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Statherin SV2 and Its Analogue. Synthesis and Evaluation of Antimicrobial Activity*

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Statherins are natural peptides derived from human saliva. They may take part in transport of calcium and phosphate and most probably are partly responsible for protection and recalcification of tooth enamel. The solid phase synthesis of statherin SV2 and its dephosphorylated analogue, their purification by solid phase extraction (SPE), as well as the evaluation of their antibacterial activity are presented.

Key words: statherin, solid phase peptide synthesis, solid phase extraction, antimicrobial activity

Statherin is a multifunction low-molecular-weight protein produced by salivary glands. Its molecule consists of 43 amino acid residues [1]:

^{1}DS(OH_2PO_3)S(OH_2PO_3)EEKFLRRIGRFGYGYGPYQPVPEQPLYPQPYQPQYQQYTF^{43}

It has high affinity to hydroxyapatite and can substantially inhibit the primary and secondary precipitation of calcium phosphate salts in both gland and mixed saliva [2,3]. In our previous research it was established that statherin and its short fragments reveal antibacterial activity [4]. In human saliva statherin is accompanied by statherin SV2 [5]. Its molecule is smaller and consists of 33 amino acid residues:

^{1}DS(OH_2PO_3)S(OH_2PO_3)EEYGYGPYQPVPEQPLYPQPYQPQYQQYTF^{33}

In this study we present the solid phase synthesis of statherin SV2 and its dephosphorylated analogue, as well as the evaluation of their antibacterial activity.

EXPERIMENTAL

Chemical part

Starting materials: N^{α}-9-Fluorenylmethyloxycarbonyl amino acids and N^{α}-Fmoc-O-benzyl-L-phosphoserine [Fmoc-Ser(PO(OBzl)OH)-OH] were obtained from Novabiochem (Switzerland), tri-

*Abbreviations: The symbols of amino acids, peptides and their derivatives are in accordance with the 1983 Recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature [*Eur. J. Biochem.*, **138**, 9 (1984)] and European Peptide Society [*J. Peptide Sci.*, **5**, 465 (1999)].

fluoroacetic acid (TFA), 1,3-diisopropylcarbodiimide (DIC), piperidine, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), N-hydroxybenzotriazole (HOBt), Triton, triisopropylsilane (TIS), dimethylformamide (DMF), N-methyl-2-pyrrolidone (NMP), methylene chloride (DCM), and acetonitrile were purchased from Fluka AG (Switzerland). All solvents and reagents used for solid-phase synthesis were of analytical grade. TentaGel resin; AC-Phe-Fmoc (0.22 mmol Fmoc-Phe/g of resin) was obtained from Rapp Polymere (Germany).

Analytical procedure: HPLC analyses of the peptides were performed using a Beckman Gold System chromatograph with a Knauer C8 column (5 μm particle size, 4.6×250 mm plus 4.6×45 mm precolumn). Solvent systems: (A) 0.1% trifluoroacetic acid in water, (B) acetonitrile in the presence of TFA 0.1%, linear gradient 0–50% B for 50 min. Acetonitrile was of an HPLC grade. The mass spectra (MS-ESI) were obtained on a Finnigan MAT 95S spectrometer. The instrument was equipped with an electrospray ionization source.

Synthesis of the peptides: The peptides were synthesized manually on a 0.1 milimole scale by the solid phase method using the Fmoc/Bu^t procedure [6]. The side-chain protecting groups of the amino acids were: trityl for Gln; *tert*-butyl ether for Tyr, Ser and Thr; *tert*-butyl ester for Asp and Glu. SV2 and its analogue were synthesized using the Fmoc method, according to the following procedure: (i) 5 and 15 min deprotection steps using 20% piperidine in the mixture DMF/NMP (1:1, v/v) or 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in DMF in the presence of 1% Triton; (ii) the coupling reactions carried out with the protected amino acid diluted in a mixture DMF/NMP (1:1, v/v) in the presence of 1% Triton using DIC as the coupling reagent in the presence of HOBt (Fmoc-AA:DIC:HOBt:1:1:1) for 1.5 h. The completeness of each coupling reaction was monitored by the chloranil test [7]. The protected peptidyl resins were treated with the mixture: 95% trifluoroacetic acid (TFA), 2.5% water and 2.5% TIS for 1 h. The cleaved peptide was precipitated with diethyl ether and lyophilized.

Purification of peptides: Peptides were purified by the solid-phase extraction (SPE). The SPE column was prepared by transferring 1 g of sorbent (Kromasil C8, 5 μm particle size, 100 Å pore size) to a 10-ml plastic polypropylene syringe equipped with porous disks at the bottom and at the top. The column was conditioned just prior to use by washing with 2×5 ml acetonitrile, 2×5 ml acetonitrile/water (1:1) and 2×5 ml water/0.1% trifluoroacetic acid. A 1-ml sample of stock solution of the peptide (10 μmol peptide) was transferred to a prewashed SPE cartridge. After washing with aqueous acetonitrile (5–20%) the peptides were eluted from the cartridge with 3–5 ml of 25, 26, 27, 28, 29, 30% acetonitrile/water in the presence of TFA 0.1%. Finally, the column was washed with 2×5 ml acetonitrile. The cartridge was not allowed to dry throughout the purification, and the flow was such that 1 ml of the liquid passed through the cartridge in about 15–20 s. The sample was forced through the column using a vacuum flask and a rubber stopper. The vacuum pulls a solution through the SPE column. The solution was collected in a test tube located inside the flask. The purity of the products was tested by the reversed-phase high performance liquid chromatography (RP-HPLC). RP-HPLC was performed on a Beckman "System Gold" chromatograph with Kromasil 5 μm C8 100 A (4.6×250 mm) column, flow rate was 1ml/min, absorbance at 226 nm. The mobile buffer consisted of A: 0.1% TFA in water and B: 0.1% TFA in acetonitrile. The peptides were characterized with a linear gradient 0–50% of B for 50 min. Fractions containing pure peptides (> 95–98%) were pooled and lyophilized.

Microbiological part

Materials and methods: Anaerobes. The investigations included 25 strains of anaerobic bacteria isolated from gingival pocket: *Prevotella denticola* (1 strain), *Prevotella intermedia* (7), *Porphyromonas gingivalis* (3), *Bacteroides forsythus* (1), *Bacteroides fragilis* (1), *Fusobacterium nucleatum* (3), *Peptostreptococcus micros* (2), *Peptostreptococcus magnus* (1), *Propionibacterium acnes* (3), *Actinomyces israelii* (1), *Clostridium spp.* (2) and 6 standard strains: *Bacteroides fragilis* ATCC 25285, *B. ovatus* ATCC 8482, *B. vulgatus* ATCC 8483, *Fusobacterium nucleatum* ATCC 25585, *Propionibacterium acnes* ATCC 11827, *Peptostreptococcus anaerobius* ATCC 27337 (Table 2). The susceptibility (MIC) of anaerobic bacteria was determined using the plate technique in agar. Statherin SV2 and its analogue were dissolved in sterile distilled water (immediately before the experiment) to obtain the following concentrations: 100, 50, 25 and 12.5 μg/ml. Peptide solutions were added to Brucella agar supplemented with 5% defibrinated sheep's blood, menadione and hemin [8,9]. The plates were inoculated using a Steers multipoint inoculator. The inoculum contained 10^6 CFU/spot (CFU - Colony Forming Units). In

each experimental series, the growth of strains on the culture medium without the compounds investigated was checked. The incubation was performed for 48 h at 37°C (310 K) in anaerobic jars containing a mixture of 10% CO_2, 10% H_2 and 80% N_2, in the presence of a palladium catalyst and an indicator of anaerobiosis. The concentration, at which no macroscopic growth of the microbes, was observed on the medium and was regarded as the lowest concentration inhibiting the growth of microbes (MIC). The examinations of the susceptibility of microbes to the peptides were carried out twice.

Aerobes. The investigations included 23 strains aerobes isolated from patients with infections in the oral cavity or respiratory tract. The material was inoculated into the culture medium and incubated under aerobic conditions at 37°C for 24–72 hours. Aerobes were identified according to the classification given in the literature. The following aerobes strains were examined: *Escherichia coli* (7 strains), *Acinetobacter baumannii* (1 strain), *Klebsiella pneumoniae* (4), *Pseudomonas aeruginosa* (4), *Pseudomonas stutzeri* (1), *Staphylococcus aureus* (4), *Bacillus spp.* (2) and 8 standard strains: *Escherichia coli* ATCC 25922, *Acinetobacter baumannii* ATCC 19606, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Proteus vulgaris* ATCC 13315, *Bacillus subtilis* NCTC 8236, *Bacillus cereus* ATCC 10876 (Table 3). The susceptibility (MIC) of aerobic bacteria, like in the case of anaerobic bacteria, was determined by the plate technique in agar. Statherin SV2 and its analogue were dissolved in sterile distilled water (immediately before the experiment) to obtain the following concentrations: 100, 50, 25 and 12.5 μg/ml. The plates were inoculated using a Steers multipoint inoculator. The inoculum contained 10^6 CFU/spot. In each experimental series, the growth of the strains on the culture medium without the compounds investigated was checked. The incubation was performed for 24 h at 37°C (310 K) in aerobic jars. After this time MIC was measured.

Table 1 Physico-chemical characteristics of statherin SV2 and its analogue.

Peptide*	Molecular mass [Da] (MS-ESI)		RP HPLC	
	Calculated	Found $[M + H]^+$	R_T [min]	k′
SV2	4149.3	4150.5	34.67	11.25
[Ser^2, Ser^3] SV2	3988.3	3989.1	35.58	11.57

*Amino acid composition of the peptides is consistent with expectation.

Table 2. Susceptibility (MIC) of anaerobes isolated from the gingival pockets to statherin SV2 and its analogue.

Anaerobes	Number of strains	Minimal inhibitory concentration (MIC) – μg/ml							
		Statherin SV2				[Ser^2, Ser^3] Statherin SV2			
		≤ 12.5	25	50	≥ 100	≤ 12.5	25	50	≥ 100
Gram-negative									
Prevotella denticola	1				1				1
Prevotella intermedia	7	1		2	4	1	2		4
Porphyromonas gingivalis	3			1	2	2			1
Bacteroides forsythus	1			1		1			
Bacteroides fragilis	1			1		1			
Fusobacterium nucleatum	3		1		2	2			1
Gram-positive									
Peptostreptococcus micros	2	1	1			2			
Peptostreptococcus magnus	1		1			1			
Propionibacterium acnes	3		1	1	1		2		1
Actinomyces israelii	1	1				1			
Clostridium spp.	2				2		1		1
TOTAL	**25**	**3**	**4**	**6**	**12**	**11**	**5**		**9**

Table 2 (continuation)

Standard strains								
Bacteroides fragilis ATCC 25285	1			1				1
Bacteroides ovatus ATCC 8482	1			1				1
Bacteroides vulgatus ATCC 8483	1			1				1
Fusobacterium nucleatum ATCC 25585	1			1				1
Propionibacterium acnes ATCC 11827	1			1				1
Peptostreptococcus anaerobius ATCC 27337	1		1			1		

Table 3. Susceptibility (MIC) of aerobes isolated from the oral cavity and respiration tract to statherin SV2 and its analogue.

Aerobes	Number of strains	Minimal inhibitory concentration (MIC) – μg/ml							
		Statherin SV2				[Ser^2, Ser^3] Statherin SV2			
		≤ 12.5	25	50	≥ 100	≤ 12.5	25	50	≥ 100
Gram-negative									
Escherichia coli	7				7				7
Acinetobacter baumannii	1				1				1
Klebsiella pneumoniae	4				4				4
Pseudomonas aeruginosa	4				4				4
Pseudomonas stutzeri	1				1				1
Gram-positive									
Staphylococcus aureus	4				4				4
Bacillus spp.	2				2				2
TOTAL	**23**				**23**				**23**
Standard strains									
Escherichia coli ATCC 25922	1				1				1
Acinetobacter baumannii ATCC 19606	1				1				1
Klebsiella pneumoniae ATCC 13883	1				1				1
Pseudomonas aeruginosa ATCC 27853	1				1				1
Staphylococcus aureus ATCC 25923	1				1				1
Proteus vulgaris ATCC 13315	1				1				1
Bacillus subtilis NCTC 8236	1				1				1
Bacillus cereus ATCC 10876	1				1				1

RESULTS AND DISCUSSION

Statherin SV2 and its dephosphorylated analogue were synthesized by the solid phase method. The Fmoc/Bu^t procedure was applied. Products were purified by the solid phase extraction. The purity of the peptides synthesized was 95–98%, as determined by HPLC.

The results of previous studies show, that statherin and its C-terminal fragments inhibited the growth of anaerobes isolated from the gingival pockets [4]. At concentrations from 12.5 μg/ml to 100 μg/ml statherin inhibited the growth of 51% of all the strains of anaerobes examined. *Peptostreptococcus* strains were the most susceptible to statherin and its C-terminal fragment YTF (MIC ≤ 12.5 μg/ml). Statherin SV2 and its analogue exhibited antibacterial activity against obligate anaerobes isolated from gingival pockets (Table 2). At concentration from 12.5 μg/ml to 25 μg/ml the analogue of statherin SV2 inhibited the growth of 64% of all strains of the anaerobes examined. In the case of statherin SV2, the inhibition was lower (28%). At concentrations ranging from 12.5 to 25 μg/ml, the analogue of statherin SV2 inhibited the growth of 9 (51%) of Gram-negative anaerobes. In the case of statherin SV2, the growth inhibition was observed for 2 (13%) strains. Among nine strains of Gram-positive anaerobes seven (78%) were susceptible to the growth inhibition by the statherin analoque used at concentration from 12.5 to 25 μg/ml. In the case of statherin SV2, the growth was observed for five (56%) strains (Table 2). In contrast to anaerobic bacteria, all strains of aerobes examined were unsusceptible to statherin SV2 and its analogue at concentrations tested (Table 3).

Anaerobic bacteria are present predominantly in the physiological flora of the oral cavity. The amount of anaerobes in the oral cavity is approximately 10^8 CFU/ml and varies depending on the anatomical region. Being a sort of opportunistic flora, anaerobes play a role in various infections within the oral cavity (among others in oral mucosa inflammation and ulceration as well as in gingivitis or dental pulp inflammation). They are also responsible for peridental diseases, peridental abscess and the following complications. These infections are often associated with the pathogenic activity of microaerophiles or aerobes. All these bacteria produce lots of various enzymes, toxic substances and endotoxins that facilitate damage and tissue penetration as well as destruction of protective mechanisms of the host. A lot of these microbes digest protein and do not digest hydrocarbons.

CONCLUSIONS

Statherin SV2 and [Ser^2, Ser^3]statherin SV2 exhibited antibacterial activity against obligate anaerobes isolated from gingival pockets. Dephosphorylated analogue of statherin SV2 showed higher antibacterial activity particulary at low concentrations (12.5–25 μg/ml). Gram-positive anaerobes were especially susceptible to the dephosphorylated analogue of statherin SV2.

All strains of aerobes examined were unsusceptible to statherin SV2 and its analogue at concentrations tested.

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REFERENCES

1. Schlesinger D.H. and Hay D.I., *J. Biol. Chem*., **252**, 1689 (1977).
2. Raj P.A., Johnsson M., Levine M.J. and Nancollas G.H., *J. Biol. Chem*., **267**, 5968 (1992).
3. Ramasubbu N., Thomas L.M., Bhandary K.K. and Levine M.J., *Crit. Rev. Oral Biol. Med*., **4**, 363 (1993).
4. Kochańska B., Kędzia A., Kamysz W., Maćkiewicz Z. and Kupryszewski G., *Acta Microbiol. Pol*., **49**, 243 (2000).
5. Jensen J.L., Lamkin M.S. and Oppenheim F.G., *J. Dent. Res*., **71**, 1569 (1992).
6. Atherton E. and Sheppard R.C., The Peptides: Analysis, Synthesis, Biology (Eds. Udenfriend S. and Meienhofer J.) Academic Press, NY, 1987, vol. 9, p. 1.
7. Hancock W.S. and Battersby J.E., *Anal. Biochem*., **71**, 260 (1976).
8. Jousimies-Somer H.R. and Finegold S.M., Anaerobic Gram-Negative Bacilli and Cocci. In: Manual of Clinical Microbiology (Balows A. et al. eds.) (5th ed.). Am. Soc. Microbiol., Washington 1991, p. 538.
9. Baron E.J. and Finegold S.M., Bailey and Scotts Diagnostic Microbiology (8^{th} ed.), C.V. Mosby Co., St. Louis, 1990, p. 529.