Porphyromonas gingivalis Cysteine Proteinase Inhibition by κ -Casein Peptides[▽]

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Received 7 April 2010/Returned for modification 17 May 2010/Accepted 13 December 2010

***Porphyromonas gingivalis* is a major pathogen associated with chronic periodontitis, an inflammatory disease of the supporting tissues of the teeth. The Arg-specific (RgpA/B) and Lys-specific (Kgp) cysteine proteinases of *P. gingivalis* are major virulence factors for the bacterium. In this study κ -casein(109–137) was identified in a chymosin digest of casein as an inhibiting peptide of the *P. gingivalis* proteinases. The peptide was synthesized and shown to inhibit proteolytic activity associated with *P. gingivalis* whole cells, purified RgpA-Kgp proteinase-adhesin complexes, and purified RgpB proteinase. The peptide κ -casein(109–137) exhibited synergism with Zn(II) against both Arg- and Lys-specific proteinases. The active region for inhibition was identified as κ -casein(117–137) using synthetic peptides. Kinetic studies revealed that κ -casein(109–137) inhibits in an uncompetitive manner. A molecular model based on the uncompetitive action and its synergistic ability with Zn(II) was developed to explain the mechanism of inhibition. Preincubation of *P. gingivalis* with κ -casein(109–137) significantly reduced lesion development in a murine model of infection.**

Chronic periodontitis is one of the most common infectious diseases in the world affecting 5 to 20% of most populations (36, 38). The proliferation of specific Gram-negative bacteria in subgingival plaque causes a chronic inflammatory response leading to the breakdown of the tissues supporting the teeth, commonly resulting in tooth loss (35, 42). *Porphyromonas gingivalis* has been shown to be a major pathogen associated with chronic periodontitis (42). The main virulence factors of *P. gingivalis* are its outer membrane cysteine proteinases with Arg-specific (RgpA and RgpB) and Lys-specific (Kgp) activity (30, 32, 41). The proteinases exist on the cell surface or are secreted into the culture medium as noncovalently associated proteinase-adhesin complexes (RgpA-Kgp complexes) or as a discrete Arg-specific proteinase (RgpB) (6, 30, 39, 47). They play an important role in acquisition of nutrients and evasion of the host defenses (30, 44). They are also implicated in adhesion to host tissue, hemagglutination, and the processing of bacterial cell surface proteins (44).

These proteinases are classified in the C25 family of Clan CD of cysteine proteinases which have little sequence similarity to other cysteine proteinases. Therefore, the development of nontoxic inhibitors with high specificity is a feasible prospect. There have been several molecules, both naturally derived and synthetic, which have been reported to inhibit the *P. gingivalis* proteinases. Natural inhibitors have been identified through the screening of bioactive products, whereas several synthetic inhibitors have been designed and synthesized based on cleavage site specificity and the pharmacophoric map of the active site using Rational Protease Inhibitor Design (RAPiD)

methods (13, 20). The natural inhibitors of the *P. gingivalis* proteinases found to date are polyphenolic structures, such as those in cranberry juice and green tea catechins (8, 12, 34). These inhibitors exhibit a range of both Arg- and Lys-specific proteinase inhibition from low to moderate affinity (from >100 μ M to 1.1 μ M) (8, 12, 34). Synthetic inhibitors such as KYT-1 and KYT-36 exhibit high inhibitory potency with less than 1 μ M inhibiting *P. gingivalis* proteinases (20). However, these inhibitors have substantial safety and regulatory hurdles for human use and have not been further developed.

Bovine milk caseins are a rich natural source of specific peptides with biological activity (27). Proteinase inhibitor activity has been identified in both human and bovine milk proteins, including lactoferrin, cystatins, β -casein, β -lactoglobulin, and α_{S1} -antitrypsin (4). Bovine milk has the added advantage of being nontoxic, easily extracted in large quantities for commercial production, and subsequently biodegradable.

Zn(II) is a potential coinhibitor that has been shown to increase the inhibitory potency of several proteinase inhibitors such as benzamidine and chlorhexidine (12, 26). Zn(II) enhances inhibitor binding by acting as a bridge, forming a tetrahedral geometry when complexed with Cys, connecting the active site of the proteinase to the inhibitor (3, 19). Cys, Ser, and His have been reported to have the highest affinities toward Zn(II) (45). Since the *P. gingivalis* cysteine proteinases have Cys and His residues within the catalytic site, the potentiating effect of added Zn(II) may also be of therapeutic interest.

In the present study, we identified that κ -casein(109–137) had *P. gingivalis* proteinase inhibitory activity in a screening of peptides derived from bovine casein digested with chymosin. The bioactivity of this peptide was characterized by producing synthetic peptides and assessing their inhibitory activity against *P. gingivalis* whole cells, purified outer membrane RgpA/Kgp

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[▽] Published ahead of print on 20 December 2010.

FIG. 1. Amino acid sequence of κ-casein(106-169) and the shorter derivatives synthesized using microwave synthesis.

proteinase complexes, and purified RgpB. The peptide was then tested in a murine lesion model.

MATERIALS AND METHODS

Chymosin digestion of casein and screening of peptides for *P. gingivalis* gipain proteinase activity. Casein was obtained from Alacid acid casein produced by New Zealand Milk Products (Wellington, New Zealand). Casein was dissolved in deionized H₂O at 2.5% (wt/vol) and adjusted to pH 8.0 using 1 M NaOH. The pH was adjusted to pH 6.3 prior to the addition of chymosin (Renin R4877; Sigma-Aldrich, St. Louis, MO) at an enzyme-substrate (E:S) ratio of 1:10,000 (wt/wt) and incubation for 2 h at 37°C. The hydrolysis was terminated by addition of 5 M HCl to lower the pH to 3. The hydrolysate was centrifuged (10,000 × g, 5 min), and the supernatant was collected and fractionated using a Superose-12 column connected to an ÄKTA explorer FPLC system (Amersham Pharmacia Biotech, Sunnyvale, CA), followed by an analytical C₁₈ reversed-phase (RP) column connected to an Agilent Preparative 1100 high-performance liquid chromatography (RP-HPLC) instrument (Agilent, Palo Alto, CA). Collected fractions were screened for proteinase inhibitory activity and identified by mass spectrometry (MS) and tandem MS (MS/MS) analysis (Bruker Daltonics, New South Wales, Australia).

Nonglycosylated κ-casein(106-169) was obtained by chymosin digestion as previously described (28). κ-casein(106-137) was obtained by hydrolysis of nonglycosylated κ-casein(106-169) dissolved in 50 mM ammonium acetate (pH 4.0) buffer with endoprotease-Glu-C from *Staphylococcus aureus* strain V8 (Roche, Penzberg Germany) at 37°C (E:S ratio, 1:200) for 24 h. The hydrolysis was terminated by increasing the pH to 6.0 by the addition of 2 M NaOH, and peptides were separated using analytical C₁₈-RP-HPLC. Collected fractions were analyzed, and peptides were identified by MS/MS analysis.

Peptide synthesis. The peptides κ-casein(106-137), κ-casein(109-137), κ-casein(117-137), κ-casein(117-123), and κ-casein(127-137) (Fig. 1) were synthesized using standard Fmoc (9-fluorenylmethoxy carbonyl) chemistry protocols on a Liberty Microwave peptide synthesizer (CEM Corp., Matthews, NC) as described previously (2). Crude peptides were purified using a semipreparative C₁₈-RP-HPLC system. Purified samples were analyzed using an Esquire-HCT electrospray ionization-MS (ESI-MS) system (Bruker Daltonics). The purity of the peptides was determined using analytical C₁₈-RP-HPLC. Purified fractions were lyophilized and stored at -20°C.

Bacterial strains and growth conditions. Glycerol or freeze-dried cultures of *Porphyromonas gingivalis* W50, ATCC 33277, and HG66 were grown anaerobically and maintained by passage at 37°C on horse blood agar (Oxoid, Adelaide, Australia). Cells from passages 3 to 7 were used to inoculate 20 and 200 ml of brain heart infusion (Oxoid) broth (37 g/liter), supplemented with hemin (5 mg/liter) and L-cysteine (0.5 g/liter). For *P. gingivalis* 33277, vitamin K₃ (menadiolone; 5 mg/liter) was also added. Growth was determined by measurement of the culture optical density at a wavelength of 650 nm. Culture purity was determined by Gram staining and microscopic analysis. *P. gingivalis* cells were harvested during exponential growth phase by centrifugation (8,000 × g, 20 min, 4°C) and washed once with TC150 buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl₂ [pH 8.0]) containing 0.5 g of L-cysteine/liter. The washed cells were resuspended in 2 ml of TC150 buffer (with 0.5 g of L-cysteine/liter) and kept at 4°C to be used immediately in the proteolytic assays.

Purification of *P. gingivalis* RgpA-Kgp proteinase adhesin complexes and RgpB. *P. gingivalis* W50 cells were grown to late exponential phase in 2-liter batch cultures, and the RgpA-Kgp proteinase adhesin complexes were purified from Triton X-114 extracts by using Arg-Sepharose affinity chromatography based on

the procedure described previously (37). RgpB was purified from *P. gingivalis* strain HG66 according to previously published procedures (9, 39).

Determination of *P. gingivalis* Arg- and Lys-specific proteolytic activity using chromogenic substrates. Bacterial proteinase inhibitory activity was determined by using an assay developed for whole-cell Arg- and Lys-specific proteolytic activity of *P. gingivalis* that was modified and adapted to be performed using sterile 96-well microtiter plates (Corning, Inc., Corning, NY) with reduced incubation volumes (31, 32). The Arg- and Lys-specific proteolytic activities were determined by using synthetic chromogenic substrates; *N*-α-benzoyl-Arg-p-nitroanilide (BAPNA) and *N*-(p-tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt (GPK-NA) were both obtained from Sigma. The Arg- and Lys-specific reaction buffer contained 2 mM BAPNA or GPK-NA, respectively, dissolved in 30% (vol/vol) isopropanol, 0.93 mM L-cysteine, 400 mM Tris-HCl (pH 8.0), and 100 mM NaCl. The casein peptides were diluted with TC150 buffer depending on the concentration required. A total of 10 μl of 10 mM L-cysteine (pH 8.0) and either *P. gingivalis* whole-cell suspension, purified RgpA-Kgp proteinase-adhesin complexes (0.01 μg/μl), or purified RgpB (0.00116 μg/μl) was added to a final volume of 100 μl. Samples were then incubated for 15 min at 37°C before the addition of Arg- or Lys-specific reaction buffer (total volume, 200 μl). The activity was determined by measuring the absorbance at 405 nm at 10-s intervals for ~20 min at 37°C and pH 8.0 using the Perkin-Elmer 1420 Multilabel Counter VICTOR3 (Waltham, MA). A stock of 8 mM ZnCl₂ solution was prepared in deionized H₂O and used to dissolve the lyophilized casein peptides at the concentrations required for the specific assays.

After incubation, the assay contents were analyzed by RP-HPLC using an analytical (C₁₈) column and a linear gradient of 0 to 100% buffer B to determine whether there was casein peptide degradation during the assay. Eluted fractions were analyzed by ESI-MS and MS/MS analysis.

Determination of *P. gingivalis* proteolytic activity with fluorescently labeled bovine serum albumin (BSA). Bacterial proteinase inhibitory activity was also determined using DQ Green BSA (Molecular Probes, Eugene, OR) with modifications from previously published procedures (17, 48). *P. gingivalis* 33277 whole cells, harvested during exponential growth (optical density at 650 nm of 0.6), were used for the assay with 5.6×10^9 CFU/ml per well. The assay mixture contained *P. gingivalis* cells (100 μl of culture), TC150, and inhibitors (final volume, 80 μl), and DQ Green BSA (20 μl; 200 μg/ml). We used 1 mM TLCK (*N*-α-p-tosyl-L-lysine chloromethyl ketone)-treated cells as controls. TLCK is known to inhibit both Rgp and Kgp activity (14, 39). The assay mixtures were incubated in the dark for 2 h at 37°C prior to measuring the fluorescence (emission, 535 nm; excitation, 485 nm) using a fluorometer (the Perkin-Elmer 1420 Multilabel Counter VICTOR3). The fluorescence value obtained from the negative control (i.e., the TLCK-treated cells) was subtracted from all values. All assays were performed in triplicate with two to three biological replicates unless stated otherwise.

Statistical analysis. Proteinase activity data were subjected to a single factor analysis of variance (ANOVA). When the ANOVA indicated statistical significant difference ($P < 0.05$) between the means of tested inhibitors, a modified Tukey test was conducted on the data to distinguish which inhibitors were significantly different (15, 49).

FIC index. The fractional inhibitory concentration (FIC) index was used to determine the interaction of the peptide inhibitor and Zn(II) (5). The FIC index was calculated as follows: $\text{FIC index} = [(A + B)/A] + [(A + B)/B]$, where *A* is the effect of the synthetic peptide, *B* is the effect of zinc chloride, and *A + B* is the effect of the combination of both. An FIC index of ≤0.5 is interpreted as a synergistic effect wherein the combined activities are greater than the sum of the individual activities (1, 33). In contrast, an FIC index of >4 is interpreted as an antagonistic effect where the combined activity is less than the sum of individual activities (33).

Determination of type of inhibition and inhibition constants. Inhibition kinetics were determined using purified RgpB (0.116 μg/well) in the chromogenic substrate assay as described above. Initial reaction rates were obtained at substrate (BAPNA) concentrations of 0.15, 0.25, or 1 mM. Inhibitor (peptide) concentrations ranged from 0 to 100 μM.

Murine lesion model. The murine lesion model was conducted essentially as described previously (32). Exponentially growing *P. gingivalis* 33277 cells were harvested by centrifugation (7,500 × g, 30 min, 4°C) and resuspended at 5.6×10^9 cells/ml in TC150 buffer or TC150 buffer containing 500 μM TLCK or 500 μM κ-casein(109-137) for 2 h. *P. gingivalis* cells were then harvested (7,500 × g, 30 min, 4°C) and resuspended in TC150 to a cell density of 7.5×10^{10} cells/ml. BALB/c mice 6 to 8 weeks old were subcutaneously inoculated in the abdomen with 7.5×10^9 inhibitor-treated or untreated *P. gingivalis* cells (100 μl), and the lesion sizes were measured after 2 days. The control group was sham inoculated with 100 μl of TC150 buffer. Lesion sizes were statistically analyzed by using the

TABLE 1. Activity of Arg- and Lys-specific proteinases from whole cells of *P. gingivalis* 33277, with various concentrations of synthetic casein peptides measured using chromogenic substrates (BAPNA and GPK-NA)

Proteinase	Concn (μ M)	Mean activity (U/10 ¹¹ cells) $\pm$ SD (%) ^a	
		Arg specific	Lys specific
Control	0	17.99 $\pm$ 1.11 (100)	4.41 $\pm$ 1.07 (100)
κ -casein(109–137)	50	12.66 $\pm$ 2.83 ^{A,B} (70)	1.69 $\pm$ 0.64 ^A (41)
	100	6.89 $\pm$ 2.51 ^A (38)	0.76 $\pm$ 0.30 ^A (19)
	200	1.48 $\pm$ 1.18 ^A (8)	0.10 $\pm$ 0.06 ^A (3)
κ -casein(117–123)	200	20.04 $\pm$ 1.72 ^E (111)	ND
	500	16.10 $\pm$ 0.57 ^{C,D} (90)	ND
	1,000	16.52 $\pm$ 3.26 ^{C,D} (92)	ND
κ -casein(127–137)	200	18.26 $\pm$ 1.78 ^{D,E} (102)	ND
	500	17.39 $\pm$ 0.33 ^{C,D} (97)	ND
	1,000	14.83 $\pm$ 1.19 ^{A,B,C} (82)	ND

^a Superscript letters indicate significance as follows: A, significantly different ($P < 0.05$) from the control; B, C, D, and E, significantly different ($P < 0.05$) from other values not similarly marked. ND, not determined.

Kruskal-Wallis test and Mann-Whitney U-Wilcoxon rank-sum test. All protocols were approved by the University of Melbourne Ethics Committee for Animal Experimentation.

Molecular modeling. The program FUGUE (40) was used to identify possible structural motifs for the peptide against a curated protein database, HOMSTRAD, which contains 1,034 protein families and 10,230 aligned structures (29). FUGUE scans the database of structural profiles, calculates the sequence-structure compatibility scores, and produces a list of potential homologues and alignments. Specificity, sensitivity, and ranking are calculated according to the Z-score. Z-score thresholds of 6 indicate 99% specificity, and Z-score thresholds of 5 indicate 95% specificity. A model of the inhibitor peptide was constructed by using SYBYL/Tripes. Potential metal-binding sites in the inhibitor peptide were identified by the locus of the metal atom positions relative to the backbone and C β atom positions of the model peptide, using in-house software.

RESULTS

***P. gingivalis* Arg- and Lys-specific proteinase inhibitory activity of κ -casein peptides.** From a screening of a chymosin digest of bovine casein for inhibitory activity against the *P. gingivalis* Arg-specific proteinase, a peptide κ -casein(109–137) was identified which at $\sim$ 100 μ M inhibited activity by 85%. The inhibitory activity of this casein-derived peptide was confirmed by synthesizing and testing it against *P. gingivalis* whole cells from two strains (W50 and 33277), purified RgpA-Kgp proteinase adhesin complexes, and purified RgpB. The purity of the synthetic peptide was determined by RP-HPLC and ESI-MS. The measured mass of the synthetic peptide was 3,095.6 Da corresponding to κ -casein(109–137) with a calculated mass of 3,096.4 Da.

At a concentration of 200 μ M, synthetic κ -casein(109–137) exhibited 92 and 97% inhibition of whole-cell Arg- and Lys-specific proteinase activity, respectively (Table 1). Subsequently, purified RgpA-Kgp proteinase adhesin complexes were used in the proteolytic assays, where κ -casein(109–137) exhibited similar inhibitory effects at half the peptide concentration (Table 2).

A fluorescent substrate, DQ Green BSA, was also used to measure the inhibitory activity of the casein peptides against *P.*

TABLE 2. Activity of purified RgpA-Kgp proteinase adhesin complexes of *P. gingivalis* W50 with various concentrations of synthetic casein peptides measured using chromogenic substrates (BAPNA and GPK-NA)

Proteinase	Concn (μ M)	Mean activity (U/mg) $\pm$ SD (%) ^a	
		Arg specific	Lys specific
Control	0	4.13 $\pm$ 1.11 ^D (100)	4.67 $\pm$ 1.01 (100)
κ -casein(109–137)	20	1.80 $\pm$ 0.06 ^{A,C} (43)	2.90 $\pm$ 0.55 ^A (62)
	50	0.87 $\pm$ 0.38 ^{A,B} (21)	1.80 $\pm$ 0.91 ^A (39)
	100	0.33 $\pm$ 0.28 ^{A,B} (8)	0.28 $\pm$ 0.18 ^{A,B} (6)
κ -casein(117–137)	50	1.95 $\pm$ 0.38 ^{A,C} (46)	1.34 $\pm$ 0.25 ^{A,C} (29)
	100	0.41 $\pm$ 0.11 ^{A,B} (10)	0.98 $\pm$ 0.053 ^{A,C} (21)
	200	0.33 $\pm$ 0.02 ^{A,B} (8)	0.41 $\pm$ 0.014 ^{A,B} (9)
κ -casein(117–123)	200	3.68 $\pm$ 1.69 ^D (89)	4.62 $\pm$ 0.51 (99)
κ -casein(127–137)	200	4.49 $\pm$ 2.05 ^D (109)	3.86 $\pm$ 0.09 ^{A,D} (83)
κ -casein(106–137)	200	3.51 $\pm$ 1.77 ^D (85)	4.15 $\pm$ 0.64 ^{A,D} (89)

^a Superscript letters indicate significance as follows: A, significantly different ($P < 0.05$) from the control; B, C, and D, significantly different ($P < 0.05$) from other values not similarly marked.

gingivalis whole-cell proteinase activity. Prior to the addition of the peptides, the assay was optimized for the number of cells per well and incubation time. A 2-h incubation period with 10⁹ cells per well was selected for the assay since the rate of protein hydrolysis was linear under these conditions (data not shown). The nonglycosylated κ -casein(106–169) peptide derived from casein inhibited 60% of the proteinase activity at 500 μ M peptide concentration in the fluorescence assays. The synthetic shorter fragment, κ -casein(106–137), had little effect on the Arg- and Lys-specific proteinase activities in chromogenic assays at 200 μ M concentration while inhibiting $\sim$ 37% proteinase activity at a 500 μ M peptide concentration in the fluorescence assays (Table 2 and Fig. 2).

FIG. 2. Arg- and Lys-specific proteinase activities of *P. gingivalis* 33277 whole cells measured by using fluorescent BSA substrate (DQ Green BSA) and 500 μ M casein peptides. κ -casein(106–169) was naturally derived while the other κ -casein peptides were synthetic. The error bars were calculated as the standard deviations of three technical replicates and two biological replicates. Lowercase letters indicate significance: a, significantly different ($P < 0.05$) from the control values; b, c, d, and e, significantly different ($P < 0.05$) from other values not similarly marked. A 100% proteinase activity equals 4.5×10^5 relative fluorescence units/ 5.6×10^9 bacterial cells.

TABLE 3. Fractional inhibitory constant indices to assess the synergy of inhibition of proteinases (from *P. gingivalis* 33277 whole cells) by κ -casein(109–137) peptide with or without Zn(II)

Activity type	Mean activity (U/10 ¹¹ cells) $\pm$ SD				FIC index
	No inhibitor	κ -casein (109–137) (100 μ M)	Zn(II) (400 μ M)	κ -casein + Zn(II) (100 and 400 μ M)	
Arg specific	18.1 $\pm$ 5.69	6.92 $\pm$ 2.51	12.1 $\pm$ 3.32	1.95 $\pm$ 1.29	0.44
Lys specific	4.4 $\pm$ 1.07	0.76 $\pm$ 0.30	2.2 $\pm$ 1.31	0.31 $\pm$ 0.18	0.54

To determine whether the peptide was interacting with the RgpA-Kgp proteinase-adhesin complexes through the adhesins or binding directly to the catalytic domain of the proteinases, κ -casein(109–137) was incubated with purified RgpB. The peptide inhibited RgpB activity by 61.4% at a concentration of 100 μ M and by 87.4% at 200 μ M. Inhibition of RgpB activity indicated that the peptide binds directly to the catalytic domain and not the adhesin domains of the larger RgpA-Kgp complexes since RgpB does not have the adhesin domains. These experiments verified that the casein peptide, which does not contain any Arg residues, was not being used as a preferred substrate since RgpB cleaves at the carboxyl side of Arg residues only. RP-HPLC analysis of the proteinase assay contents after incubation confirmed that there was no peptide degradation during the assay. Only two peaks were observed in the chromatographic profiles: one of the peptides, κ -casein(109–137) that eluted at the expected retention time, which was identified by ESI-MS and MS/MS analysis, and one of the chromogenic substrates.

In order to delineate the residues essential for inhibition, a series of peptides were synthesized without the Lys-rich N-terminal region: κ -casein(117–123), κ -casein(127–137), and κ -casein(117–137) (Fig. 1). These synthetic peptides were screened for *P. gingivalis* proteinase inhibition in whole-cell assays with fluorescent BSA and in proteolytic assays using purified RgpA-Kgp complexes with a chromogenic substrate. Only κ -casein(117–137) exhibited inhibitory activity with inhibitory potencies similar to those of the longer κ -casein(109–137) (Table 2 and Fig. 2). This demonstrated the lack of importance of the Lys residues in the N-terminal region for proteinase inhibition.

Determination of the inhibition mechanism. In order to determine the inhibition constant of κ -casein(109–137) and its inhibition mechanism, a kinetic analysis was performed using purified RgpB. Lineweaver-Burk plot analysis indicated uncompetitive inhibition of the RgpB proteinase by the peptide with a K_i' value of 40.2 μ M. The K_i' value signifies the dissociation constant for binding of the inhibitor to the ES complex according to the following equation:

Zinc chloride as a coinhibitor. To investigate the potential of Zn(II) to increase the inhibitory potency of the peptides, whole-cell and purified RgpA-Kgp complexes were assayed with κ -casein(109–137) and ZnCl₂ in a 1:4 ratio. The κ -

casein(109–137) peptide at 100 μ M inhibited Arg-specific activity by 60%, while ZnCl₂ at 400 μ M exhibited 30% inhibition. However, when these two were combined, the inhibition increased to 90% with an FIC index of 0.44, indicating synergism (Table 3). Similar results were observed with the Lys-specific proteolytic assay, with the κ -casein–ZnCl₂ mixture increasing the inhibitory potency to 90% (Table 3).

Molecular modeling. The program FUGUE (40) was used to identify possible structure motifs for the peptide against a curated protein database HOMSTRAD (29). A Z-score of 3.87 was obtained for κ -casein(109–137) peptide, indicating 90% confidence for an α -helical structure. The possible structural motifs for κ -casein(109–137) include an α -helix spanning residues 109 to 126, a turn, and another α -helix spanning residues 129 to 137. Based on these motifs, a model of the κ -casein(109–137) peptide was constructed using the SYBYL software. A model of the κ -casein(109–137) inhibiting RgpB was developed based on the crystal structure of the RgpB (PDB 1cvr) and the proposed model of κ -casein(109–137) with the potential metal binding sites. Figure 3 shows the proposed model highlighting the residues of RgpB active site involved in interacting with the peptide inhibitor, κ -casein(109–137), Zn(II) and the synthetic substrate (BAPNA). This model highlights the electrostatic interactions between residues His²¹¹

FIG. 3. Molecular model of κ -casein(109–137) binding to the RgpB-BAPNA complex in the presence of Zn(II) (a) and residues of the RgpB active site involved in interacting with κ -casein (109–137), Zn(II), and the substrate (BAPNA) in the molecular model (b). The electrostatic interactions between His²¹¹ and Glu¹⁵² of RgpB and between Asp¹¹⁵ (Asp⁷) and Glu¹¹⁸ (Glu¹⁰) of κ -casein (109–137) with Zn(II) are highlighted. The hydrophobic interactions between Ile¹²² (Ile¹⁴) of the peptide with the BAPNA substrate are also highlighted. Trp²⁸⁴ and Cys²⁴⁴ of RgpB form interactions with the Arg residue and the amide bond of the substrate, respectively.

FIG. 4. Effect of pretreatment with proteinase inhibitors on lesion size of mice inoculated with *P. gingivalis* 33277. (a) Lesion size at day 2; BALB/c mice were inoculated with *P. gingivalis* 33277 (7.5×10^9 viable cells) that had been preincubated with TC150 (control) or 500 μ M TLCK or 500 μ M κ -casein(109–137). Lesion sizes are expressed as mm² and were statistically analyzed by using the Mann-Whitney U-Wilcoxon rank-sum test. (b) Inhibition of *P. gingivalis* 33277 whole-cell proteinase activity by 2 h of incubation with TC150, 500 μ M TLCK, or 500 μ M κ -casein(109–137).

and Glu¹⁵² of RgpB and residues Asp¹¹⁵ and Glu¹¹⁸ of κ -casein(109–137) with Zn(II). Residue Ile¹²² of κ -casein(109–137) forms a hydrophobic interaction with the substrate BApNA. Residues Trp²⁸⁴ and Cys²⁴⁴ of RgpB also interact with the Arg residue and the amide bond, respectively, of the BApNA substrate.

Effect of κ -casein(109–137) on lesion size induced by *P. gingivalis* in the mouse. *P. gingivalis* 33277 cells untreated or treated with 500 μ M TLCK or 500 μ M κ -casein(109–137) were injected subcutaneously into BALB/c mice (7.5×10^9 cells per mouse). Mice inoculated with untreated *P. gingivalis* cells developed a maximal lesion size by day 2 after inoculation (Fig. 4a). Preincubation of *P. gingivalis* with both TLCK and κ -casein(109–137) resulted in significantly ($P < 0.05$) smaller lesions at day 2 compared to mice inoculated with untreated *P. gingivalis* (Fig. 4a). Prior to inoculation the proteinase activity of aliquots of untreated, TLCK- or κ -casein(109–137)-treated *P. gingivalis* 33277 cells was determined by using the DQ Green BSA proteinase assay. Treatment of *P. gingivalis* cells with 500 μ M TLCK or κ -casein(109–137) also significantly reduced *P. gingivalis* whole-cell proteinase activity (Fig. 4b).

DISCUSSION

Chronic periodontitis is a major public health problem and has been associated with the bacterium *P. gingivalis* and its secreted Arg- and Lys-specific cysteine proteinases (30, 35). Currently, no therapy exists to specifically target the *P. gingivalis* proteinases, indicating an opportunity to develop a safe and specific therapeutic for *P. gingivalis*-induced periodontal disease.

The κ -casein(109–137) peptide derived from the bovine milk

protein casein demonstrated significant proteinase inhibitory activity against *P. gingivalis* whole cells, purified RgpA-Kgp complexes, and purified RgpB using synthetic substrates. The proteinase inhibition results were confirmed in whole-cell proteolytic assays using a protein substrate, fluorescently labeled BSA. The higher concentration of casein peptides required to inhibit the cleavage of fluorescent BSA in the assay was attributed to the high density of bacterial cells used in the assay and the presence of other *P. gingivalis* proteinases.

Since the peptide κ -casein(109–137) contains N-terminal Lys residues it could potentially be a preferred substrate of the Lys-specific proteinase Kgp relative to the synthetic substrate, GPK-NA. However, 200 μ M κ -casein(109–137) inhibited purified RgpB by >80%, which only has a specificity for Arg residues. Therefore, the peptide could not be a preferred substrate for RgpB. Furthermore, the κ -casein(117–137) peptide synthesized without the N-terminal residues 109 to 116 containing the Lys residues still retained Kgp proteinase inhibitory activity (Table 2), and analysis of assay contents postincubation further indicated that the peptide was not being degraded, indicating that it was also not a substrate for the Kgp proteinase.

The murine lesion model has been used extensively to determine the virulence characteristics of *P. gingivalis* and other oral bacteria (7, 23, 32, 37), and TLCK, an inhibitor of the RgpA/B and Kgp proteinases, has been demonstrated previously to reduce lesion size induced by *P. gingivalis* in this model (22). These studies demonstrated that the RgpA/B and Kgp proteinases have a major role in *P. gingivalis* virulence, tissue invasion, and lesion development. Treatment of *P. gingivalis* with κ -casein(109–137) prior to injection resulted in a 57% decrease in whole-cell proteinase activity and a 75% reduction in lesion size at day 2 that was not significantly different to the reduction (89%) resulting from treatment with TLCK at the same concentration (Fig. 4). These results indicate that the casein peptide can reduce the virulence of *P. gingivalis* by inhibition of the cell surface proteinases RgpA/B and Kgp.

An FIC index is normally used to calculate synergy between drug molecules. FIC calculations have been recently applied to short antimicrobial peptides such as magainin and gaegurin against Gram-negative and Gram-positive bacteria and, according to this index, κ -casein(109–137) exhibited synergism with ZnCl₂ (16, 24, 25, 46).

Based on the enzyme kinetics data, κ -casein(109–137) is an uncompetitive inhibitor. Consequently, the peptide only binds to the enzyme-substrate complex, preventing product formation. The inhibition constant of the peptide is 40.2 μ M and, in the case of uncompetitive inhibitors, the K_i value is equal to the 50% inhibitory concentration (IC₅₀) (10, 21). A comparison to the K_i values of other *P. gingivalis* cysteine proteinase inhibitors shows that this peptide has similar or better K_i values compared to synthetic chlorhexidine (K_i [RgpB] = 262 μ M), doxycycline (K_i [RgpB] = 54 μ M), and the naturally derived green tea catechins (IC₅₀ [RgpB] = 3 to 20 μ M and IC₅₀ [Kgp] = 100 μ M) (12, 18, 34).

In the present study, structural prediction analysis suggested that κ -casein(109–137) has a possible α -helix turn α -helix conformation. Circular dichroism and nuclear magnetic resonance studies of κ -casein(105–131) in solution indicate that this region of the peptide only forms a random coil (11). However,

this does not preclude the fact that the peptide may form a secondary structure in the right conditions, particularly when bound to another protein. A molecular model of the binding of κ -casein(109–137) peptide to the enzyme substrate complex (RgpB-BAPNA) in the presence of Zn(II) is proposed (Fig. 3). Zn(II) is naturally present in the environment and is a common ion normally associated with His, Glu, Asp, and Cys residues, playing a role in the structure and/or catalytic mechanism of proteins (3). In catalytic sites, Zn(II) generally forms complexes with water and interacts with any two nitrogen, oxygen, or sulfur donors, with His being the main amino acid involved (3). His²¹¹ acts as a proton acceptor and donor in the catalysis reaction, deprotonating the thiol group of Cys²⁴⁴ and donating a proton to the nitrogen atom of the scissile bond of the substrate, resulting in the release of the C-terminal fragment of the substrate (43). It is postulated that this “proton shuttle” is hindered when there is a high concentration of Zn(II) ions or when Zn(II) is stabilized by interaction with the κ -casein(109–137) peptide. The presence of excess Zn(II) or the binding of the peptide prevents the exchange of Zn(II) in the active site with unbound Zn(II) in the proteinase’s environment. Consequently, this postulated model is consistent with the synergistic inhibition of proteinase activity displayed by the peptide in the presence of excess Zn(II).

Conclusion. The casein peptide κ -casein(109–137) is a significant inhibitor of the *P. gingivalis* cysteine proteinases (RgpA/B and Kgp) and significantly reduces lesion development in a murine model of infection. The peptide inhibits in an uncompetitive manner, and the Lys-rich residues in the N-terminal region are not essential for proteinase inhibitory activity. The peptide acts synergistically with Zn(II) in inhibiting proteinase activity. Molecular modeling studies are consistent with the experimental evidence of the binding mechanism of the peptide in the presence of substrate and Zn(II).

ACKNOWLEDGMENTS

Elena Toh was a recipient of the University of Melbourne Sir John and Lady Higgins Research Scholarship.

We thank Peiyan Shen for technical assistance.

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