}/K_m)_{(lim)}$ presenting the theoretical limiting value of $k_{cat}/K_m$, the best fit corresponded to the groups with $pK_a$ values of $(4.4 \pm 0.1)$ and $(7.8 \pm 0.1)$. These are in excellent agreement with those obtained with recombinant human cathepsin S [32].

## 4. Discussion

From the data obtained, it is evident that stefin B is a reversible, tight-binding inhibitor of cathepsin S ($K_i = 8.0$ pM). In addition, stefin B is also a very fast acting inhibitor of cathepsin S. The association rate con-

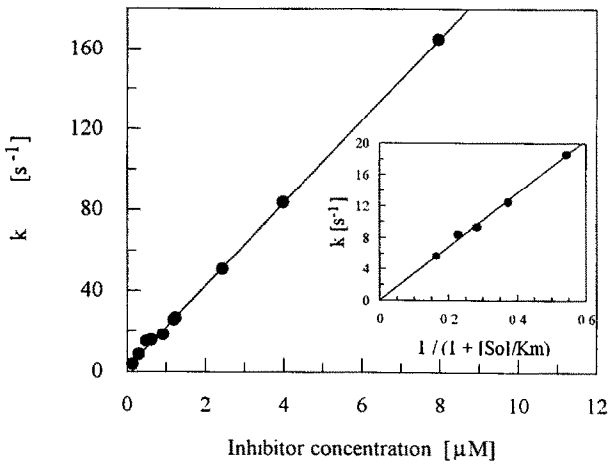


Fig. 2. Dependence of the pseudo-first-order rate constant $k$ from stefin B concentration for cathepsin S–stefin B interaction at 25°C and pH 6.0, and at 20 $\mu$M Z-Phe-Arg-AMC. The solid line was calculated using linear regression analysis. Inset: substrate dependency of pseudo-first-order rate constant. The stefin B and cathepsin S concentrations were 500 nM and 50 nM, respectively.

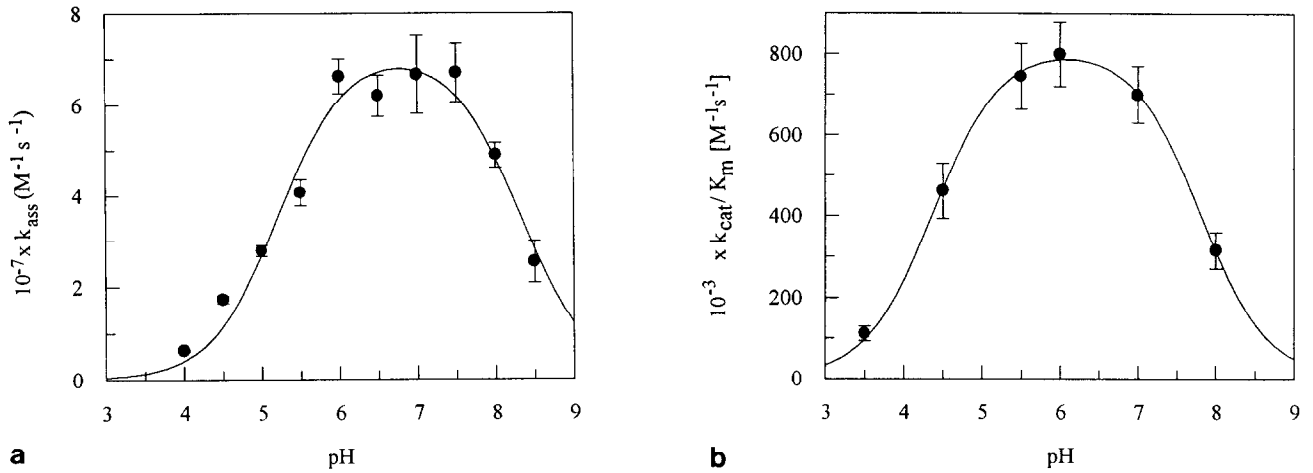


Fig. 3. pH-dependence of the second-order rate constant, $k_{ass}$, for the interaction between cathepsin S and stefin B (A) and pH activity profile for cathepsin S (B). All the measurements were performed at 25°C as described in section 2. The data were fitted to Eqn. 4 (A) or 5 (B). The solid lines are the theoretical curves corresponding to best fits.

stant for their interaction is extremely high ($6.1 \times 10^7$ $M^{-1} \cdot s^{-1}$), but still comparable to the values obtained for some other CPI-cysteine proteinase interactions [9,24, 25,33,34]. Brömme et al. [23] suggested the occurrence of a fast pre-equilibrium, which we could not confirm experimentally. The linearity of increase of the pseudo-first-order rate constant for complex formation with increasing inhibitor concentration indicated lack of the fast pre-equilibrium step, which is consistent with the present knowledge about the cysteine proteinases and their interactions with the cystatin-like inhibitors [9,24,25]. It is also in agreement with the crystal structure of stefin B–papain complex [10] and the docking experiments for the interaction based on the crystal structure of chicken cystatin [12], where it has been shown that only negligible conformational changes are accompanying the enzyme–inhibitor interaction. In addition, the competitive nature of inhibition was confirmed consistently with previous experiments on some other cystatins [9,11].

The effect of pH on the association rate was investigated between pH 3.5 and 8.5. The lack of inhibition at pH 3.5 was caused by the instability of stefin B under the experimental conditions (Žerovnik, E., unpublished results). At higher pH values a bell-shaped pH-dependence of stefin B–cathepsin S interaction was observed with at least two ionizable groups involved in the interaction, consistently with the interaction between papain and chicken cystatin [24]. Similarly a bell-shaped curve was observed also for substrate hydrolysis, but with different ionizable groups involved. This results indicate, that the inhibitor binding is controlled by different ionizable groups of the enzyme than is the substrate hydrolysis, although we can not completely exclude the involvement of ionizable groups that can affect the conformation of stefin B.

Under normal conditions lysosomal cysteine proteinases are known to act mainly intracellularly [35]. However, in several disease states they have also been detected extracellularly in significant amounts [36]. A strict control of their action is therefore needed also to prevent this harmful extracellular proteolysis. This may be particularly important in the case of cathepsin S, which differs from other lysosomal papain-like enzymes by its remarkably enhanced pH-stability under neutral conditions [20]. On the other hand, the binding of stefin B to cathepsin S was found to be unaffected around neutral pH, which is of great physiological importance. In addition, stefin B is known to be generally distributed amongst different cell types [2] and it has been detected also in different fluids in concentrations which are not insignificant [34]. According to Bieth [37] and our kinetic data we might assume, that stefin B-induced inhibition of cathepsin S in vivo is pseudo-irreversible ($k_{ass}[I_o] >> k_{diss}$). This is even more true for the total CPIs-induced inhibition where we should take in account besides stefin B also the large inhibitory potential of cystatin C and kininogens [33,34]. Therefore we might conclude that the inhibitory pathway is much more important in the regulation of cathepsin S activity than its spontaneous pH-induced inactivation, and that also stefin B is involved in this process.

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