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# Potential Applicability of Chymotrypsin-Susceptible Microcin J25 Derivatives to Food Preservation▽

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**Microcin J25 (MccJ25) is a 21-residue ribosomally synthesized lariat peptide antibiotic. MccJ25 is active against such food-borne disease-causing pathogens as *Salmonella* spp., *Shigella* spp., and *Escherichia coli*, including *E. coli* O157:H7 and non-O157 strains. MccJ25 is highly resistant to digestion by proteolytic enzymes present in the stomach and intestinal contents. MccJ25 would therefore remain active in the gastrointestinal tract, affecting normal intestinal microbiota, and this limits the potential use of MccJ25 as a food preservative. In the present paper, we describe a chymotrypsin-susceptible MccJ25 derivative with a mutation of $Gly^{12}$ to Tyr that retained almost full antibiotic activity and efficiently inhibited the growth of pathogenic *Salmonella enterica* serovar Newport and *Escherichia coli* O157:H7 in skim milk and egg yolk. However, unlike the wild-type MccJ25, the MccJ25(G12Y) variant was inactivated by digestive enzymes both in vitro and in vivo. To our knowledge, our results represent the first example of a rational modification of a microcin aimed at increasing its potential use in food preservation.**

*Escherichia coli* microcin J25 (MccJ25) is a plasmid-encoded antibiotic peptide consisting of 21 amino acid residues ($G^1$-G-A-G-$H^5$-V-P-E-Y-$F^{10}$-V-G-I-G-$T^{15}$-P-I-S-F-$Y^{20}$-G) (4, 12). Four genes (*mcjA*, *mcjB*, *mcjC*, and *mcjD*) are required for MccJ25 synthesis, export, and immunity (14, 15). The *mcjA* gene encodes a 58-amino-acid MccJ25 precursor, which is processed by the products of *mcjC* and *mcjB* (7). Once synthesized, the mature MccJ25 is excreted to the medium by McjD, an ABC-type transporter (6, 14). The tertiary structure of MccJ25 was elucidated as a lariat peptide (1, 10, 17). It contains an eight-residue ring ($G^1$ to $E^8$) and a tail ($Y^9$ to $G^{21}$) whose C-terminal end is sterically trapped within the ring. MccJ25 amino acids $F^{10}$ to $P^{16}$ form a β-hairpin structure, comprising two β-strands ($F^{10}$-$V^{11}$ and $T^{15}$-$P^{16}$) and a β-turn ($V^{11}$ to $G^{14}$).

MccJ25 is active on gram-negative bacteria related to the producer strain, and among them are several human pathogens (11, 12, 16). It was previously shown that the *E. coli* RNA polymerase (5, 18) and the bacterial respiratory chain (2, 9) are the targets for MccJ25 action. MccJ25 is active on pathogenic strains of *Salmonella* spp., *Shigella* spp. (12), and *E. coli*, including O157:H7 (11) as well as non-O157 strains (data not shown), that frequently cause outbreaks of food-borne diseases. In addition, Sable et al. (11) showed that MccJ25 was the most active microcin against 12 out of 15 diarrheagenic *E. coli* strains tested. These authors also demonstrated that MccJ25 inhibits *E. coli* O157:H7 in biological products such as milk, egg yolk, and meat extract. These findings suggest that MccJ25 could be an efficient complement to nisin for food preservation. However, the potential usefulness of MccJ25 is compromised by the fact that it is highly resistant to digestion by proteolytic enzymes of the stomach (pepsin) and intestinal

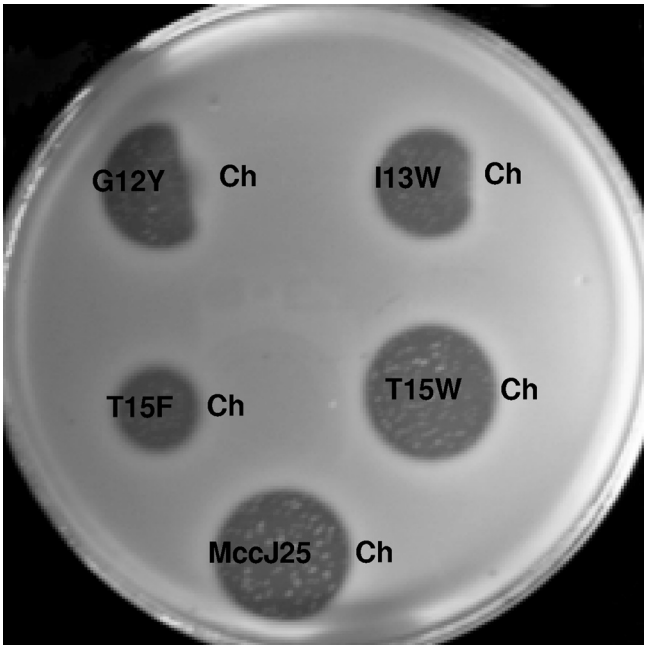


FIG. 1. Susceptibilities of the MccJ25-derivative peptides to chymotrypsin. Cell-free supernatants of strains producing native MccJ25 and MccJ25 variants with G12Y, I13W, T15W, and T15F mutations were exposed to a chymotrypsin solution (Ch) as indicated in the text.

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TABLE 1. Comparison of the sensitivities of *S*. Newport and *E. coli* O157:H7 to MccJ25 and chymotrypsin-susceptible variants

| Strain | Sensitivity to[a]: | | |
|---|---|---|---|
| | MccJ25 | MccJ25(G12Y) | MccJ25(I13W) |
| *S*. Newport | 0.22 | 0.22 | 0.44 |
| *E. coli* O157:H7 | 14.6 | 14.6 | 29.2 |

[a] Each result is given as the peptide concentration (μM) of the last dilution which produced a clear or turbid inhibitory spot.

(trypsin, chymotrypsin, and carboxypeptidases) contents. Thus, the antibiotic would most likely remain active in the intestine, and this could lead to disturbance of the normal microbiota. Therefore, for potential use of MccJ25 as a food additive, it would be desirable to render MccJ25 susceptible to at least one of these proteases. In the present work, we describe a chymotrypsin-susceptible MccJ25 derivative that remains fully active on *S*. Newport and *E. coli* O157:H7 in biological products, namely milk and egg yolk. In addition, we demonstrate that the peptide is inactivated by rat intestinal contents.

**Selection of MccJ25 derivatives susceptible to chymotrypsin digestion.** Recently, a complete collection of mutants with point substitutions in the MccJ25-encoding part of *mcjA* was constructed and characterized (8). Despite the complex three-dimensional structure and multistep processing/maturation of MccJ25, a surprisingly large proportion of precursor McjA polypeptide derivatives were found to mature, and most microcin variants retained their antibacterial function. In particular, it was found that substitutions in the β-turn and surrounding amino acids were well tolerated. Since chymotrypsin is selective for peptide bonds with aromatic or large hydrophobic side chains on the carboxyl side, we selected for further analysis the functional MccJ25 derivatives with the following mutations in this region: I13W, T15F, G12Y, and T15W. Previous work indicates that cleavage of the polypeptide backbone of MccJ25 in the β-turn region abolishes its antibacterial activity (3, 13), so we expected that if chymotrypsin-sensitive microcin derivatives were found, they would be inactivated by proteolytic digestion. To determine the sensitivity of MccJ25 derivatives to chymotrypsin, 10 μl of cultured medium of cultures of cells producing wild-type or substitution-containing microcins was spotted onto M9 plates and 10 μl of 0.5 mg/ml chymotrypsin solution was placed 1 cm away from the cultured medium drops. After the drops had dried, the plates were overlaid with 4 ml of soft agar inoculated with $10^7$ cells of a clinical isolate of *Salmonella enterica* serovar Newport (16). After overnight incubation at 37°C, the growth inhibition zones around the drops of MccJ25-containing media were recorded (Fig. 1). Circular growth inhibition zones were observed around drops containing wild-type MccJ25 as well as drops containing MccJ25 (T15F) or MccJ25(T15W). In contrast, growth inhibition zones around drops containing MccJ25(G12Y) and MccJ25(I13W)

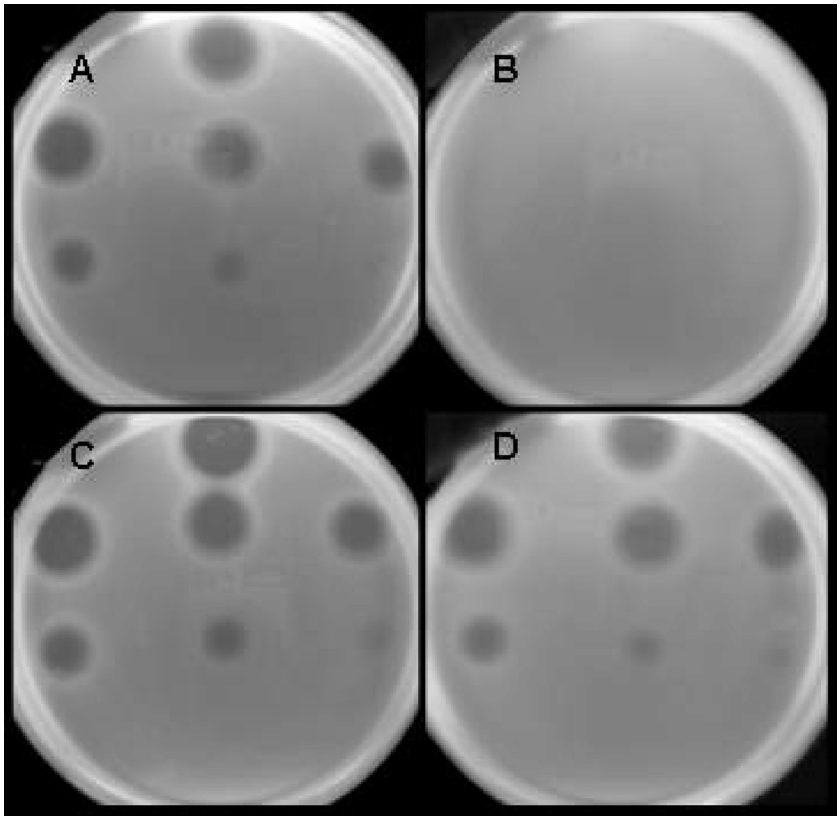


FIG. 2. MccJ25(G12Y) cleavage by digestive enzymes. MccJ25(G12Y) and MccJ25 were incubated with either intestinal contents (IC) (panels B and D, respectively) or boiled IC (panels A and C, respectively) at 37°C for 24 h. All samples were then assayed for residual activity by serial twofold dilution.

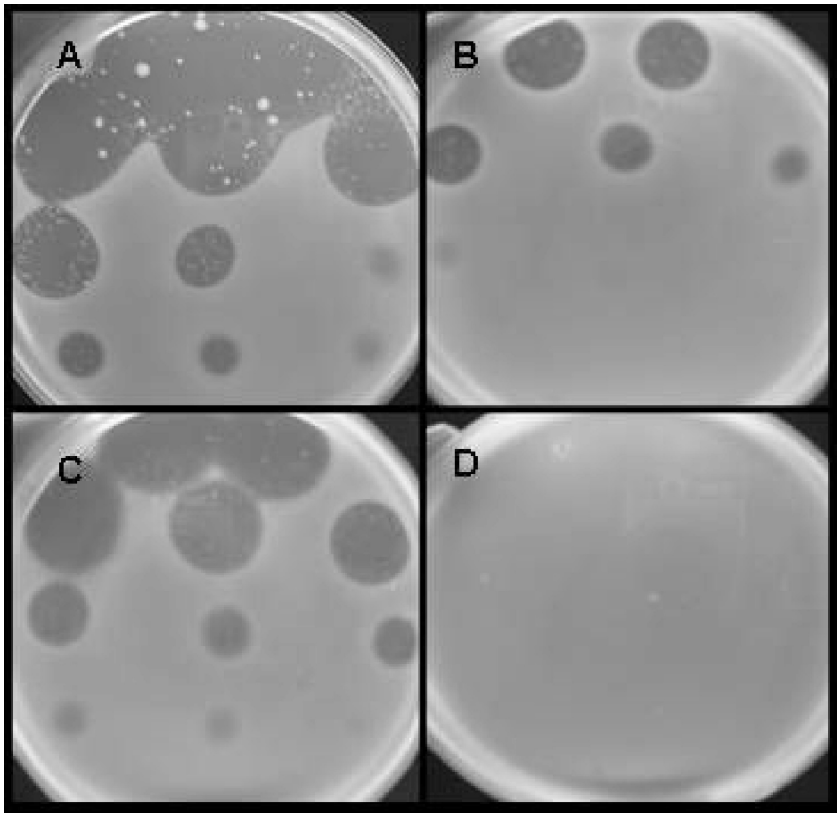


FIG. 3. In vivo inactivation of MccJ25(G12Y) by digestive enzymes. The antimicrobial activities of the native MccJ25 and MccJ25(G12Y) were determined before (panels A and C, respectively) and after (panels B and D, respectively) passage through the intestinal lumen. The experiment was done three times, with similar results. The results shown are from one representative experiment.

peptides contained a crescent-shaped distortion at the side closest to the chymotrypsin drop. We interpret the shapes of growth inhibition zones around these derivatives as suggesting that chymotrypsin that diffuses out of its spot digests and detoxifies them, decreasing the efficiency of indicator strain growth inhibition. The other two MccJ25 derivatives were apparently not affected by chymotrypsin.

**Antimicrobial activity of MccJ25 derivatives.** Sensitivity to purified microcins was tested by a spot-on-lawn assay as follows. To avoid aggregation (4), concentrated methanolic solutions of wild-type or variant microcins (2 mM) were prepared and serial doubling dilutions in double-distilled water containing 0.1% Tween 80 were made. Aliquots (10 μl) of each dilution were spotted onto M9 medium plates supplemented with 0.2% tryptone. After the drops had dried, the plates were overlaid with 4 ml of soft agar (0.6%) containing the indicator strain. The plates were incubated for 12 h at 37°C before examination. The concentration (μM) of the last dilution giving a clear or turbid-growth inhibition zone was defined as the MIC.

Sensitivities of the *S.* Newport and *E. coli* O157:H7 (clinical isolate) strains to native MccJ25, MccJ25(G12Y), and MccJ25 (I13W) were compared and are reported in Table 1. In agreement with previous data, the MccJ25(G12Y) peptide displayed antibiotic activity similar to that of the native MccJ25. This microcin derivative was also most susceptible to chymotrypsin digestion (Fig. 1). For this reason, MccJ25(G12Y) was selected for the rest of the experiments.

**MccJ25(G12Y) cleavage by digestive enzymes.** Experiments described hereafter were performed on female albino rats of a locally bred strain (originally Sprague-Dawley stock). Per the guidelines of the American Physiological Society and as approved by the animal board of the Consejo Nacional de Investigaciones Científicas y Técnicas, only rats weighing 220 to 250 g were used in this study.

To obtain intestinal contents, the rats were anesthetized with ketamine (0.3 mg/kg of body weight) and xylazine (Rompun) (16 mg/kg of body weight). A midline abdominal incision was made, and the end of the small intestine was identified and ligated to a polyethylene catheter through which the intestinal efflux was collected. The sensitivity of MccJ25(G12Y) to digestive enzymes was assayed by incubating 5 μl of the peptide solution (1 mg/ml) in 20 mM phosphate buffer, pH 7.0, with 45 μl of the intestinal contents at 37°C for 24 h. As a control, an aliquot of intestinal contents was heated in boiling water for 10 min (to inactivate the digestive enzymes) and also incubated with MccJ25(G12Y). All samples were assayed for residual antimicrobial activity as described in "Antimicrobial activity of MccJ25 derivatives" (above). MccJ25(G12Y) lost its inhibitory activity after incubation with intestinal contents (Fig. 2B), whereas native MccJ25 remained fully active (Fig. 2D). No effect was observed when both peptides were incubated with

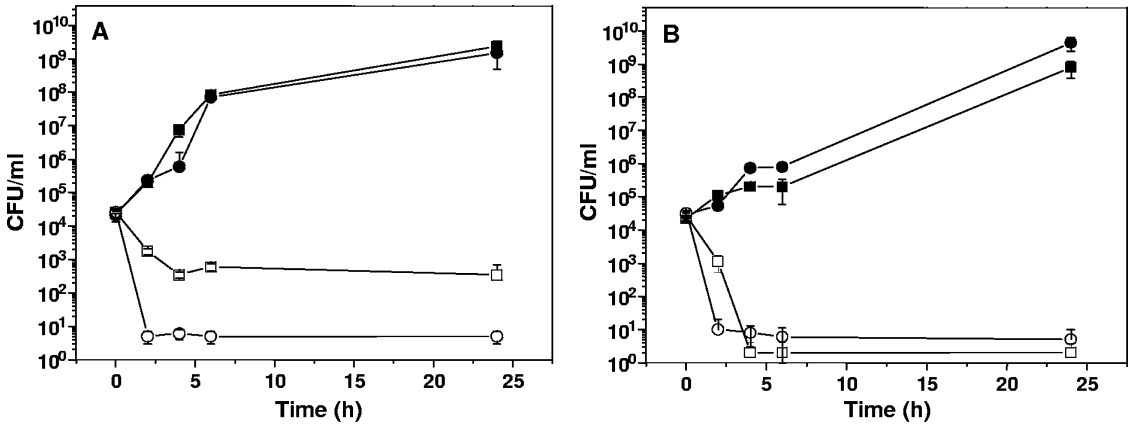


FIG. 4. Antimicrobial activity in food products. Diluted egg yolk (panel A) and milk (panel B) were inoculated with either *S*. Newport (circles) or *E. coli* O157:H7 (squares) in the presence (open symbols) or absence (filled symbols) of MccJ25(G12Y) and then incubated at 37°C. CFU from serial dilutions of both food samples were determined at several times. Error bars represent standard deviations from five experiments.

boiled intestinal contents (Fig. 2A and C). The most likely explanation for these results is that loss of activity of MccJ25 (G12Y) was caused by a digestive enzyme.

**In vivo inactivation of MccJ25(G12Y) by digestive enzymes.** For the following experiments, rats were housed in three group cages (three animals per cage). Two groups of animals were orally given 0.2 ml of a solution of MccJ25 or MccJ25(G12Y) (both at 0.5 mg/ml), and a third group was inoculated with the same volume of sterile water as a control. Efflux from the intestine was recovered as described above. The collection of intestinal contents began immediately after administration of microcin and continued for 60 min. Intestinal samples were then subjected to antimicrobial activity detection. The original antimicrobial activities of the two peptide solutions used in the assay were also determined.

Figure 3A and B show that considerable native MccJ25 activity was recovered after MccJ25 passage through the intestinal lumen. The observed decrease in activity could, at least in part, be explained by the dilution with intestinal fluid. On the contrary, no MccJ25(G12Y) activity appeared after passage of that protein through the intestine (Fig. 3C and D). In this case, the possibility of a dilution effect could be discarded, since when the experiment was repeated with a 10-fold-concentrated sample, no activity was detected (data not shown). Samples collected from the control group (inoculated with water) did not show any antimicrobial activity.

**Antimicrobial activity in food products.** To evaluate the antimicrobial activity of MccJ25(G12Y) in biological products, 1 ml of sterile skim milk (10%, wt/vol) and diluted egg yolk (1:5 mixture of sterile egg yolk and sterile distilled water) were inoculated with about $10^4$ cells/ml of either *S*. Newport or *E. coli* O157:H7, in the presence or absence of MccJ25(G12Y) (1 μg/ml), and incubated at 37°C. At various times, aliquots of both food samples were withdrawn and viable bacterial counts were determined. The MccJ25(G12Y) peptide efficiently inhibited the growth of both pathogenic strains in egg yolk and milk (Fig. 4A and B, respectively). This result suggests that the antibiotic can exert its activity in food samples and demonstrates its stability in biological products.

The thermostability of MccJ25(G12Y) was also assessed. To this end, a sample of peptide in phosphate buffer (1 mg/ml) was autoclaved (121°C for 20 min) and then cooled and tested for antimicrobial activity. The microcin derivative showed high heat resistance similar to that of wild-type microcin (data not shown). This property may allow the use of this peptide in processes requiring high temperature.

As far as we know, this study represents the first example of a rational modification of any microcin with the aim of a potential use in food preservation. This strategy could open the way to attempting similar modifications in other peptide antibiotics which are naturally resistant to digestive enzymes.

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