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# Pancreatic secretory trypsin inhibitor acts as an effective inhibitor of cysteine proteinases gingipains from *Porphyromonas gingivalis*

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*Background and Objective:* *Porphyromonas gingivalis* has been implicated as the major pathogen of periodontitis in adults. This organism produces an array of virulence factors, of which cysteine proteinases, referred to as gingipains K and R, are believed to play a crucial role in pathogenicity. The aim of this study was to investigate the susceptibility of gingipains K and R to inhibition by a pancreatic secretory trypsin inhibitor.

*Material and Methods:* Enzyme activities were measured spectrophotometrically using chromogenic turnover substrates. To estimate the value of the association constant ($K_a$), constant amounts of enzyme were reacted with increasing amounts of inhibitor to reach equilibrium. The $K_a$ was calculated by fitting the experimental data to the given equation.

*Results:* In this study it was shown that gingipains are susceptible to pancreatic Kazal-type trypsin inhibitors (pancreatic secretory trypsin inhibitors). Bovine pancreatic secretory trypsin inhibitor, having an Arg residue at the $P_1$ position of the reactive site, specifically inhibited the activity of the Arg-specific cysteine proteinase gingipain R, whereas porcine inhibitor, possessing a Lys residue at the $P_1$ position, exhibited activity only against the Lys-specific cysteine proteinase gingipain K. The $K_a$ values for the inhibitor–proteinase interaction were $1.6 \times 10^6$ M$^{-1}$ and $2.0 \times 10^4$ M$^{-1}$ for gingipain R and gingipain K, respectively.

*Conclusion:* This finding is the first demonstration of the inhibitory potency of the Kazal-type specific trypsin inhibitors against cysteine proteinases. These discoveries open new possibilities for the use of naturally occurring inhibitors, displaying activity across enzyme families, as a model in designing new molecules of therapeutic significance.

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Pancreatic secretory trypsin inhibitor is a single-chain, low-molecular-weight protein comprising 56 amino acid residues and is mainly produced in pancreatic acinar cells and secreted into the pancreatic juice. It was initially found and purified from bovine pancreas by Kazal *et al.* (1); now, however, it is known that such proteins are present in the pancreases of all animals studied. Considerable amounts of pancreatic secretory trypsin inhibitor

have also been found in a variety of extrapancreatic tissues (2,3). Pancreatic secretory trypsin inhibitor, belonging to a well-defined family of Kazal-type serine proteinase inhibitors, is distinguished from other members of this family by a narrow specificity for the inhibition of trypsin, with very little or no inhibitory capacity against other serine proteinases, even those exhibiting trypsin-like specificity (4). It is worth noting that a protein of significant similarity to pancreatic secretory trypsin inhibitor, having the Arg or Lys residue substituted at the $P_1$ position with Leu, thus possessing antichymotrypsin activity, has been isolated from duck pancreas (5). The primary physiological role of pancreatic secretory trypsin inhibitor has been postulated to be the prevention of trypsin-catalyzed premature activation of trypsinogen in the pancreas or pancreatic duct (4). However, the findings reported by Marchbank *et al.* (3), Matsuda *et al.* (6) and Ogawa (7), of raised serum pancreatic secretory trypsin inhibitor content in patients with severe inflammation, tissue destruction and malignant diseases, indicated that this protein may be involved in various functions outside the pancreas. A recent report by Tsuzuki *et al.* (8), on a novel role of pancreatic secretory trypsin inhibitor as a protein capable of inhibiting proteolytic activity of granzyme A, a tryptase produced in cytotoxic lymphocytes, strongly supports this hypothesis.

*Porphyromonas gingivalis*, a gram-negative, black-pigmented, anaerobic bacterium, has been implicated as the major pathogen of periodontitis in adults (9). This organism produces an array of virulence factors, of which cysteine proteinases, referred to as gingipains K and R, exhibiting Lys-Xaa and Arg-Xaa specificity, respectively, are believed to play a crucial pathogenic role (10). For hydrolytic activity, gingipains require reducing agents for activation and calcium for stabilization (11). Iodoacetamide, leupeptin, EDTA and chloromethylketones with Lys or Arg residues at the $P_1$ position were found to be strong inhibitors of gingipains (12). Gingipains are involved in both the destruction of periodontal tissues and the interruption of host defense mechanisms through the degradation of immunoglobulins and complement factors, leading eventually to disease progression (13).

As gingipains are regarded as a key virulence determinant of *P. gingivalis*, it has been suggested that the development of potent, gingipain-specific inhibitors might be a helpful tool in a therapeutic strategy to prevent or treat periodontal diseases (14–16). We found that of the naturally occurring protein inhibitors, pancreatic secretory trypsin inhibitor, surprisingly, showed an ability to quench the activity of gingipains. In this study we investigated the effect of pancreatic secretory trypsin inhibitor on the activities of gingipain R and gingipain K and showed that its specificity strongly depends on the type of its reactive site.

## Material and methods

### Materials

Porcine and bovine pancreases, obtained from a slaughterhouse not later than 2 h after slaughter, were placed on ice and, after removal of fat, were stored frozen at −20°C until analyzed.

The chemicals used were as follows: bovine β-trypsin (EC3.4.21.4), dimethyl sulfoxide, Tris, divinyl sulfone, phenylmethanesulfonyl fluoride, *N*-α-benzoyl-DL-arginine *p*-nitroanilide (BApNA), *Z*-Lys-*p*-nitroanilide and leupeptin from Sigma (St Louis, MO, USA); Sepharose 4B from Pharmacia LKB Biotechnology (Uppsala, Sweden); and 4-nitrophenyl 4-guanidinobenzoate hydrochloride from Fluka Chemie AG (Buchs, Switzerland). Gingipains R and K, as well as chicken ovoinhibitor, were generous gifts from Prof. Jan Potempa (Jagiellonian University, Krakow, Poland) and Prof. Jacek Otlewski (University of Wroclaw, Wroclaw, Poland), respectively. All other reagents were of analytical grade.

### Purification of inhibitors

Kazal-type pancreatic secretory trypsin inhibitors were purified from porcine and bovine pancreases using the method of Wilimowska-Pelc *et al.* (19), which consists of the following steps: extraction of the pancreas with 80% methanol in 0.3 M HCl, acetone precipitation of the extracted proteins followed by perchloric acid fractionation, affinity chromatography on anhydrotrypsin-Sepharose 4B, and reverse-phase high performance liquid chromatography on a Nucleosil 300 $C_{18}$ column.

### Protein assay

Protein content was determined either by the method of Smith *et al.* (18), with bicinchoninic acid using bovine serum albumin as the standard, or spectrophotometrically at 280 nm. For the bovine and porcine pancreatic secretory trypsin inhibitors, the extinction coefficients of $E_{280} = 6.5$ and 5.18, respectively, were used.

### Standardization of enzymes and inhibitors

The concentration of active enzymes in a stock solution of trypsin (dissolved in 1 mM HCl, 20 mM $CaCl_2$) was determined by spectrophotometric titration with *p*-nitrophenyl *p*-guanidinobenzoate (19). The standardized trypsin solution was used to titrate pancreatic secretory trypsin inhibitor and chicken ovoinhibitor [$I_0$]. The concentrations of gingipain R and gingipain K [$E_0$] were measured by titration with leupeptin.

### Proteinase activity and inhibitor measurements

Enzyme activities were measured spectrophotometrically at 22°C using the chromogenic turnover substrates BApNA for trypsin and gingipain R and *Z*-Lys-*p*-NA for gingipain K. All stock solutions of substrate were prepared in dimethylsulfoxide.

To determine the concentrations of pancreatic secretory trypsin inhibitor and chicken ovoinhibitor, a suitable amount of inhibitor was allowed to complex with trypsin ($2.0 \times 10^{-7}$ M) in 50 mM Tris-HCl, 20 mM $CaCl_2$, 0.005% Triton X-100 buffer, pH 8.3,

in a final volume of 1 mL. The residual enzyme activity was measured by the addition of BApNA ($2 \times 10^{-4}$ M); after 20 min of incubation the reaction was stopped with 50 μL of glacial acetic acid and the release of 4-nitroaniline was measured at 412 nm.

To measure enzyme–inhibitor interactions, gingipain R ($2.6 \times 10^{-8}$ M) and gingipain K ($9 \times 10^{-7}$ M) were incubated for 10 min in 200 mM Tris-HCl buffer, pH 7.6, containing 50 mM Gly–Gly, 10 mM $CaCl_2$ and 10 mM Cys-HCl to activate the enzymes. Then, the activated proteinase was allowed to react with increasing amounts of inhibitor ($5 \times 10^{-8}$ M to $2.5 \times 10^{-6}$ M for gingipain R and $3 \times 10^{-7}$ M to $3.6 \times 10^{-5}$ M for gingipain K) for 15 min in a final volume of 2.0 mL. The residual activity of the enzyme present at equilibrium was measured following the addition of the respective substrates, BApNA ($5 \times 10^{-4}$ M) or Z-Lys-p-NA ($1 \times 10^{-4}$ M), by recording the release of 4-nitroaniline for 60 s at 412 nm with an HP8452A diode array spectrophotometer. A control reaction, in the absence of inhibitor, was carried out under the same conditions.

### Measurements of equilibrium association constants

The equilibrium association constants were estimated using the method described by Empie & Laskowski (20). The reactions were conducted in a polystyrene cuvette in a final volume of 2.0 mL in 200 mM Tris-HCl buffer, pH 7.6, containing 50 mM Gly–Gly, 10 mM $CaCl_2$ and 10 mM Cys-HCl. The enzyme concentrations $[E_0]$ used complied with the equation: $2 < [E_0] \times K_a < 50$ (where $K_a$ is the association constant), and the concentration of inhibitor $[I_0]$ ranged from 0 to $2 \times [E_0]$. Constant amounts of enzyme were reacted with increasing amounts of inhibitor at 22°C for a predetermined period of time to reach equilibrium, followed by the addition of a small volume of a stock solution of substrate whose final concentration in the reaction did not exceed $0.2 \times K_m$, where $K_m$ is the Michaelis–Menten constant. The hydrolysis of *p*-nitroanilides was monitored at 412 nm for 60–120 s and the free enzyme content was calculated. The $K_a$ was calculated by fitting the experimental data to the following equation:

$$E = \frac{1}{2}\left([E_0] - [I_0] - K_a^{-1} + \sqrt{([E_0] + [I_0] + K_a^{-1})^2 - 4[E_0][I_0]}\right),$$

where [E] is the residual enzyme concentration, and $[E_0]$ and $[I_0]$ are the total enzyme and inhibitor concentrations, respectively.

### Chemical modification

Modification of Arg residues in bovine pancreatic secretory trypsin inhibitor was performed with a 30-fold molar excess of cyclohexanedione, according to Patthy & Smith (21). The protein, at a concentration of 1 mg/mL in 0.2 M borate buffer, pH 9.0, and 50 mM cyclohexanedione in the same buffer were mixed at a 1 : 30 molar ratio and incubated at 37°C. Aliquots were removed at given time intervals and residual gingipain R activity was measured.

## Results and Discussion

The unabated interest in proteinase inhibitors stems from the fact that these substances are regarded as being very important factors in controlling proteolysis. In animals they control a variety of critical events associated with, for example, blood coagulation, complement activation, cell migration, hormone transportation, tumor suppression, prohormone conversion, fibrinolysis and the inflammatory reaction (22,23). The physiological significance of proteinase inhibitors is, however, much broader and is not limited to the cited functions.

While screening the naturally occurring protein inhibitors against proteolytic enzymes present in a crude preparation obtained from *P. gingivalis* culture by means of acetone precipitation and ammonium sulfate fractionation, we found that bovine pancreatic secretory trypsin inhibitor was capable of partially reducing their activity against casein in a dose-dependent manner. At least 85% of the proteolytic enzymes produced by *P. gingivalis* are cysteine proteinases, referred to as gingipains K and R (15,24), and it was therefore of interest to investigate which of them is sensitive to a specific inhibitor of trypsin, a serine proteinase.

To investigate the inhibitory effect of Kazal-type trypsin inhibitors on gingipain activity, each of the enzymes (gingipain R and gingipain K) was separately incubated with various concentrations of the studied inhibitor and the residual enzyme activity was determined. The inhibitors used in this study included bovine and porcine pancreatic secretory trypsin inhibitors and the III and IV domains of chicken ovoinhibitor.

As shown in Fig. 1, gingipain R was sensitive only to the bovine pancreatic secretory trypsin inhibitor, which inhibited its activity to ≈ 33% at 0.5 μM, 66% at 1 μM and 87% at

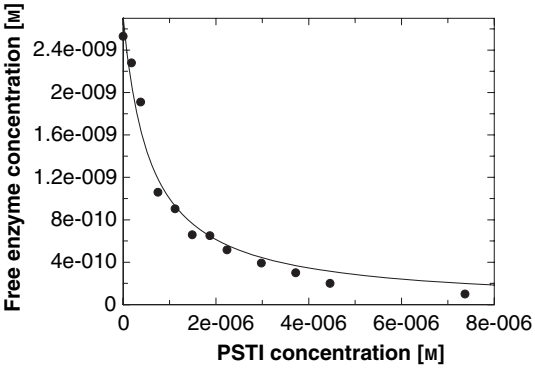


*Fig. 1.* Inhibition curve of gingipain R by bovine pancreatic secretory trypsin inhibitor (PSTI) in 200 mM Tris-HCl, 50 mM Gly–Gly, 10 mM $CaCl_2$ and 10 mM Cys HCl buffer, pH 7.6.

2.5 μM (Fig. 1). In contrast, the porcine pancreatic secretory trypsin inhibitor had no effect on gingipain R activity, even at a concentration 100-fold higher than that of the enzyme. The latter, however, exhibited some inhibitory activity against gingipain K (Fig. 2). The association constant for the binding of gingipain R to bovine pancreatic secretory trypsin inhibitor was $1.6 \times 10^6$ M$^{-1}$. The inhibition of gingipain K by the porcine pancreatic secretory trypsin inhibitor was significantly lower and was calculated to be $5.1 \times 10^4$ M$^{-1}$ (Table 1).

The pancreatic secretory trypsin inhibitors, regardless of origin, are known to be effective and very specific inhibitors of trypsin, for which peptide bonds formed by both Arg-Xaa and Lys-Xaa are almost equally susceptible to cleavage. There is, however, one notable difference between the bovine and porcine inhibitors, namely that the protein of bovine origin has an Arg residue at the $P_1$ position of the reactive site, whereas the protein of porcine origin has a Lys residue at the $P_1$ position of the reactive site. This strongly suggests that this difference in the structure of the reactive sites of the inhibitors reflects their specificity. The Arg residue at the inhibitor reactive site is essential for binding gingipain R, an Arg-specific proteinase, whereas the Lys residue is needed to bind Lys-specific enzyme. To prove this hypothesis, the Arg residue in the bovine pancreatic secretory trypsin inhibitor was subjected to chemical modification. The data presented in Fig. 3 show that indeed the anti-gingipain R activity of the inhibitor is very sensitive to Arg modification, indicating that this amino acid residue resides in the gingipain R-binding site of the inhibitor. This means that the same reactive site of the inhibitor is responsible for inhibiting both trypsin, a serine proteinase, and gingipain, a cysteine proteinase.

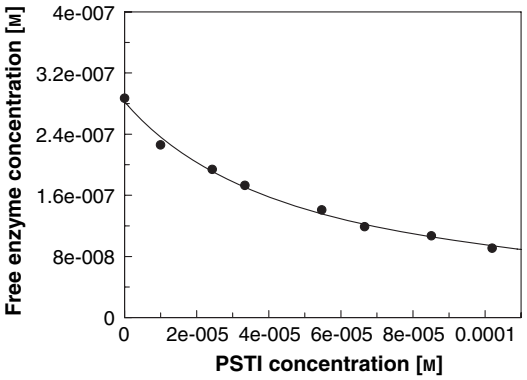


*Fig. 2.* Inhibition curve of gingipain R by bovine pancreatic secretory trypsin inhibitor (PSTI) in 200 mM Tris-HCl, 50 mM Gly–Gly, 10 mM $CaCl_2$ and 10 mM Cys HCl buffer, pH 7.6.

*Table 1.* Association equilibrium constants (M$^{-1}$) for the interaction of gingipains with Kazal-type serine proteinase inhibitors

| Enzyme | PSTI bovine | PSTI porcine | Ovoinhibitor[a] |
|---|---|---|---|
| Rgp | $1.6 \times 10^6$ | NI | $9.3 \times 10^4$ |
| kgp | NI | $5.1 \times 10^4$ | NI |

[a]III–IV domain of chicken ovoinhibitor.
NI, no inhibition; PSTI, pancreatic secretory trypsin inhibitor.

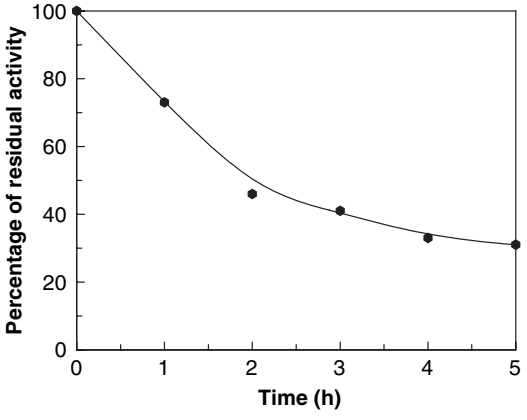


*Fig. 3.* Effect of cyclohexanedione on the inhibitory activity of bovine pancreatic secretory trypsin inhibitor (PSTI). Inhibitor was incubated with a 30 molar excess of cyclohexanedione, for the given time-periods, in 0.2 M borate bufffer, pH 9.0, at 37°C and aliquots were removed and assayed for residual activity against gingipain R.

R gingipain was found to be inhibited also by a fragment of chicken ovoinhibitor, another member of the Kazal-type serine proteinase inhibitor family. The preparation used in the experiment consisted of the III and IV domains, which, like the bovine pancreatic secretory trypsin inhibitor, have Arg residues at the $P_1$ position. This interaction with gingipain R was, however, significantly weaker ($K_a = 9.3 \times 10^4$ M$^{-1}$) than that of bovine pancreatic secretory trypsin inhibitor (Table 1). The difference between the two inhibitors could arise from the difference in the reactive site sequence. The $P_2'$ and $P_3'$ sites of pancreatic secretory trypsin inhibitor contain Tyr and Asn residues, respectively, whereas the ovoinhibitor III and IV domains have Leu and Ser or Leu and Leu, respectively, in the corresponding positions (Fig. 4).

To date, no information is available on the proteinaceous inhibitors of gingipains; nevertheless, there are a series of synthetic compounds posses-

| | $P_3$ | $P_2$ | $P_1$ | $P_1$' | $P_2$' | $P_3$' | $P_4$' |
|---|---|---|---|---|---|---|---|
| PSTI bovine | Cys | Pro | **Arg** | Ile | Tyr | Asn | Pro |
| PSTI porcine | Cys | Pro | **Lys** | Ile | Tyr | Asn | Pro |
| Ovoinhibitor III domain | Cys | Pro | **Arg** | Ile | Leu | Ser | Pro |
| Ovoinhibitor IV domain | Cys | Pro | **Arg** | Ile | Leu | Leu | Pro |

*Fig. 4*. Sequence alignment of bovine pancreatic secretory trypsin inhibitor (PSTI)- and ovoinhibitor-reactive sites.

sing considerable inhibitory activity. In addition to being inhibited by iodoacetamide, leupeptin, EDTA and chloromethylketones, as mentioned in the Introduction, gingipains were shown to be inhibited strongly by the small peptide analogs carbobenzoxy-Lys-Arg-CO-Lys-N-$(CH_3)_2$ and carbobenzoxy-Glu(NHN$(CH_3)$Ph)-Lys-CO-NHCH$_2$Ph with $K_a$ values in the order of $10^{11}$–$10^{10}$ M$^{-1}$ (25) and, to a lesser extent, by tetracycline (26) and benzamidine derivatives (17), which inhibit gingipain R with $K_a$ values of $1 \times 10^4$ M$^{-1}$ and $3.3 \times 10^4$ M$^{-1}$, respectively. To our knowledge, the data presented here show for the first time that specific trypsin inhibitors of the Kazal type have inhibitory potency against cysteine proteinases, particularly against gingipains. Recently, Sasaki *et al*. (27) described a similar phenomenon for the Kunitz-type serine proteinase inhibitor (BmTIsint) separated from *Boophilus microplus*, which exhibited activity against cysteine proteinase cathepsin L with a $K_a$ value of $9.2 \times 10^6$ M$^{-1}$. These discoveries open new possibilities for the use of naturally occurring inhibitors displaying activity across enzyme families as a model for designing new molecules of therapeutic significance.

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