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Note

Adsorption of cyclic peptides analogous to gramicidin S and gratisin onto octadecylsilica stationary phase and bacterial cells

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Gramicidin S-1 (GS) [1–3] is an antibiotic cyclodecapeptide with a rigid β-pleated sheet conformation [4]. A characteristic feature of this conformation is the orientation of the side-chains in such a way that the two charged Orn side-chains are situated on one side of the molecule and the four hydrophobic Val and Leu side-chains on the other. This arrangement is apparently maintained by a rigid ring structure containing two type II′ β-turns composed of D-Phe-Pro. From numerous investigations of the relationship between the structure and the antibiotic activity of this antibiotic, it has been proposed that this specific feature of the molecule is essential to the antibiotic activity [5].

With regard to the mechanism of the action of GS on bacterial cell membranes, Semenov et al. [6] and Ivanov [7] reported that an important step in the function of GS is the formation of a surface-active complex due to electrostatic interaction of amino groups of the Orn residues in GS with phospholipids in the membranes of the cells; and that for the formation of such a complex, two positive charges of Orn residues must be arranged at a definite distance from one another

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on the same side of the plane of the ring of the cyclopeptide. Kaprel'yants et al. [8] reported that, in addition to the electrostatic interaction, the hydrophobic interaction of GS with the membrane also plays an important role.

In general, the affinity of peptides containing hydrophobic side-chains for the octadecylsilica (ODS) gel stationary phase used in reversed-phase high-performance liquid chromatography (HPLC) is believed to be caused by a hydrophobic interaction [9,10]. Thus, our interest is in whether the ODS stationary phase can be regarded as a model of the hydrophobic surface of target bacterial cells. To further elucidate the mode of action of GS and the related peptides, the affinity of some synthetic cyclic peptides analogous to GS and gratisin (GR) [11,12] for an ODS column and for bacterial cells was investigated.

EXPERIMENTAL

Synthesis of peptides

The primary structures of the synthesized peptides [13–16] are shown in Table I. The homogeneities of the peptides were confirmed by means of thin-layer chromatography (TLC), HPLC, elemental analysis, mass spectra and amino acid analysis of their acid hydrolysates.

HPLC analysis

HPLC was carried out using a Twincle system (Jasco, Japan) consisting of a Twincle pump, a VL-611 injector and a Uvidec 100-III detector. A Finepak SIL C_{18} column (5 μm, 250 $\times$ 4.6 mm I.D., Jasco) was used: flow-rate, 1 ml/min; solvent, methanol–5% aqueous sodium perchlorate (3:1); monitoring wavelength, 220 nm.

Measurement of adsorption of the peptides onto bacterial cells

Staphylococcus aureus IAM 12544 and *Escherichia coli* IAM 1239 were used for the experiments. They were cultured in a liquid medium of nutrient broth (Eiken) at 30°C for 18 h. The cells were collected by centrifugation and washed twice with physiological saline. The cells were diluted to $6.00 \cdot 10^8$ cells per ml (*S. aureus*) or $6.35 \cdot 10^8$ cells per ml (*E. coli*) in the saline, and an aliquot (90 μl) was mixed with the peptide solution (10 μl, 3 or 5 mg/ml in methanol). The mixture was allowed to stand for 10 min at room temperature and then centrifuged. An aliquot (10 μl) of the supernatant was analysed by HPLC equipped with a Shimadzu Chromatopac C-R3A integrator. From the amount of the peptide determined from the peak area, the total amount of the peptide left in the supernatant was calculated.

RESULTS AND DISCUSSION

Table I shows the retention times in HPLC analysis of some synthetic cyclic peptides and their antibiotic potencies. GS-1, -a, -b, -c and -d, all of which had the same amino acid composition, showed different affinities for the ODS column. These differences, when represented as differences in the retention time

TABLE I

RETENTION TIME AND ANTIBIOTIC ACTIVITY

The organisms examined were *Staphylococcus aureus* ATCC 6538P, *Streptococcus pyogenes* N.Y. 5, *Micrococcus flavus* ATCC 10240, *Corynebacterium diphtheriae* P.W. 8, *Bacillus subtilis* ATCC 6633 and *Escherichia coli* NIHJ-JC 2. Minimum inhibitory concentration (μg/ml) was determined by an agar dilution method with 10^6 organisms per ml. GS and GR possess antibiotic activity against bacteria tested except *E. coli*. The symbols −, +, ++ and +++ represent relative potencies of the synthetic peptides against the sensitive bacteria.

Peptide	Retention time at 30°C (min)	Activity
Gramicidin S analogues [13,14]		
GS-a Cyclo(-D-Val-Orn-Leu-Phe-Pro-)$_2$	8.5	−
GS-b Cyclo(-D-Val-Orn-Leu-D-Phe-Pro-)$_2$	10.4	−
GS-c Cyclo(-Val-Orn-Leu-Pro-D-Phe-)$_2$	12.4	−
GS-d Cyclo(-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-Pro-D-Phe-)	27.4	++
GS-1 Cyclo(-Val-Orn-Leu-D-Phe-Pro-)$_2$	73.8	+++
Gratisin peptides [15,16]		
GR-7 Cyclo(-Val-Orn-Leu-D-Ala-Pro-D-Tyr-)$_2$	6.4	−
GR-12 Cyclo(-Val-Pro-Orn-Leu-D-Phe-D-Tyr-)$_2$	7.2	−
GR-11 Cyclo(-Val-Orn-Pro-Leu-D-Phe-D-Tyr-)$_2$	7.6	−
GR-2 Cyclo(-Val-Orn-Leu-D-Phe-Pro-Tyr-)$_2$	10.0	±
GR-5 Cyclo(-Val-Orn-Leu-D-Tyr-Pro-D-Phe-)$_2$	10.4	+
GR-1 Cyclo(-Val-Orn-Leu-Phe-Pro-Tyr-)$_2$	12.7	−
GR-3 Cyclo(-Val-Orn-Leu-Phe-Pro-D-Tyr-)$_2$	12.7	−
GR-10 Cyclo(-Val-Orn-Leu-Pro-D-Phe-D-Phe-)$_2$	16.5	++
GR-8 Cyclo(-Val-Orn-Leu-D-Phe-Pro-D-Ala-)$_2$	17.2	++
GR-4 Cyclo(-Val-Orn-Leu-D-Phe-Pro-D-Tyr-)$_2$	19.2	++
GR-6 Cyclo(-Val-Orn-Leu-D-Phe-Pro-D-Phe-)$_2$	28.4	+++
GR-9 Cyclo(-Val-Orn-Leu-D-Phe-D-Tyr-Pro-)$_2$	30.0	+++
GR-13 Cyclo(-Val-Orn-Leu-D-Phe-D-Phe-Pro-)$_2$	35.6	+++

(GS-a, GS-b, GS-c < GS-d < GS-1), cannot be explained without considering the conformations of the molecules, because the sum of the hydrophobicities of side-chains of the constituent amino acid residues is the same in each peptide. The elution order should reflect the degree of effective hydrophobicity, which varies according to the ring structure determined by the primary structure of each peptide.

Among the gratisin peptides, GR-1, -2, -3, -4, -5, -9, -11 and -12 all have the same amino acid compositions, as do GR-6, -10 and -13. Wide variations observed in the retention times indicated that the GR peptides also have various degrees of effective hydrophobicity, in other words, various ring structures.

A linear relationship was found between the logarithm of the retention volumes and the reciprocal of the column temperature (30, 40, 50 and 60°C) (Fig. 1). This showed that Van't Hoff's equation could be set up for the adsorption of the GS and GR peptides on the stationary phase in the temperature range. A similarity between the inclinations of the respective lines for the various peptides showed that the heats of transfer of the solutes from the stationary to mobile

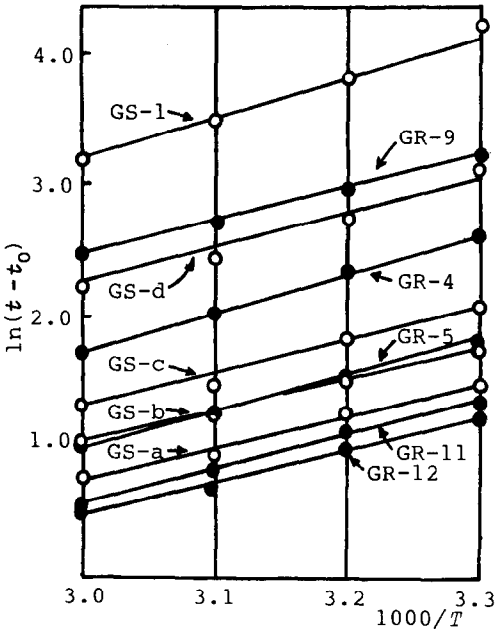

Fig. 1. Effect of temperature on retention time: t = retention time of solute; t_0 = retention time of solvent; T = absolute temperature.

phase were similar. This finding indicated that the peptides examined were adsorbed on the ODS column through a common mechanism, and that comparison of the degree of effective hydrophobicity between the peptides was possible by use of retention time as the parameter.

Between the retention times of the GS and the GR peptides and their circular dichroic (CD) spectra, no apparent relationship was observed. This fact may indicate that the CD spectra of the antibiotics reflect mainly the ring features near the Pro residue [13–15], but not always the whole structures of the molecules. It is to be noted that, in the case of both the GS and the GR peptides, the more active peptides were eluted more slowly from the ODS column (Table I). This means the antibiotics with stronger activity exhibit a higher degree of effective hydrophobicity.

Yonezawa and co-workers [17,18] measured the amount of ^{14}C-labelled GS peptides adsorbed on cells, and found that the active analogues were adosrbed more tightly on the cells than were the inactive ones. We have now developed a very convenient method for quantitative estimation of the adsorption by means of HPLC analysis (Fig. 2, see also Experimental). Table II shows the amounts of some peptides adsorbed on *S. aureus* and *E. coli*. The data also indicate that the peptides with stronger activity were adsorbed on the cells more tightly, and a distinct correlation was observed among the affinities of the peptides for the ODS stationary phase, that for the bacterial cells and the antibiotic potencies.

In view of the mechanisms proposed [6–8] for the antibiotic action of GS, our data strongly suggest that the GS and GR peptides were adsorbed on the ODS

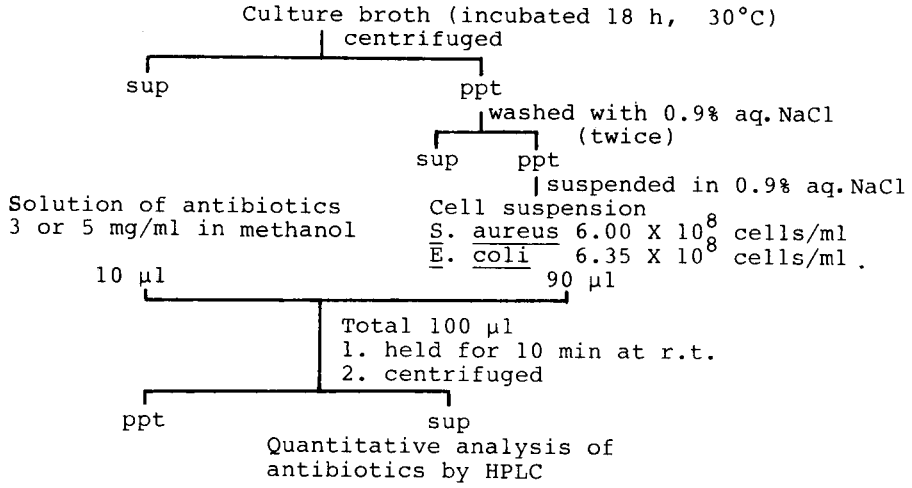

Fig. 2. Measurement of adsorption of the peptides on bacterial cells.

stationary phase and on bacterial cells by similar mechanisms, in which the cyclic peptides were adsorbed on the adsorbents by hydrophobic interaction orienting the non-polar face of the molecules with a ring structure. An alteration to the ring structure led to variation in the affinity, which variation further affected the antibiotic potency. The electrostatic interaction between the positively charged Orn side-chains and negatively charged parts of the membrane would be effective after the antibiotics were adsorbed on the cell to settle in some hydrophobic environment, because such charged regions would be easily hydrated in an aqueous medium thus losing their chances for interacting with each other.

Of course, ODS stationary phase and surfaces of bacterial cells are very different physically. Indeed, our data suggest that the hydrophobic interaction plays a

TABLE II

ADSORPTION OF ANTIBIOTICS ON BACTERIAL CELLS

Exp. 1: initial concentration of peptide is 0.3 mg/ml; Exp. 2: initial concentration of peptide is 0.5 mg/ml.

Antibiotic	Activity	Adsorbed amount (%)			
		S. aureus		*E. coli*	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
GS-c	–	14	2	3	5
GS-d	++	22	15	6	7
GS-1	+++	47	42	36	28
GR-11	–	5	0	8	7
GR-4	++	35	20	23	11
GR-9	+++	42	21	27	18

predominant role when GS and GR peptides exhibit their antibiotic activity, but the interaction is not all that is required for exhibition of the activity. The facts that the peptides tested were also adsorbed on *E. coli*, for which they were not effective [15], show that further work is necessary to understand fully the mechanisms of the action of the antibiotics on bacterial cells.

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