Salivary histatin 5 is a potent competitive inhibitor of the cysteine proteinase clostripain

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Abstract Histatin 5 is a low molecular weight salivary protein which is known to exhibit inhibitory activity against several proteinases, including the cysteine proteinases gingipains. The purpose of this study was to characterize the effect of salivary histatin on the proteolytic activity of the cysteine proteinase clostripain derived from the pathogen *Clostridium histolyticum*. Using a synthetic nitroanilide substrate, we studied in detail the inhibition of clostripain by histatin 5 and compared the effect of this peptide to that of leupeptin, a known competitive inhibitor of clostripain. It was found that the concentration of histatin 5 required to inhibit 50% of clostripain activity was 23.6 ± 1.6 nM. Kinetic analysis revealed that histatin 5 is a competitive inhibitor of clostripain with an inhibition constant (K_i) of 10 nM. The K_i for the inhibition of clostripain activity against nitroanilide substrate by leupeptin was found to be 60 nM, significantly higher than that of histatin 5. Thus, histatin 5 inhibits clostripain more effectively than leupeptin and other cysteine protease inhibitors studied here. No significant proteolysis of histatin 5 was observed when histatin 5 was incubated at physiologic concentrations with clostripain. The potent inhibition of clostripain by histatin 5 points towards the possibility that this protein may prevent establishment of clostridial infections and therefore may have significant potential for the treatment of diseases associated with this enzyme. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Histatin 5 is a member of a family of low molecular weight histidine-rich salivary proteins secreted by human parotid and submandibular/sublingual glands. The major family members are histatin 1, 3 and 5 which contain 38, 32 and 24 amino acid residues, respectively, and their primary structures have been determined [1]. Histatins are known to exhibit bactericidal and fungicidal activities against several oral pathogens, although this property of histatins may reflect only one of a number of functional roles that these proteins have in vivo [1–5]. Other antibacterial functions have been attributed to his-

tatins based on its inhibitory effect on bacterial proteinases [6,7]. Histatin 5, the smallest protein among the protein family inhibits trypsin-like and collagenase activities of *Porphyromonas gingivalis* [6,7] as well as a cysteine proteinase from *Clostridium histolyticum* [6].

The anaerobic bacterium *C. histolyticum* produces toxins and proteolytic enzymes which are important virulence factors implicated in gas gangrene syndrome, a severe pathologic condition [8]. In this condition, collagenases and cysteine proteinases produced in large quantities are associated with lethal, necrotizing and muscle digesting activities leading to massive tissue destruction [9]. The cysteine proteinase clostripain has been isolated and characterized as an important proteolytic enzyme from *C. histolyticum*. Structural studies have shown that clostripain is composed of two polypeptide chains which are non-covalently associated [10–12]. Like many other cysteine proteinases, clostripain requires calcium ions for stability and activity [13]. The substrate specificity has been demonstrated to be similar to that of trypsin, in which hydrolysis is restricted to substrates containing arginine or lysine. However, proteolysis by clostripain occurs in preference at the carboxyl terminal side of arginine residues [14].

Although preliminary studies have shown that histatin 5 inhibits clostripain, very little is known about the kinetic mechanism of this inhibition [6]. Therefore, the aim of the present study was to further characterize the inhibition of clostripain by salivary histatin 5 by determining the effect of this protein on the kinetic parameters K_M and k_{cat}, and to compare its activity with leupeptin. We found that histatin 5 was more effective than leupeptin, the strongest inhibitor of clostripain previously reported.

2. Materials and methods

The materials used in this study were purchased from commercial sources as follows: synthetic histatin 5 (DSHAKRHHGYKRKF-HEKHHSHRGY, MW 3037) was purchased from American Peptide Company (Sunnyvale, CA, USA). The preparation was subjected to reverse phase high performance liquid chromatography (HPLC) analysis using an acetonitrile gradient to verify purity [1]. Clostripain, benzamidine, iodoacetic acid, phenylmethylsulfonyl fluoride (PMSF), *N*-tosyl-Lys-chloromethylketone (TLCK), E-64 (*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane)), leupeptin, *N*-benzoyl-DL-arginine *p*-nitroanilide (BAPNA), 3-(*N*-morpholino)propane-sulfonic acid (MOPS), *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) and *N,N*-dimethylformamide were obtained from Sigma (St. Louis, MO, USA). $CaCl_2 \cdot 2H_2O$ and dithiothreitol (DTT) were purchased from Fisher (Pittsburgh, PA, USA).

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Clostripain activity was assayed spectrophotometrically. Clostripain was activated in 10 mM MOPS, pH 7.4, containing 1 mM $CaCl_2$ and 2.5 mM DTT for 3 h at 25°C. After activation, clostripain (46 nM) was added to 600 µl of enzyme buffer (50 mM TES pH 7.4) containing the substrate BAPNA (80 µM) in the presence and absence of histatin 5 (0.012–0.35 µM). The reaction was carried out in cuvettes of 1 cm light-path at 25°C and the formation of product (p-nitroaniline) was monitored by the increase in absorbance at 410 nm using a Milton Roy Spectronic 1201 Spectrometer (Londonderry, NH, USA). The velocity of the reaction was obtained from the initial slope of plots of absorbance versus time. All data were corrected for the absorbance of the enzyme–substrate mixture recorded immediately upon mixing at time zero.

The K_M and k_{cat} were determined at 25°C using BAPNA at concentrations ranging from 40 to 160 µM with final concentrations of 46 nM clostripain either in the absence or in the presence of two different concentrations of histatin 5. Initial rates of hydrolysis were calculated at five different concentrations of substrate and the type of inhibition and Michaelis–Menten parameters were determined from Lineweaver–Burk plots [15] by using the equations derived from the linear regression analysis of each curve. The k_{cat} value was calculated applying the following equation: $k_{cat} = V_{max}/[E]$ where [E] is the enzyme concentration in the assay [16].

The inhibition constant (K_i) was estimated either by using the equation applicable to reversible, competitive type inhibitors, $K_i = IC_{50}/(1+S/K_M)$ [17,18] or extrapolated from a Dixon plot [15]. Three different concentrations of BAPNA (60, 80 and 106 µM) were used in the absence or in the presence of increasing concentrations of histatin 5 to determine the K_i from a Dixon plot.

To analyze whether histatin 5 is susceptible to proteolytic degradation under the conditions used for the inhibition assays, activated clostripain was incubated at room temperature with histatin 5 in the absence of BAPNA for different periods of time. The reaction mixture consisted of 46 nM clostripain and 10 µM histatin 5 dissolved in TES buffer pH 7.4 at a final volume of 800 µl. Aliquots of 150 µl were collected at different time points and mixed with 40 µl of acetic acid to terminate the reactions. Subsequently, samples were filtered through 0.45 µm filters (Millipore, Bedford, MA, USA) and subjected to reverse phase HPLC. Sample application to a TSK-GEL 5 µm ODS-120T column (4.6×250 mm) equipped with an Ultrasphere 5 µm ODS guard column was carried out in 0.1% trifluoroacetic acid in water (eluent A) and separations were performed with a linear gradient of 0.09% trifluoroacetic acid to 30% acetonitrile/water (eluent B) over 40 min at a flow rate of 1 ml/min. The protein elution profile was monitored at 230 nm.

3. Results and discussion

Inhibition of clostripain by histatin 5 was investigated using the synthetic substrate BAPNA. A typical inhibition curve was obtained when the activity of clostripain was measured in the presence of histatin 5 (Fig. 1). The IC_{50} value for this inhibition was found to be 23.6 ± 1.6 nM ($n = 3$).

To compare the effect of histatin 5 with other known proteinase inhibitors, the activity of clostripain was measured in the presence of 35 µM concentrations of inhibitors of cysteine proteinases (iodoacetic acid, E-64 and leupeptin), of trypsin-like proteinases (benzamidine and TLCK) and of a serine proteinase (PMSF). Histatin 5, TLCK and leupeptin completely abolished clostripain activity while iodoacetic acid, a known inhibitor of other cysteine proteinases [19], had limited inhibitory activity against clostripain at the concentration used (Table 1). As expected, the well established serine proteinase inhibitor PMSF failed to inhibit clostripain. These results demonstrate that histatin 5 is a potent inhibitor of clostripain.

In order to study the kinetics of clostripain inhibition by histatin 5 and determine the properties of this inhibition, enzyme activity was measured with clostripain in the absence, and in the presence of 12 and 24 nM histatin 5. These con-
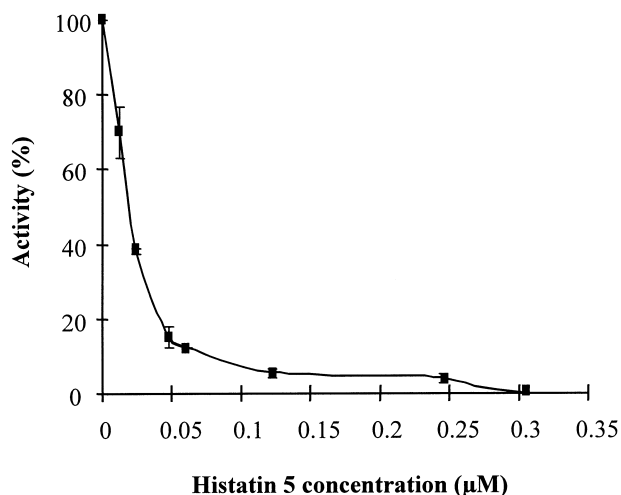

Fig. 1. Effect of histatin 5 on clostripain activity. Histatin 5 was added to the enzyme–substrate mixture and the velocity of cleavage of BAPNA was recorded spectrophotometrically as an increase in absorbance at 410 nm. Activity is expressed as a percent of control solutions containing enzyme without inhibitor. The error bars indicate the standard deviations for three experiments.

centrations of histatin 5 were chosen from the plot of activity versus inhibitor concentration (Fig. 1). The initial velocity (AU/min) of the reaction was obtained for each concentration of substrate used and the data were presented in a Lineweaver–Burk plot.

As shown in Fig. 2A the reciprocal plots of the control and in the presence of inhibitor intercept at a common point on the y-axis indicating that the V_{max} is unchanged in the presence of histatin 5. Furthermore, the K_M values determined from the intercepts at the x-axis demonstrate that the K_M values increase when the histatin 5 concentration increases (Fig. 2A). These kinetic features are characteristic of competitive enzyme inhibition. Means and standard deviations of the kinetic parameters from three separate experiments are given in Table 2. Analysis of V_{max} and k_{cat} values obtained for clostripain revealed that these values were not altered in the presence of histatin 5. Increases were observed in the K_M and decreases in the k_{cat}/K_M values, consistent with the competitive inhibition.

Kinetic experiments were also carried out with the positive control, leupeptin, which verified that this peptide is a competitive inhibitor of clostripain (Fig. 2B) [13].

Table 1
Effect of inhibitors on the activity of clostripain[a]

Inhibitor	Class of inhibitor			Clostripain inhibition (%)
	trypsin-like	cysteine	serine	
Histatin 5				100
Iodoacetic acid		X		13
E-64		X		68
Leupeptin		X		100
Benzamidine	X			28
TLCK	X			100
PMSF			X	0

[a]Inhibitors were added to the enzyme–substrate mixture at a final concentration of 35 µM and the activity was measured spectrophotometrically as an increase at 410 nm.
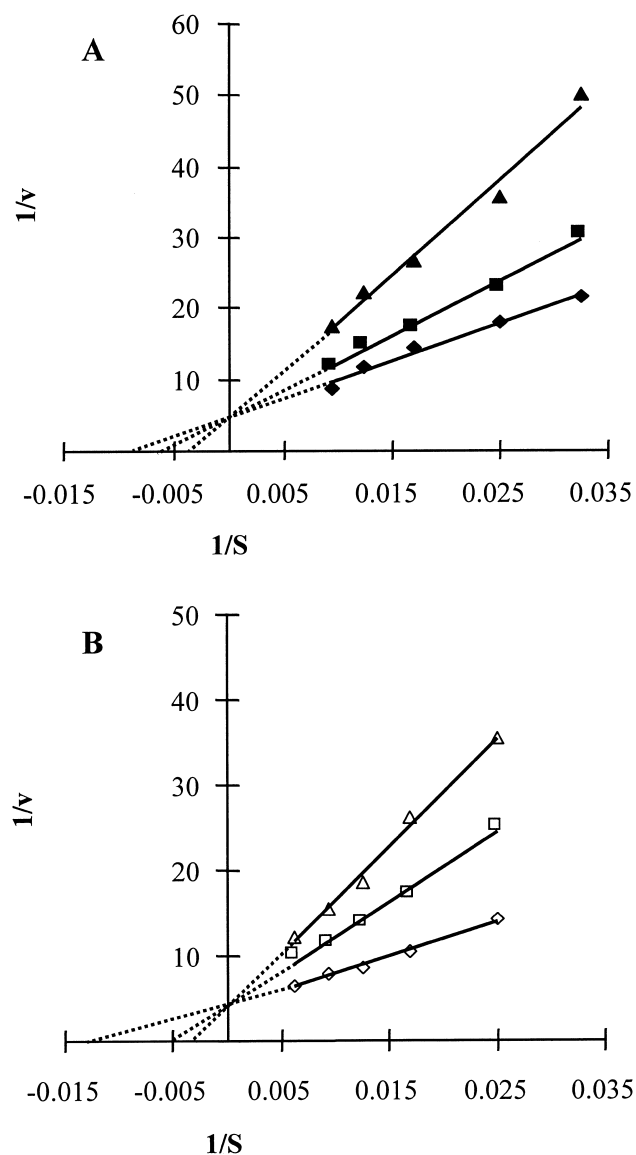
Fig. 2. Lineweaver–Burk plots of the inhibition of clostripain by histatin 5 (A) and by leupeptin (B). Enzyme assays were carried out with clostripain alone ($\blacklozenge$, $\lozenge$) and in the presence of either histatin 5 at 12 nM ($\blacksquare$) and 24 nM ($\blacktriangle$) or leupeptin at 100 nM ($\square$) and 200 nM ($\triangle$). Equations were obtained from linear regression analysis of each line and were used to calculate the kinetic parameters given in Table 2. Dashed lines are extensions of linear regression lines and indicate that the inhibition of clostripain by histatin 5 and leupeptin is competitive. Individual graphs represent the results of triplicate experiments.

The inhibition constant, K_i, for histatin 5 against clostripain was determined. For this purpose, enzyme assays were performed at three different concentrations of substrate (60, 80 and 106 μM) in the presence and absence of histatin 5. The K_i

was obtained graphically using a Dixon plot for competitive inhibition. In this plot, $1/v$ (velocity) is plotted against inhibitor concentration at varying concentrations of substrate and a series of straight lines was obtained converging to the point that represents $K_i = -[I]$. The K_i value for the inhibition of clostripain by histatin 5 was 10 nM. Furthermore, when the K_i was calculated using the equation ($K_i = IC_{50}/(1 + S/K_M)$) applicable for competitive inhibition, similar values were obtained. The K_i (60 nM) for the inhibition of clostripain by leupeptin was determined in a similar manner (data not shown).

Since the competitive nature of the inhibition of clostripain by histatin 5 indicates its interaction with the active site, it is important to assess whether this inhibitor might be a potential substrate for the enzyme, particularly in view of the fact that histatin 5 contains arginine and lysine residues which could be sites of cleavage by clostripain. When histatin 5 was incubated at a physiologic concentration (10 μM) with 46 nM clostripain, the same enzyme concentration used in the inhibition assays, no significant turnover could be observed, even after 30 min of incubation. The concentrations of clostripain and histatin 5 used in the initial rate assays were 46 and 24 nM, respectively. Under these standard assay conditions, peptides that might be formed due to possible histatin 5 proteolysis by clostripain were not detectable in HPLC analyses. Even at concentrations of clostripain and histatin 5 of 0.92 and 0.48 μM, respectively, the reaction mixture showed no evidence for histatin 5 degradation by clostripain. Thus, it appears that the inhibitory effect of histatin 5 on clostripain activity against BAPNA is not due to histatin 5 acting as a competing substrate under the described conditions.

In summary, this study provides new information about the kinetics of the inhibition of clostripain by histatin 5. Our results clearly show that histatin 5 has a high inhibitory activity toward clostripain as reflected in the K_i value found for this inhibition. To our knowledge, this is the most potent inhibitor of clostripain ever reported in the literature. Earlier studies have indicated leupeptin to be the most potent inhibitor of clostripain [13] but the present study found that histatin 5 has a six-fold lower K_i than that of leupeptin. Therefore, it is highly possible that under physiologic conditions histatin acts as a natural inhibitor of this enzyme. Taken together, these results point toward a novel property of histatin 5 besides its well-known anticandidal activity. Furthermore, since histatin 5 is a salivary peptide and therefore naturally exposed to epithelial surfaces it is proposed that this protein could be used for the prevention or treatment of topical infections such as those caused by *C. histolyticum*.

Table 2
Kinetic parameters of clostripain in the presence of histatin 5[a]

	V_{max} (AU/min)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} M^{-1} ×10^{-6})
Clostripain only	0.25 ± 0.02	133 ± 9.0	5.4 ± 0.4	0.04 ± 0.001
Histatin 5 (12 nM)	0.23 ± 0.005	184 ± 14.5	5.0 ± 0.2	0.027 ± 0.002
Histatin 5 (24 nM)	0.24 ± 0.02	314 ± 7.2	5.2 ± 0.4	0.017 ± 0.001

[a]Parameters were calculated using the equations obtained from the Lineweaver–Burk plots. Standard deviations given are for three separate experiments.

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