Divalent Metal Cations Increase the Activity of the Antimicrobial Peptide Kappacin

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**Kappacin, nonglycosylated κ-casein(106-169), is a novel antimicrobial peptide produced from κ-casein found in bovine milk. There are two major genetic forms of kappacin, A and B, and using synthetic peptides corresponding to the active region, κ-casein(138-158), of these forms, we have shown that the Asp148 to Ala148 substitution is responsible for the lesser antibacterial activity of κ-casein-B(106-169). Kappacin was shown to have membranolytic action at concentrations above 30 μM at acidic pH when tested against artificial liposomes. There was little membranolytic activity at neutral pH, which is consistent with the lack of antibacterial activity of kappacin against Streptococcus mutans at this pH. Kappacin specifically bound two zinc or calcium ions per mol, and this binding enhanced antibacterial activity at neutral pH. Nuclear magnetic resonance analysis indicated that a κ-casein-A(138-158) synthetic peptide undergoes a conformational change in the presence of the membrane solvent trifluoroethanol and excess divalent metal ions. This change in conformation is presumably responsible for the increase in antibacterial activity of kappacin detected in the presence of excess zinc or calcium ions at neutral pH. When tested against the oral bacterial pathogen S. mutans cultured as a biofilm in a constant-depth film fermentor, a preparation of 10 g/liter kappacin and 20 mM ZnCl2 reduced bacterial viability by 3 log10 and suppressed recovery of viability. In contrast 20 mM ZnCl2 alone reduced bacterial viability by ∼1 log10 followed by rapid recovery. In conclusion, kappacin has a membranolytic, antibacterial effect that is enhanced by the presence of divalent cations.**

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Kappacin, the nonglycosylated, phosphorylated forms of bovine caseinomacropeptide [CMP; κ-casein(106-169)], has been shown to have antibacterial activity in vitro against both gram-negative and gram-positive bacteria (15). CMP is a 64-amino-acid polypeptide released from bovine κ-casein by chymosin hydrolysis of the peptide bond between Phe105 and Met106. It comprises the 106 to 169 C-terminal fragment of κ-casein and contains all the posttranslational modification sites found in κ-casein. CMP is both variably phosphorylated and glycosylated (23, 25, 31). CMP is completely phosphorylated at Ser149 and partially phosphorylated (∼10%) at Ser127 as determined by matrix-assisted laser desorption ionization-time of flight (MALDI)-post source delay (PSD) mass spectrometry (31). Additionally, there are at least six genetic variants of κ-casein, with variants A and B being by far the most common (5). Genetic variants A and B differ only at residues 136 and 148, where the hydrophilic residues Thr136 and Asp148 of variant A are substituted by the hydrophobic residues Ile136 and Ala148 in variant B. The active region of kappacin was demonstrated to be residues 138 to 158 with phosphorylation of Ser149 essential for activity, as determined using the synthetic peptides Ser(P)149-κ-casein-A(138-158) and κ-casein-A(138-158) (15). The MIC of kappacin genetic variant A [κ-casein-A(106-169)] against Streptococcus mutans was 100 μM, while variant B [κ-casein-B(106-169)] was less active, with a MIC of 154 μM (1.04 mg/ml) (15).

The mechanism by which kappacin inhibits bacterial growth is still unclear. Kappacin was found to be most effective against S. mutans at slightly acidic growth pH. The nonglycosylated κ-casein-B(130-158) has been proposed to form an amphipathic α-helix, especially in the presence of trifluoroethanol (TFE; 23a). This characteristic could help to explain its antibacterial activity if it functions as a surface-active agent, creating pores in the cell membrane. This mode of action has been proposed for the majority of the cationic antimicrobial peptides isolated to date, although there is still debate as to whether membrane penetration may be a precursor for some peptides to reach intracellular targets (8). However, kappacin is an anionic peptide that does not exhibit sequence similarity with the better-known cationic antibacterial peptides, and apart from a possible propensity to form an amphipathic helical structure, does not possess any of the other characteristics of these peptides. Kappacin does share some characteristics with the more recently discovered anionic antibacterial peptides, especially enkelytin. This peptide, like kappacin, contains a number of glutamyl residues, and phosphorylation is essential for antibacterial activity (10, 11, 29).

The aim of this study was to elucidate the antibacterial activity and mechanism of kappacin, determine the effect of divalent cations on activity and structure, and determine its activity against biofilms of the oral pathogen S. mutans, alone and in combination with zinc ions.
MATERIALS AND METHODS

Kappacin preparation. Casein-HCl (Bonlac Foods, Melbourne, Australia) was dissolved by slow addition with constant stirring to deionized water at 50°C and addition of NaOH to pH 8.0 to give a final concentration of 21.5 g/liter. Once the casein had dissolved, the temperature was lowered to 37°C and the pH was adjusted to 6.3 by the slow addition of 1 M HCl to avoid precipitation of casein. To begin the hydrolysis Rennet (90% chymosin: EC 3.4.23.4; 145 international milk clotting units [IMCU]/ml; single strength; Chr. Hansen) was added to a final concentration of 1.2 IMCU/g casein and the solution was stirred at 37°C for 1 h. The pH of the solution was maintained at 6.3 ± 0.2 by the addition of 1 M NaOH. Hydrolysis was stopped by the addition of trichloroacetic acid to a final concentration of 4%, and the precipitated proteins were pelleted by centrifugation.

(Caseinomacropeptide [CMP]) was concentrated and washed with H2O using diafiltration with a 3,000-Da cutoff membrane (SIOYS, Amicon/Millipore). The retentate was analyzed by high-pressure liquid chromatography (HPLC) and mass spectrometric analysis (see below) prior to use in the biofilm antibacterial assay.

This preparation was further fractionated into glycosylated κ-casein(106-169) forms, nonglycosylated κ-casein-A(106-169), and nonglycosylated κ-casein-B(106-169) by reversed phase HPLC using a C18 column and elution with 90% acetonitrile/0.1% trifluoroacetic acid, as described previously (15). The identity of each fraction was confirmed by mass spectrometric analysis using a Voyager linear MALDI mass spectrometer (Applied Biosystems, Mass.) and N-terminal sequence analysis as described previously (15).

Solid-phase peptide synthesis and purification. Peptides corresponding to Ser(P)149-κ-casein-A(138-158), Ser(P)149-κ-casein-B(138-158), and κ-casein-B (138-158) were synthesized using standard solid-phase peptide synthesis protocols as described previously (15). Peptides were purified by reversed phase HPLC using a C18 column and identified by mass spectrometric analysis as described previously (15).

Liposome preparation and assay. Liposome swelling assays were carried out essentially as described by Nikaide et al. (20) using acetonate-washed crude egg phosphatidylcholine (type X-E, Sigma). The phosphatidylcholine was partitioned as described by Folch et al. (9) except that 1 mM EDTA was used instead of water and toluene replaced benzenc to remove all traces of water from the lipid. Liposomes were formed by suspending the lipid in 5 mM Tris-HCl, pH 8.0, with 15% dextran T40 (Amersham Biosciences). Dextran T40 is a large impermeant polymer of glucopyranosyl residues with an average molecular weight of 40 kDa. The pH of the solution was adjusted to either 6.5 or 7.2 by the addition of HCl.

Nonglycosylated κ-casein-A(106-169) in 5 mM Tris-HCl, pH 6.5 or 7.2, was added to the liposome suspension in the presence of an isotonic solution of sucrose (42 mM) to give final concentrations between 0 and 100 μM. The initial rate of swelling was determined by measuring the rate of change in optical density at a wavelength of 400 nm.

Divalent metal cation binding assay. CaCl2 or ZnCl2 at specified concentrations were added to the peptide solutions and the solutions were stirred at 37°C for 1 h. The pH was adjusted by dropwise addition of 1 M HCl. A spectrum was determined by Scatchard analysis. Peptides corresponding to κ-casein-A(106-169), residues 138 to 158, peptide, Ser(P)149-κ-casein-B(138-158), and nonglycosylated κ-casein-B(138-158) did not inhibit biofilm formation. The concentration was 44 μM.

RESULTS

Antibacterial activity of the genetic variants of κ-casein (106-169). To determine whether the difference in the relative antibacterial activities of the major genetic variant of kappacin [nonglycosylated κ-casein(106-169)] was due to the amino acid sequence difference in the previously identified active region of κ-casein-A(106-169), residues 138 to 158, peptides corresponding to the sequences Ser(P)149-κ-casein-B(138-158) and the nonglycosylated κ-casein-B(138-158) were synthesized and their activity was compared to that of Ser(P)149-κ-casein-A(138-158) when tested against planktonic S. mutans.

The purity of the synthetic Ser(P)149-κ-casein-B(138-158) was determined by reversed-phase HPLC, and a single peak was observed (Fig. 1A). Analysis of this peak by mass spectrometry gave a single peak with an observed m/z of 2,233.9, which corresponded to the calculated mass for the synthetic peptide, Ser(P)149-κ-casein-B(138-158) of 2235.4 (Fig. 1B). The purity of synthetic κ-casein-B(138-158) was determined in the same manner (not shown). The calculated MIC for the synthetic peptide Ser(P)149-κ-casein-B(138-158) tested against S. mutans at a growth pH of 6.28 in the microplate growth assay was 44 μM. Synthetic κ-casein-B(138-158) did not inhibit growth at the concentrations tested.

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Mechanism of action. The interaction of kappacin with membranes was studied using a liposome swelling assay. The addition of nonglycosylated \( \kappa \)-casein-A(106-169) to liposomes suspended in buffer at pH 6.5 caused a concentration-dependent increase in the rate of liposome swelling. There was a large increase in the rate of liposome swelling between 30 and 40 \( \mu \)M (Fig. 2). A maximum rate of 3.5 \( \times \) 10\(^{-3}\) absorbance units (AU) s\(^{-1}\) was obtained by the addition of 50 \( \mu \)M \( \kappa \)-casein-A(106-169). Addition of higher concentrations produced no further increase in rate. When \( \kappa \)-casein-A(106-169) was tested against liposomes at pH 7.2, it was much less active than at pH 6.5. A maximum rate of 1.66 \( \times \) 10\(^{-3}\) AU s\(^{-1}\) was obtained at the highest concentration tested, 100 \( \mu \)M.

**Interaction of kappacin with divalent metal cations.** There was no inhibition of \( S. \) mutans growth by either of the synthetic active-region peptides, Ser(P)\(^{149}\)\-casein-A(138-158) and Ser(P)\(^{149}\)\-casein-B(138-158), or the genetic variants of the purified \( \kappa \)-casein(106-169) when tested at a growth pH of 7.20 at concentrations of up to 300 \( \mu \)M (Table 1). When the two genetic variants of \( \kappa \)-casein(106-169) were tested for bacterial growth-inhibitory activity at pH 7.20 in the presence of an equimolar concentration of the antibacterial divalent cation \( \text{Zn}^{2+}\), a synergistic effect was observed (Fig. 3). Zinc ions alone had a MIC of 200 \( \mu \)M, which masked the synergistic effect of kappacin and zinc when tested at ratios above 1:1. Interest-

**TABLE 1. MICs of the two genetic variants of kappacin and the synthetic peptide Ser(P)\(^{149}\)\-casein-A(138-158) tested singly and in combination with a 1:1 ratio of zinc or calcium against \( S. \) mutans at pH 7.20**

<table>
<thead>
<tr>
<th>Peptide and metal ion</th>
<th>MIC (( \mu )M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \kappa )-Casein-A(106-169)</td>
<td>NI</td>
</tr>
<tr>
<td>( \kappa )-Casein-A(106-169) and ( \text{Zn}^{2+})</td>
<td>161</td>
</tr>
<tr>
<td>( \kappa )-Casein-A(106-169) and ( \text{Ca}^{2+})</td>
<td>248</td>
</tr>
<tr>
<td>( \kappa )-Casein-B(106-169)</td>
<td>NI</td>
</tr>
<tr>
<td>( \kappa )-Casein-B(106-169) and ( \text{Zn}^{2+})</td>
<td>200</td>
</tr>
<tr>
<td>( \kappa )-Casein-B(106-169) and ( \text{Ca}^{2+})</td>
<td>NI</td>
</tr>
<tr>
<td>Ser(P)(^{149})-casein-A(138-158)</td>
<td>NI</td>
</tr>
<tr>
<td>Ser(P)(^{149})-casein-B(138-158)</td>
<td>NI</td>
</tr>
<tr>
<td>Ser(P)(^{149})-casein-A(138-158) and ( \text{Zn}^{2+})</td>
<td>149</td>
</tr>
<tr>
<td>( \text{ZnCl}_2)</td>
<td>200</td>
</tr>
<tr>
<td>( \text{CaCl}_2)</td>
<td>NI</td>
</tr>
</tbody>
</table>

*NI, no growth inhibition at concentrations of up to 250 \( \mu \)M.

FIG. 1. A. Reversed-phase HPLC chromatogram of purified, synthetic Ser(P)\(^{149}\)\-casein-B(138-158). B. Mass spectrometric analysis of reversed-phase HPLC-purified peptide using MALDI-TOF-MS. The major peak observed with an \( m/z \) of 2,233.9 corresponded to the synthesized peptide, Ser(P)\(^{149}\)\-casein-B(138-158).

FIG. 2. Effect of \( \kappa \)-casein-A(106-169) on the rate of liposome swelling at pH 6.5. \( \kappa \)-Casein-A(106-169) in 5 mM Tris-HCl was added to a liposome suspension in the presence of an isotonic solution of sucrose, and the initial rate of swelling was determined spectrophotometrically at 400 nm.

FIG. 3. Effect of \( \kappa \)-casein-A(106-169) (\( \bigcirc \)), \( \kappa \)-casein-B(106-169) (\( \Delta \)), ZnCl\(_2\) (X), and Zn\( \times \)k-casein-B(106-169) in a 1:1 ratio (\( \bullet \)) and Zn\( \times \)k-casein-A(106-169) in a 1:1 ratio (\( \ast \)) on \( S. \) mutans growth in THYE at pH 7.2. Growth was determined by monitoring the optical density of cultures at 620 nm in a 96-well plate.
ingly, when the zinc ions were replaced with calcium ions, an antibacterial effect was still detected with \( \kappa \)-casein-A(106-169), although no effect on \( S. \) mutans growth could be detected with \( \kappa \)-casein-B(106-169) and calcium in a 1:1 ratio up to 300 M (Table 1).

The optimal ratio of calcium to \( \kappa \)-casein-A(106-169) for bioactivity was determined by testing various ratios against \( S. \) mutans in the microplate growth assay. A ratio of 2:1 was shown to be more effective than 1:1, while increasing the calcium: \( \kappa \)-casein-A(106-169) ratio to 4:1 did not increase activity (Fig. 4).

Scatchard analysis of the binding of the divalent cation \( \text{Zn}^{2+} \) to nonglycosylated \( \kappa \)-casein-A(106-169) demonstrated that there were two binding sites for zinc on this peptide (Fig. 5). Similar results were obtained for calcium binding (data not shown).

**Structural analysis.** The amide region of the \( ^1H \) NMR spectra of synthetic Ser(P)\(^{149} \) \( \kappa \)-casein-A(138-158) in a 90\% H\(_2\)O/10\% D\(_2\)O as a function of TFE concentration is shown in Fig. 6. This region of a \( ^1H \) NMR spectrum showed that the amide resonances were not well dispersed in the absence of TFE, occurring in a 0.6 ppm region extending from about 8.15 to 8.75 (Fig. 6a). Addition of 5\% TFE resulted in a change of chemical shift for some of the resonances and a general broadening of the peaks (Fig. 6b). As more TFE was added to the sample, there were further changes in amide chemical shifts and a general sharpening of the NMR resonances. However, the range of chemical shifts was still relatively small, with a range of 0.6 ppm from about 8.1 to 8.7 at TFE concentrations up to 30\% (Fig. 6c and 6d). The addition of calcium ions in the presence of 30\% TFE caused the amide resonances of the \( ^1H \) spectrum of Ser(P)\(^{149} \) \( \kappa \)-casein-A(138-158) to spread over a range of 1.25 ppm from 7.75 to 9.0 (Fig. 6e).

**Antibacterial biofilm assay.** A kappacin preparation was used to determine the effect of the peptide on biofilm bacteria. HPLC analysis of the preparation indicated that in a 10-g/liter solution, there was 4.4 g/liter of nonglycosylated \( \kappa \)-casein-A(106-169), 1.9 g/liter of nonglycosylated \( \kappa \)-casein-B(106-169), and 3.0 g/liter of glycosylated \( \kappa \)-casein(106-169). Based on a calculated average molecular mass for glycosylated \( \kappa \)-casein(106-169) of 7,500, there was a concentration of \( 1.33 \) mM of all forms of \( \kappa \)-casein(106-169) in the preparation. After inoculation into the constant-depth film fermentor, \( S. \) mutans formed a stable biofilm (Fig. 7). In a biofilm experiment with no antibacterial treatments, viability of the \( S. \) mutans biofilm was stable for over 25 days (data not shown). Addition of 5 ml of 2 mM \( \text{ZnCl}_2 \), in 2 mM Tris-HCl, pH 6.0, at day 12 after inoculation reduced the viable count of \( S. \) mutans in the biofilm by 60\%. The number of viable cells recovered rapidly from this treatment.

The addition of 20 mM \( \text{ZnCl}_2 \) to the biofilm in an identical manner resulted in a decrease in viable cell counts of 92\%. Again, a rapid recovery of viable cell counts was observed. In contrast, addition of the 10-g/liter kappacin preparation on day 16 resulted in a rapid decrease in the \( S. \) mutans viable cell.
count such that 2 h after addition there had been a 99.5% reduction in the viable cell count. Recovery of the S. mutans biofilm was slow after kappacin addition, and 3 days after the addition of the kappacin preparation, the viable cell count was still less than 13% of the pretreatment level (Fig. 7). The addition of kappacin-zinc (10 g/liter kappacin, 20 mM Zn\textsuperscript{2+}) to the biofilm caused a similar decrease in S. mutans numbers to kappacin addition, with a 96.0% decrease in viable cell numbers in 2 h. However, 3 days after kappacin-zinc treatment, the number of viable S. mutans in the biofilm had decreased to less than 0.5% of pretreatment levels (Fig. 7). Further, the viability of S. mutans in the biofilm did not recover from the kappacin-zinc treatment over the following 15 days. The inhibition of the S. mutans viability caused by kappacin and kappacin-zinc treatments in biofilms of various ages was similar and sequential treatment of the biofilms had no effect on the magnitude of the inhibition (data not shown).

**DISCUSSION**

Kappacin, the nonglycosylated, phosphorylated form of the caseinomacropeptide [CMP; κ-casein(106-169)], has been shown to have antibacterial activity against oral bacteria (15). There are two major genetic variants of kappacin in bovine milk, and in this study we have shown that the single amino acid substitution of the hydrophilic residue Asp\textsuperscript{148} in variant A for the hydrophobic Ala\textsuperscript{148} in variant B in the active region accounts for most of the difference in activity of the A and B genetic variants of κ-casein(106-169) against S. mutans at acidic pHs.

Most cationic antibacterial peptides function by altering the permeability of the bacterial membrane (3, 17). These bacteriostatic and/or bactericidal properties are usually attributed to the formation of transmembrane channels (18, 19). Synthetic liposomes are now being used to test the membranolytic activity of antibacterial peptides (1, 21, 24). At pH 6.5, κ-casein-A(106-169) increased the permeability of liposomes, indicating that kappacin has a membranolytic mode of action (Fig. 2). However, at pH 7.2, κ-casein-A(106-169) had a much lower membranolytic activity against synthetic liposomes, which may explain the lack of kappacin growth inhibitory activity at neutral pH (Table 1).

The divalent metal ion zinc reduces growth and metabolism of oral bacteria by interacting with sulfhydryl groups on bacterial enzymes, inhibiting their activity (6). The phosphoenolpyruvate:sugar phosphotransferase system and the proton-translocating ATPase are particularly sensitive to zinc inhibition, and this inhibition reduces sugar transport and acid tolerance (2, 7, 22). Zinc is largely bacteriostatic, although very high concentrations can have a bactericidal effect (12, 22). The addition of zinc to κ-casein-A(106-169) in a 1:1 ratio at neutral pH produced an antibacterial effect against S. mutans greater than that of zinc alone (Table 1, Fig. 3). The combination of zinc with κ-casein-B(106-169) in a 1:1 ratio did not produce an MIC that was lower than that of zinc alone, but at sub-MIC concentrations this combination had some growth-inhibitory activity that was not detected with zinc or κ-casein-B(106-169) alone (Fig. 3).

The combination of zinc and the synthetic peptide Ser(P)\textsuperscript{149}κ-casein-A(138-158) in a 1:1 ratio was as growth-inhibitory activity as zinc:κ-casein-A(106-169), indicating that the divalent metal ions may interact with this region of the peptide. To determine if this increased antibacterial activity at neutral pH was due solely to the peptide’s enhancing the antibacterial activity of zinc or whether it was due to a conformational change in the peptide, calcium, a divalent metal ion with no antibacterial activity was tested with kappacin peptides. The addition of calcium ions to κ-casein-A(106-169) produced an antibacterial effect against S. mutans at neutral pH, suggesting that the presence of the divalent metal cation helped to potentiate the activity of the peptide, probably by modifying its structure. The most efficacious molar ratio of divalent metal cation to κ-casein-A(106-169) was determined to be 2:1 using calcium (Fig. 4), which was consistent with the results of Scatchard analysis, indicating that each κ-casein-
A(106–169) molecule specifically bound two divalent metal cations (Fig. 5).

In general, antibacterial peptides isolated from mucosal surfaces and secretions of higher organisms contain a high percentage of basic amino acids in an amphipathic structure (8, 26). These characteristics have been proposed to facilitate interaction between the positively charged peptide and the negatively charged bacterial membrane (8). Kappacin is an unusual antibacterial peptide in that it contains a high proportion of negatively charged amino acids. Recently, other negatively charged antibacterial peptides have been discovered, including enkelytin and chromacin. The structure of the phosphorylated form of enkelytin has not been determined, although phosphorylation, which is essential for activity, has been proposed to change the conformation of the peptide through electrostatic repulsion or by divalent metal ion binding (10, 14).

The antibacterial fragment of kappacin, Ser(P)149-k-casein (138–158), contains an internal proline, Pro150, similar to enkelytin, such that the conformation of kappacin may also be a proline-kinked amphipathic helix and therefore the mechanism of action of the two peptides may be similar. One-dimensional 1H NMR analysis of α-k-casein-A(138–158) showed that in an aqueous environment it exists in a random-coil conformation (Fig. 6). This is consistent with the work of Smith et al. (28a), who found that nonglycosylated κ-casein (106–169) in the absence of calcium had a largely random, flexible structure. Addition of up to 30% TFE, a solvent that mimics the bacterial cell membrane surface, resulted in the peptide’s becoming preferentially associated with the apolar TFE environment, although the peptide remained largely in a random-coil conformation (Fig. 6). Addition of a molar excess of calcium ions in the presence of 30% TFE produced a major change in chemical shifts and a general sharpening of the NMR resonances, suggesting a conformational preference in this environment. These data suggest that κ-casein-A(138–158) will interact with apolar phases, such as the bacterial cell membrane, and that in the presence of calcium ions the peptide adopts a specific conformation in that environment (Fig. 6).

Oral streptococci grow as a biofilm in vivo, adhering to the surface of the tooth, embedded in a matrix of bacterial and host polymers (16). Streptococci within a biofilm have also been shown to be up to 500 times more resistant to certain antimicrobial agents than planktonic cells (11, 15). The exposure of negatively charged bacterial membrane. There is synergy between the known antibacterial divalent metal cation Zn2+ and kappacin, the combination of which produces a sustained antigrowth effect against an oral streptococcal biofilm. This combination may have some utility in dental caries prevention or supragingival plaque suppression, and we are currently investigating these possibilities.

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