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Research Paper

Stability of Antimicrobial Decapeptide (KSL) and Its Analogues for Delivery in the Oral Cavity

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Purpose. To investigate the stability of KSL, an antimicrobial decapeptide, and its analogues, in human saliva and simulated gastric fluid for delivery in the oral cavity.
Materials and Methods. The degradation products of KSL in human saliva and simulated gastric fluid were separated by reversed-phase HPLC and their structures were identified by electrospray ionization-mass spectrometry. Analogues of KSL were synthesized by solid-phase synthesis procedure. Their enzymatic stabilities and antimicrobial activities were studied.
Results. KSL was degraded by the peptide bond cleavages at Lys^6–Val^7 in the human saliva and Phe^5–Lys^6 in simulated gastric fluids. Three analogues of KSL were synthesized; the Lys^6 residue was either methylated (KSL-M), or replaced with Trp (KSL-W), or the d-form of Lys (KSL-D). The KSL analogues were much more stable than the native KSL, with the rank order of stability being KSL-D > KSL-W > KSL-M > KSL in human saliva. However, in simulated gastric fluid, while KSL-D was still stable, KSL-W was significantly degraded. In addition, KSL-D significantly lost the antimicrobial activity, whereas KSL-W completely preserved the activity against several oral bacteria. In a chewing gum formulation, KSL-W showed a more sustained release profile as compared with the native KSL.
Conclusion. This study suggests that KSL-W could be used as an antiplaque agent in a chewing gum formulation.

KEY WORDS: antimicrobial peptide; antiplaque agent; chewing gum; peptide analogue; stability.

INTRODUCTION

Recently, antimicrobial peptides have received attention as alternative classes of antimicrobials because of their selectivity for prokaryotes and promise of minimizing microbial resistance (1). A novel decapeptide, Lys-Lys-Val-Val-Phe-Lys-Val-Lys-Phe-Lys–NH_2 (KSL), is an antimicrobial peptide developed by using synthetic combinatorial library technology and shows a broad range of antibacterial activity (2). Recently, this antimicrobial peptide has been shown to effectively prevent the development of oral biofilm formed by isolated human salivary bacteria *in vitro* as well as inhibit the growth of oral bacterial pathogens associating with caries development and plaque formation (3,4).

One of the approaches for the prevention and treatment of plaque-related oral diseases is the use of antimicrobial agents, such as chlorhexidine, triclosan, metal ions, quaternary ammonium compounds and essential oils (5,6). The use of a chewing gum as a vehicle for antiplaque agents is appealing from a practical and compliance standpoint because of a longer residence time in the mouth than rinses and toothpastes (7). Promising results have been obtained by using this route for the local delivery of chlorhexidine to the oral cavity (8–10). However, chlorhexidine is known to have several disadvantages which include its bitter taste, impairment of taste perception, reversible staining of teeth and tongue, and interaction with surfactants in the toothpastes (11). Recently, KSL was reported to be a promising antimicrobial agent in a chewing gum formulation (12). The chewing gum formulation showed a desirable sustained release profile over 20 to 40 min of chewing time.

Stability of the peptide is one of the important factors for successful formulation development. Peptides can undergo a variety of physical, chemical and biological degradation reactions (13–15). In the previous study, KSL was found to be stable in acidic buffer and artificial saliva. The purpose of this study was to investigate the stability of KSL in oral environments, i.e., in saliva. In order to determine the stability of the peptide released from the formulation to the oral cavity, the degradation kinetics and mechanism of KSL in human saliva were studied. Based on the degradation mechanism in saliva,

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KSL analogues were synthesized and their stabilities were investigated. In addition to the stability in human saliva, the degradability of peptide in gastrointestinal environments is also a concern, specifically the impact of the antimicrobial peptide on the microbial balance of the intestinal flora. Therefore, the peptide stability in simulated gastric fluid was studied. In addition, the effect of the KSL modification on the antimicrobial activity was investigated. The most suitable KSL analogue was selected on the basis of both salivary stability and degradability in gastric fluid as well as the antimicrobial activity. Finally, the *in vitro* release properties of the chewing gum containing the stable KSL analogue were investigated.

MATERIALS AND METHODS

Materials

KSL (MW = 1,250 Da) and its analogues were synthesized by standard solid-phase procedures using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on an automatic peptide synthesizer (Model 90, Advanced ChemTech, Louisville, KY) as described previously (3). Acetonitrile (HPLC grade) and dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Fair Lawn, NJ). Trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL). Pepsin and alpha-cyano-4-hydroxycinnamic acid (α-CHCA) were obtained from Sigma (St. Louis, MO). All other chemicals were of analytical grade and used as obtained commercially.

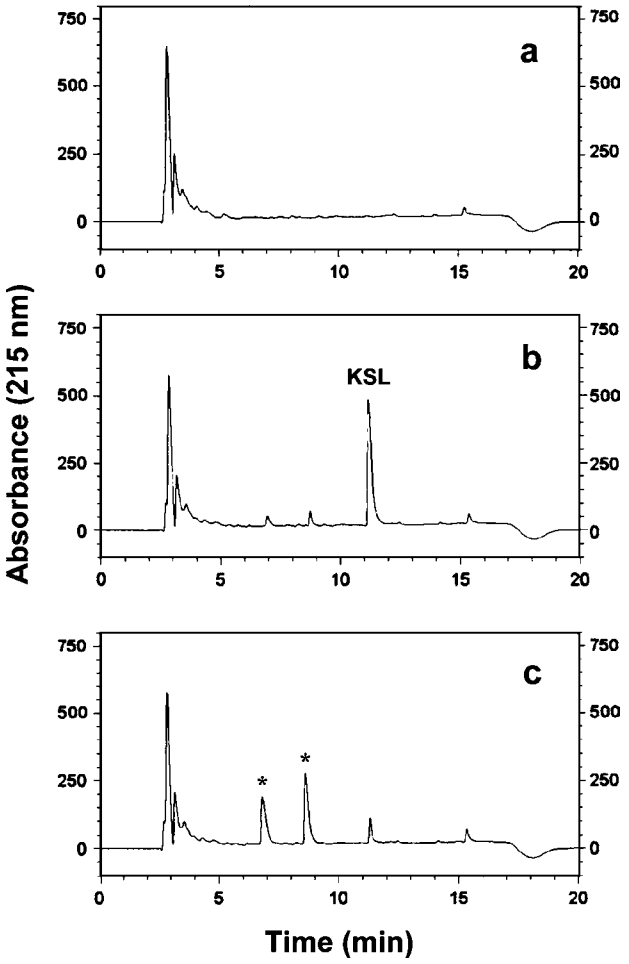

Fig. 1. HPLC chromatograms of blank human saliva (**a**), human saliva spiked with KSL (**b**), and KSL incubated in human saliva (0.5 mg/ml) at 37°C for 5 min (**c**) (*asterisk* indicates degradation product of KSL, $R_t = 6.8$ and 8.6 min).

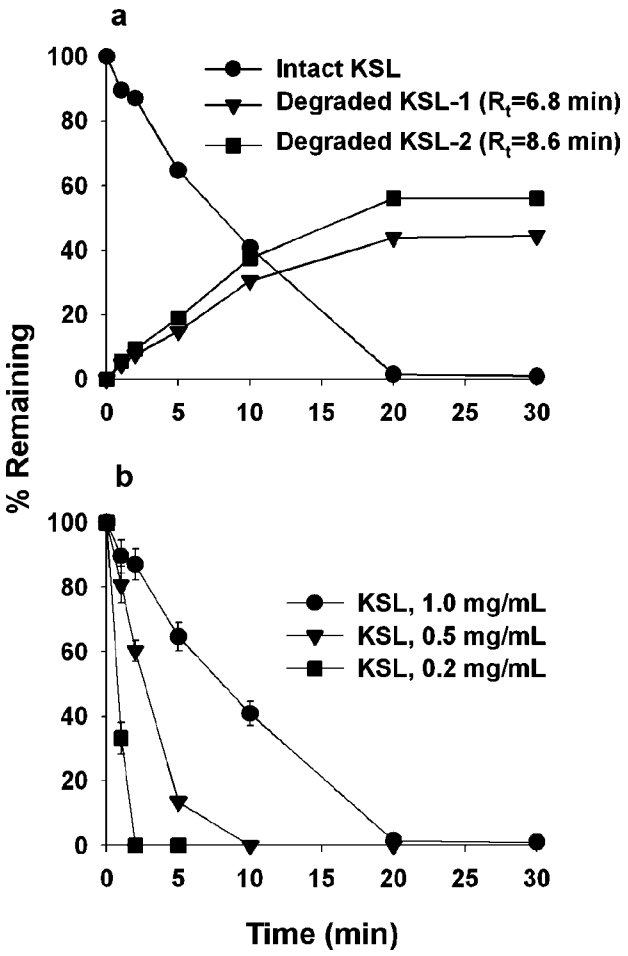

Fig. 2. Degradation of KSL in human saliva at 37°C. (**a**) Degradation of KSL (1.0 mg/ml in human saliva) and the production of the degradation compounds (peaks of $R_t = 6.8$ and 8.6 min in HPLC chromatograms of Fig. 1). (**b**) Effect of KSL amount per 1 ml of human saliva on the peptide degradation.

HPLC Analysis

KSL and its analogues were analyzed by reversed-phase HPLC using a Prosphere C-18 analytical column (4.6 × 250 mm, Alltech, Deerfield, IL) with a Prosphere C-18 guard column (4.6 × 7.5 mm, Alltech, Deerfield, IL) with a slight modification of a HPLC method described previously (12). A gradient elution was performed with mobile phase A (0.1% TFA in water) and mobile phase B (0.1% TFA in acetonitrile). KSL or its analogue was eluted with a linear gradient from 88:12 to 65:35 (mobile phase A:B) for 10 min at a flow rate of 1.2 ml/min.

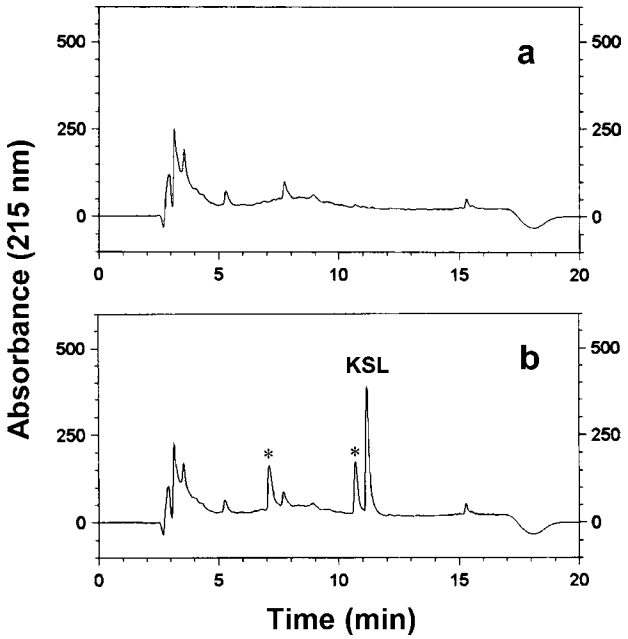

Fig. 3. HPLC chromatograms of blank simulated gastric fluid (**a**) and KSL incubated in simulated gastric fluid (0.5 mg/ml) at 37°C for 5 min (**b**) (*asterisk* indicates degradation product from KSL, $R_t = 7.1$ and 10.7 min).

Total run time was 20 min and the injection volume was 40 μl. Chromatograms were recorded by UV detection at 215 nm.

Stability Study in Human Saliva

Whole human saliva was collected from three healthy male donors in the morning prior to breakfast in accordance with institutional approved procedures. After collection, the saliva was immediately centrifuged at 12,000 rpm for 20 min and the supernatant was filtered through a 0.45 μm membrane filter. KSL or its analogue solutions were prepared using a stock peptide solution of 10 mg/ml in deionized water. Each 100 μl of KSL or its analogue solution was added to 900 μl of whole saliva to provide peptide concentrations of 0.2, 0.5, and 1 mg/ml. The peptide in saliva was incubated at 37°C for predetermined time. The samples were filtered and analyzed by reversed-phase HPLC. KSL or its analogue dissolved in artificial saliva at 37°C was used as a control. The ingredients of the artificial saliva were as follows: sodium chloride, 0.844 g; potassium chloride, 1.200 g; calcium chloride dihydrate, 0.193 g; magnesium chloride hexahydrate, 0.111 g; potassium phosphate dibasic, 0.342 g; water to make to 1,000 ml. The pH was adjusted with hydrochloric acid solution to pH 5.7 ± 0.1 (16).

Stability Study in Simulated Gastric Fluid

Simulated gastric fluid was prepared according to the procedure of the USP, National Formulary: 2.0 g NaCl and 3.2 g pepsin were dissolved in 7.0 ml HCl and distilled water was added to make 1000 ml (pH 1.2). One hundred μl of KSL solution (5 mg/ml in water) or its analogues was added to 900 μl of simulated gastric fluid (final concentration of 0.5 mg/ml). The solution was incubated at 37°C for predetermined time. The samples were filtered and analyzed by reversed-phase HPLC.

Mass Spectrometry

The degradation products of KSL in whole saliva and simulated gastric fluid were collected from reversed-phase HPLC and evaporated by Speed-Vac (Eppendorf, Hamburg, Germany) to analyze with electrospray ionization–mass spectrometry (ESI-MS). Samples were analyzed on a ThermoFinnigan LCQ mass spectrometer (ThermoFinnigan, CA). Samples were introduced by direct infusion using a syringe pump at a flow of 3 μl/min. The molecular masses of the KSL analogues were obtained by matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS, Bruker Daltonics, MA) as described previously (17). The α-CHCA in 50% acetonitrile in water containing 0.1% TFA was used as matrix. Data for 2 ns pulses of the 337 nm nitrogen laser were averaged for each spectrum in a linear mode, and a positive ion TOF detection was performed using an accelerating voltage of 20 kV.

Antimicrobial Activity Assay

Bacterial susceptibility to KSL and its analogs was determined as described previously (3). Mueller–Hinton broth (Becton Dickinson, Sparks, MD) was used as the main assay medium for most of the test organisms. Lactobacilli MRS broth at 10% (Becton Dickinson) was used as the assay medium for *Lactobacillus acidophilus*.

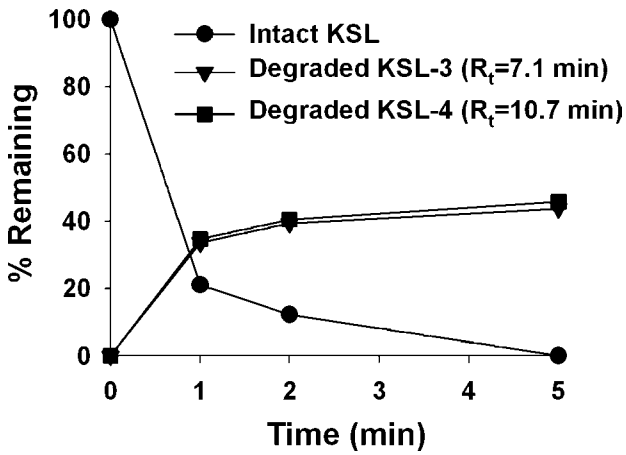

Fig. 4. Degradation of KSL (0.5 mg/ml) and the production of the degradation compounds (peaks of $R_t = 7.1$ and 10.7 min in HPLC chromatograms of Fig. 3) in simulated gastric fluid at 37°C.

Table I. Mass Spectrometric Identification of Degraded KSL in Human Saliva and Simulated Gastric Fluid using ESI-MS

Sample	Test Medium	R_t in HPLC (min)	Mass (m/z)	Peptide Sequence
KSL	–	11.3	1,250	KKVVFKVKFK (1–10)
Degraded KSL-1	Human saliva	6.8	520	VKFK (7–10)
Degraded KSL-2	Human saliva	8.6	749	KKVVFK (1–6)
Degraded KSL-3	Simulated gastric fluid	7.1	648	KVKFK (6–10)
Degraded KSL-4	Simulated gastric fluid	10.7	620	KKVVF (1–5)

Chewing Gum Preparation

The chewing gum formulations were prepared following a procedure described previously (12). The gum base was heated at a temperature between 50 and 60°C for melting. When the gum base exhibited proper fluid consistency, the peptide was added as a fine powder along with the other components. The temperature was kept constant while mixing the components with the gum base in a mortar. After mixing, the homogenous chewing gum mixture was extruded, cut into squares of approximate shape and size and hardened at room temperature overnight. The composition of a piece of the gum was as follows: 550 mg of gum base, 420 mg of sorbitol, 10 mg of mannitol, 10 mg of saccharin, and 10 mg of KSL or its analogue (total weight: approximately 1 g).

In Vitro Release Study

In vitro release study of KSL or its analogue from chewing gums was carried out using an *in vitro* chewing release apparatus consisting of two modules (AB FIA, Lund, Sweden) (18). Each module had a thermostatted glass cell in which two vertically oriented pistons holding an upper and lower chewing plate were mounted. The cells were filled with 40 ml of artificial saliva and the chewing gum was loaded onto the lower chewing surface. The chewing procedure consisted of up and down strokes of the lower surface in combination with a twisting movement of the upper surface; this action provides mastication of the chewing gum and agitation of the test medium. The temperature of the test medium was controlled at 37°C and the chew frequency was 50 ± 2 strokes per min. At predetermined time intervals, 400 μl of supernatant were removed. The artificial saliva in the cells was replaced with fresh artificial saliva kept at 37°C after each sampling. The released amount of KSL or its analogue was determined by reversed-phase HPLC.

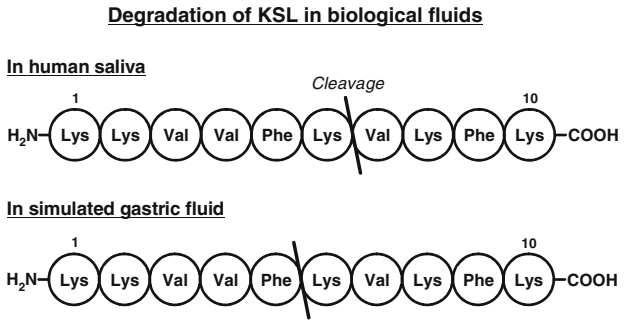

Fig. 5. Degradation sites of KSL in human saliva and simulated gastric fluid.

RESULTS AND DISCUSSION

Degradation of KSL in Human Saliva

Figure 1 shows reversed-phase HPLC chromatograms of human saliva, human saliva spiked with KSL, and KSL incubated in saliva. In a HPLC analysis of KSL incubated in saliva at 37°C for 5 min, two degradation products (degraded KSL-1 and 2) were found at retention time (R_t) of 6.8 and 8.6 min. The degradation products were well resolved from intact KSL ($R_t = 11.3$ min).

Figure 2 shows the degradation kinetics of KSL in human saliva at 37°C. As the intact KSL decreases, the degraded KSL-1 and 2, were proportionally produced. At each point, total peak area of intact KSL and two degradation compounds almost reached to initial amount of the incubated KSL (Fig. 2a). When KSL was incubated in artificial saliva containing no enzymes at the same conditions, the degradation products were not found for over 24 h (data not shown). These results suggest that the degradation of KSL is attributed to the presence of proteolytic enzymes in human saliva.

The degradation of KSL in saliva was also dependent on KSL amount. While 40% of KSL at 1 mg/ml in saliva was still intact at the end of 10 min, the KSL at 0.5 mg/ml was completely degraded in 10 min, and at 0.2 mg/ml, the peptide was completely degraded in 2 min (Fig. 2b). Half-lives ($t_{1/2}$) of the KSL at 0.2, 0.5 and 1 mg/ml were roughly estimated as 1, 4 and 11 min, respectively.

Table II. Mass Spectrometric Identification of KSL Analogues by Using MALDI-TOF MS

Sample	Modification at Lys[6]	Mass (m/z)	Peptide Sequence
KSL	–	1,250	KKVVFKVKFK
KSL-D	Replacement with d-Lys	1,250	KKVVF*D*–*K*VKFK
KSL-M	Methylation	1,264	KKVVF*K*–*CH*$_3$VKFK
KSL-W	Replacement with Trp	1,307	KKVVF*W*VKFK

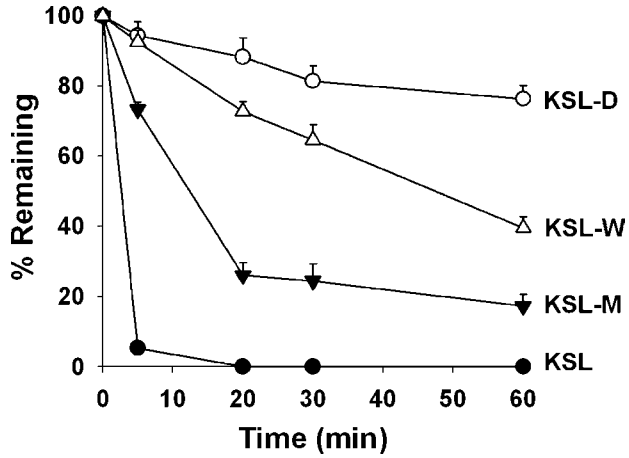

Fig. 6. Degradation of KSL and its analogues (0.5 mg/ml) in human saliva at 37°C.

Degradation of KSL in Simulated Gastric Fluid

In a HPLC analysis of KSL incubated in simulated gastric fluid at 37°C, two degradation products (degraded KSL-3 and 4) were also found at R_t of 7.1 and 10.7 min (Fig. 3). The difference in retention times from those observed in human saliva suggests a different degradation mechanism. The degradation products in simulated gastric fluid were also well resolved from intact KSL ($R_t = 11.3$ min). In a simulated gastric fluid, the KSL was completely degraded in 5 min and $t_{1/2}$ was 0.9 min (Fig. 4).

Identification of the Degradation Products of KSL

To identify the structure of the degradation products of KSL in human saliva and simulated gastric fluid, the fractions were collected from reversed-phase HPLC and analyzed by ESI-MS (Table I). The molecular masses of two degradation products (degraded KSL-1 and 2) in human saliva were *m/z* 520 and 749, which correspond to the fragments Val^7–Lys^{10} and Lys^1–Lys^6, respectively (Fig. 5). This provides strong evidence that the cleavage site is the peptide bond between Lys^6 and Val^7, which is indicative of trypsin-like enzyme activity. The degradation products (degraded KSL-3 and 4) from the KSL in simulated gastric fluid showed *m/z* 648 and 620, which means that the cleavage site is the peptide bond between Phe^5 and Lys^6. This cleavage might be caused by pepsin contained in gastric fluid (Fig. 5). In contrast to intact KSL, none of these degraded products showed any bactericidal activity against *Streptococcus mutans*, ATCC25175, the cariogenic pathogen (data not shown).

Design of Stable KSL Analogues

In order to prepare the KSL analogues that were stable in human saliva and exhibited little impact on the microbial flora of the gastrointestinal tract, the Lys^6 position in KSL, which is the degradation site in saliva, was modified or replaced with another amino acid. To be easily degraded in gastrointestinal tract, the degradation site in gastric fluid was not modified. Three KSL analogues were prepared as follows: KSL-D, the lysine at the #6 position of KSL was replaced with a d-Lys; KSL-M, the lysine at the #6 position of KSL was methylated; KSL-W, the lysine at the #6 position of KSL was replaced with tryptophan. Their structures were identified by measuring molecular mass using MALDI-TOF MS (Table II). In a KSL-D, as the l form of Lys^6 is just replaced with D form of the same amino acid, the molecular mass was the same as the intact KSL. KSL-M and KSL-W showed increased molecular mass by 14 and 57 Da, respectively.

Stability Study of KSL Analogues

All the KSL analogues were more stable than the native KSL in human saliva. Through 60 min, the order of the remaining amount of the intact peptide was KSL-D > KSL-W > KSL-M > KSL (Fig. 6). While native KSL was almost degraded with a remaining amount of 5% in 5 min, KSL-D, KSL-M and KSL-W remained 94.4, 73.2 and 92.5%, respectively. Among KSL analogues, KSL-D was the most stable through 60 min (the remaining amount of 76% during 60 min), whereas KSL-M was readily degraded and only 25% remained after 20 min. KSL-W also showed substantially

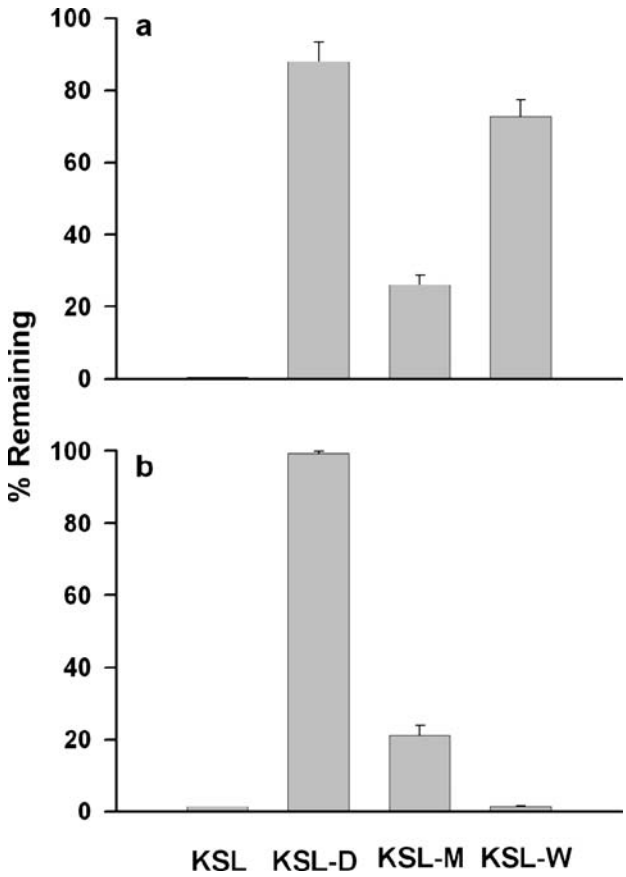

Fig. 7. Stability of KSL analogues. (**a**) The remaining amount of intact KSL and its analogues (0.5 mg/ml) after incubation in human saliva for 20 min at 37°C. (**b**) The remaining amount of intact KSL and its analogues (0.5 mg/ml) after incubation in simulated gastric fluid for 5 min at 37°C.

Table III. *In Vitro* Susceptibility of Oral Bacteria to KSL and Its Analogues

Bacterial Strains[a]	KSL	KSL-M	KSL-D	KSL-W
Streptococcus mutans ATCC 25175	12.5[b]	12.5	>200	12.5
Actinomyces naeslundii V-J1	6.25	6.25	100	3.125
Streptococcus sobrinus ATCC 33478	50	50	>200	25
Streptococcus salivarius ATCC 9222	12.5	>200	ND[c]	6.25
Lactobacillus acidophilus ATCC 29602	1.56	3.125	ND	25

[a] The number of cells used in these assays was 4×10^6 /ml.
[b] Values represent MIC expressed in micrograms per milliliter. MIC is defined as the lowest concentration of the peptide that prevents visible growth as measured by UV-Vis spectrophotometry at 600 nm.
[c] *ND* Not determined

improved stability with the remaining amount of 72 and 40% through 20 and 60 min, respectively. Therefore, the replacement of the Lys^6 position in KSL with D-Lys or tryptophan improved significantly the stability in human saliva, but methylation of the Lys^6 residue did not improve significantly the salivary stability.

Stability of the KSL analogues in simulated gastric fluid was different from that in human saliva. In simulated gastric fluid, the order of the remaining amount of the intact peptide was KSL-D > KSL-M > KSL ~ KSL-W (Fig. 7b). While the KSL-D was also stable, the KSL-M and KSL-W were significantly degraded in the gastric fluid. Figure 7 shows the comparison of the remaining amount of KSL and its analogues in human saliva for 20 min and in simulated gastric fluid for 5 min. While KSL-D was stable in both saliva and gastric fluid, KSL-W showed significantly different stability in each medium, that is, it was stable in saliva but readily degraded in gastric fluid. Consequently, KSL-W was determined to be the most suitable analogue in terms of stability in oral cavity and safety in gastrointestinal tract.

Antimicrobial Activity of KSL and Its Analogues

The minimum inhibitory concentrations (MIC) of KSL and its analogues for a selected group of oral bacteria were determined (Table III). With the exception of KSL-D, KSL-M and KSL-W exhibited similar bactericidal activity as KSL. They were effective in inhibiting growth of the cariogenic bacteria *S. mutans*, *Streptococcus sobrinus* and *L. acidophilus*. Growth of *Actinomyces naeslundii*, a primary colonizer involved in the initiation of plaque formation, was also inhibited. In addition to the stability result, this result provides that KSL-W is the most preferable KSL analogue because of the preservation of biological activity as well as the improved stability.

Release Study of KSL Analogue from Chewing Gum

In order to investigate the property of KSL-W in formulation, the chewing gum containing KSL-W was prepared and the *in vitro* release property was compared with that of native KSL chewing gum (Fig. 8). The release rate of KSL-W was slower than that of native KSL from chewing gum. Totally, almost 80% of peptide from native KSL chewing gum was released in 60 min, whereas the released amount from KSL-W chewing gum was about 45%. The slower release exhibited by KSL-W could be attributed to its higher affinity to the gum base. Incorporation of additives, which can accelerate the peptide release from chewing gum, would be necessary for an optimal release profile.

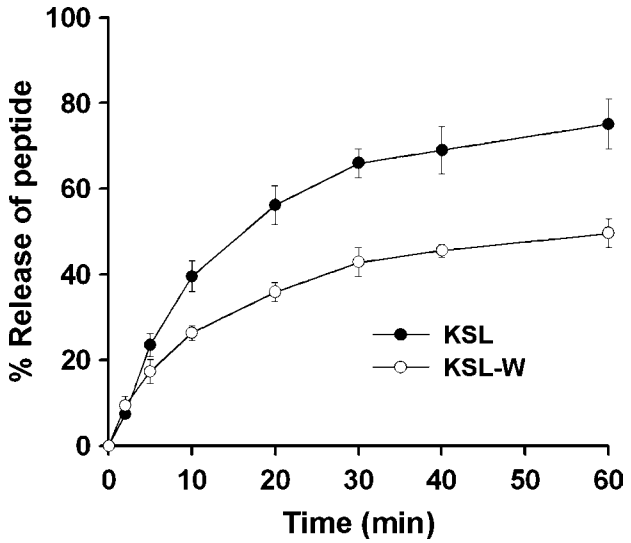

Fig. 8. *In vitro* release of KSL and KSL-W from chewing gum formulations in artificial saliva at 37°C, which were performed using a chewing apparatus.

CONCLUSIONS

KSL was found to be degraded by peptide bond cleavages at Lys^6–Val^7 in human saliva and Phe^5–Lys^6 in simulated gastric fluids. Among the analogues prepared for improving the stability, KSL-W proved to be the best in terms of stability in the oral cavity and antimicrobial activity. KSL-W showed slower release rate from chewing gum formulation compared with native KSL gum formulation. This study suggests that the KSL-W will be released from the chewing gum in a sustained manner and bioactively retained in the oral cavity to inhibit the formation of dental plaque.

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