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Studies on cyclic peptides related to gratisin by reversed-phase high-performance liquid chromatography

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ABSTRACT

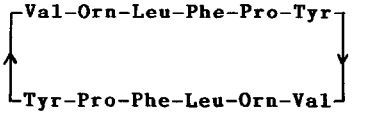
The chromatographic behaviour of several synthetic peptides related to the antibiotic peptide gratisin was investigated using reversed-phase high-performance liquid chromatography. The influence of the concentration of the peptides, column temperature and flow-rate on the chromatographic separation was examined. Of these peptides, some analogues with the D-X-D-Y-L-Pro or L-Pro-D-X-D-Y sequences and strong activities gave double peaks in the chromatogram. The isomeric conformers were separated from each of the double peaks and were in equilibrium with each other at low temperatures. It is proposed that the presence of the sequences of D-X-D-Y-L-Pro and L-Pro-D-X-D-Y in these peptides gives rise to the stabilization and various degrees of hydrophobicity of their conformers.

INTRODUCTION

An antibiotic peptide, gratisin (GR), showing a strong acitivity towards *Bacillus subtilis* 720, was isolated from *Bacillus brevis* Y-33 by Zharikova *et al.* [1]. It is a cyclododecapeptide composed of two each of Val, Orn, Leu, Phe, Pro and Tyr residues. (Note: amino acid residues with no prefix are of L-configuration unless stated otherwise. The abbreviations for amino acids and peptides are in accordance with the rules of IUPAC-IUB Commission of Biochemical Nomenclature.) The proposed primary structure of gratisin is shown in Fig. 1 [2,3]; the configuration of each amino acid residue has not yet been established. In this work many analogues of this antibiotic were synthesized (Fig. 2) and their structure–activity relationships investigated [4–6]. In studies of synthetic peptides by high-performance liquid chromatography (HPLC) using a reversed-phase column, it was found that GR peptides with the sequence D-X-D-Y-L-Pro or L-Pro-D-X-D-Y gave double peaks in the chromatogram.

Similar examples of multiple peaks on HPLC separation were found for several biologically active peptides [7–10] and model dipeptides [11] which contain the Pro residue. However, the presence of the stable conformers in cyclic peptides with D-amino acid residues around the Pro residue has not yet been seen.

This paper reports the HPLC behaviour of GR peptides and shows the role of the configuration



Gratisin (GR)

Fig. 1. Primary structure of gratisin. The configuration of each amino acid residue has not yet been established.

GR-1. cyclo(-Val-Orn-Leu-Phe-Pro-Tyr)$_2$

GR-2. cyclo(-Val-Orn-Leu-D-Phe-Pro-Tyr-)$_2$

GR-3. cyclo(-Val-Orn-Leu-Phe-Pro-D-Tyr-)$_2$

GR-4. cyclo(-Val-Orn-Leu-D-Phe-Pro-D-Tyr-)$_2$

GR-5. cyclo(-Val-Orn-Leu-D-Tyr-Pro-D-Phe-)$_2$

GR-6. cyclo(-Val-Orn-Leu-D-Phe-Pro-D-Phe-)$_2$

GR-7. cyclo(-Val-Orn-Leu-D-Ala-Pro-D-Tyr-)$_2$

GR-8. cyclo(-Val-Orn-Leu-D-Phe-Pro-D-Ala-)$_2$

GR-9. cyclo(-Val-Orn-Leu-D-Phe-D-Tyr-Pro-)$_2$

GR-10. cyclo(-Val-Orn-Leu-Pro-D-Phe-D-Phe-)$_2$

GR-11. cyclo(-Val-Orn-Pro-Leu-D-Phe-D-Tyr-)$_2$

GR-12. cyclo(-Val-Pro-Orn-Leu-D-Phe-D-Tyr-)$_2$

GR-13. cyclo(-Val-Orn-Leu-D-Phe-D-Phe-Pro-)$_2$

GR-14. cyclo(-Val-Orn-Leu-D-Tyr-D-Phe-Pro-)$_2$

GR-15. cyclo(-Val-Orn-Leu-D-Leu-D-Phe-Pro-)$_2$

GR-16. cyclo(-Val-Orn-Leu-Leu-D-Phe-Pro-)$_2$

GR-17. cyclo(-Val-Orn-Leu-Pro-D-Phe-Tyr-)$_2$

Fig. 2. Synthetic derivatives related to GR.

of the amino acid residue around the Pro residue for the formation of stable conformers.

EXPERIMENTAL

Syntheses of peptides

Peptides related to GR were synthesized by a conventional liquid-phase method. Their primary structures are shown in Fig. 2 [4–6]. The homogeneities of the synthetic peptides were confirmed by thin-layer chromatography, HPLC, elemental analysis, fast atom bombardment mass spectrometry and amino acid analysis of their acid hydrolysates.

HPLC analysis

HPLC was carried out using an 800 series system (Jasco, Tokyo, Japan) consisting of an 880 intelligent HPLC pump, an 875-UV intelligent UV–visible detector, an 860-CO column oven and a Model 7125 syringe-loading sample injector (Rheodyne, Cotati, CA, USA). A Finepak SIL C_{18} column (250 mm × 4.6 mm I.D., 10 μm particle size, Jasco) was used. The flow-rate was 1 ml/min, the mobile phase was methanol–5% aqueous sodium perchlorate (4:1, v/v) and the wavelength of detection was 220 nm. About 1 mg of each synthetic peptide was dissolved in 1 ml of the mobile phase and the resulting solution (10 μl) was injected into the chromatograph. The peak area was recorded using a Chromatopac C-R3A integrator (Shimadzu, Kyoto, Japan).

RESULTS AND DISCUSSION

The HPLC analyses of the peptides (GR-1 to GR-17) were usually performed at 20°C. GR-9, -10, -13, -14 and -15, which contain a D-X-D-Y-L-Pro or L-Pro-D-X-D-Y sequence and possess a strong activity, gave double peaks and an increased baseline between these peaks on separation by HPLC (Fig. 3), although their purity has been confirmed by other analytical methods. GR-10, containing the L-Pro-D-X-D-Y sequence, gave a profile opposite to that of GR-9, -13, -14 and -15 (D-X-D-Y-L-Pro); in the former the major peak precedes the minor peak. However, the other synthetic peptides with various sequences around the Pro residue gave a single peak and did not show such phenomena, even at lower temperatures. These results indicate that the phenomena are characteristic of GR peptides containing the D-X-D-Y-L-Pro and L-Pro-D-X-D-Y sequences.

The ratio of the areas of the double peaks were unchanged on dilution of the injected sample, suggesting that the phenomena are not caused by intermolecular aggregation.

The effect of flow-rate on the shapes of elution curves of GR-9 and -10 is shown in Fig. 4 (flow-rates 0.25, 0.5, 1.0 and 2.0 ml/min). A decrease in the flow-rate caused a broadening of the peaks. The shapes at low flow-rates reflect the interconversion of conformers in the column.

HPLC separation of GR-9 and GR-10 was performed at various temperatures (20, 30, 40, 50 and 60°C) (Fig. 5). Increasing the column temperature reduced the distance between the two peaks gradually, and they finally coalesced. These results can be explained by the more rapid interconversion between the two conformers at high temperatures.

Each of the two isomers of GR-10 and -13 was isolated by HPLC at 0°C and was separated chro-

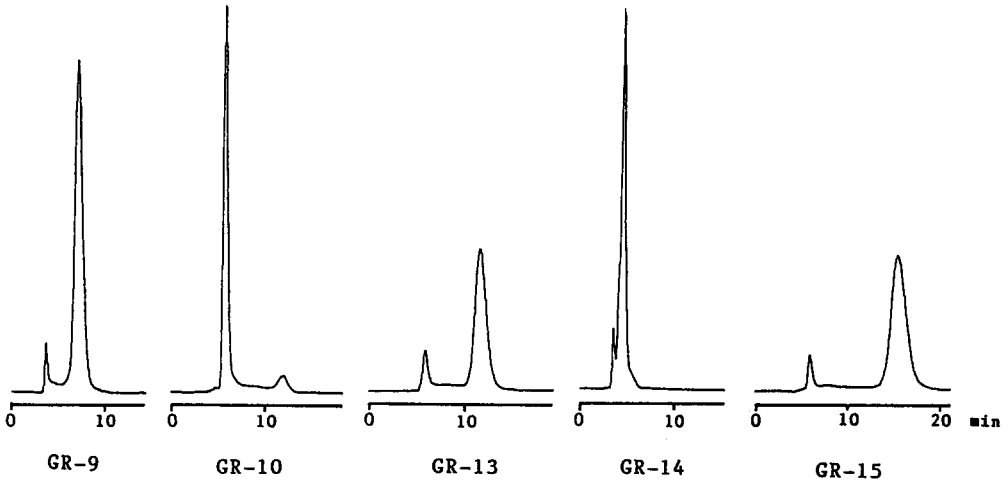


Fig. 3. HPLC profiles of GR-9, GR-10, GR-13, GR-14 and GR-15.

matographically under the original conditions after storage for various times at 0°C (Figs. 6 and 7). The chromatograms of each isomer gradually revert to the original profiles. These results further confirm the existence of two conformers which slowly interconvert to each other.

In the HPLC studies of Pro-containing dipeptides, Melander *et al.* [11] reported that the dipeptides in which the Pro residue is at the C-terminal cause peak-splitting in a reversed-phase column. These phenomena were ascribed to the slow kinetics of *cis–trans* isomerization of the X-Pro imide bond. Similar results were obtained for several biologically active peptides which contain one or more Pro residues [7–10]. In the present studies, it is noticeable that the phenomena were observed only with GR-derived peptides containing D-X-D-Y-L-Pro and L-Pro-D-X-D-Y sequences, although all of GR-1 to GR-17 have two Pro residues in each molecule. The partial structure consisting of these sequences may contribute to the stabilization of the conformers re-

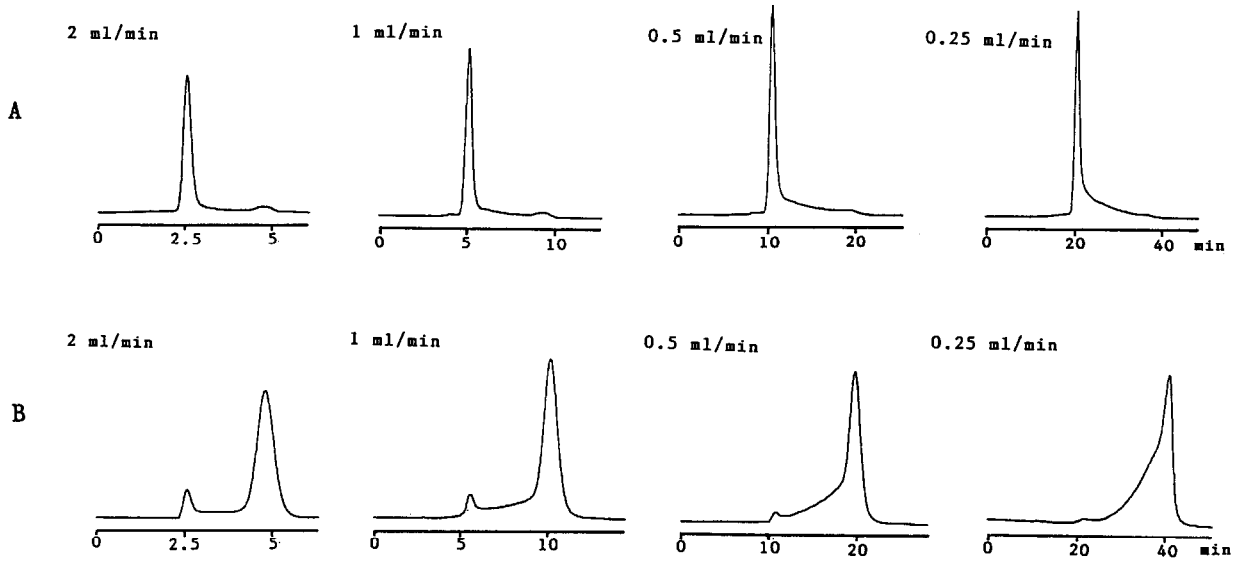


Fig. 4. Effect of flow-rate (0.25, 0.5, 1 and 2 ml/min) on the shape of chromatograms of (A) GR-10 and (B) GR-13. The column temperature was 30°C.

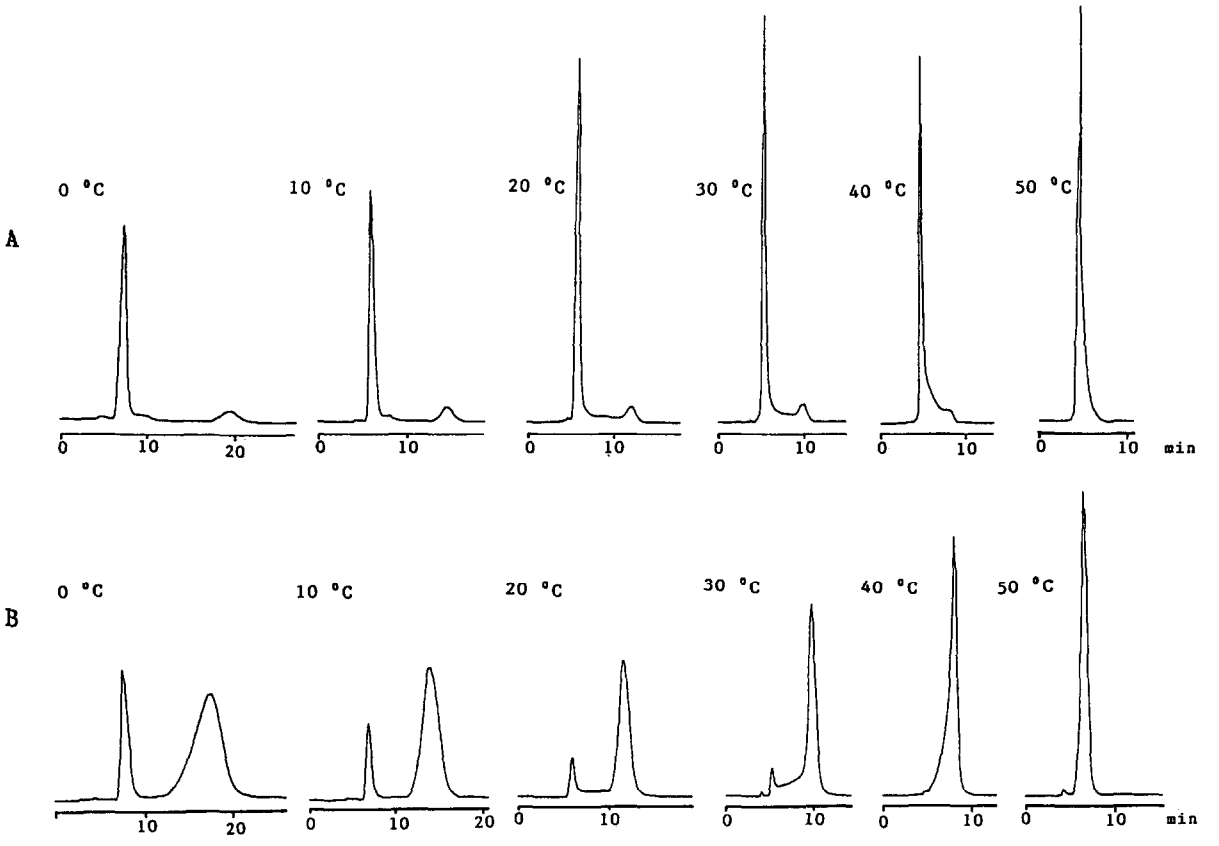


Fig. 5. Effect of column temperature (0, 10, 20, 30, 40 and 50°C) on the elution of (A) GR-10 and (B) GR-13. The flow-rate was 1 ml/min.

sulting from *cis–trans* isomerization of the D-Phe-Pro and Phe-Pro imide bonds. In addition, the differences in the elution profiles between GR-10 and GR-9 and GR-13 to GR-15 might be a consequence of the differences in the configuration of the amino acid residues preceding the Pro residues.

In the studies of the structure–activity relationship of gramicidin S (GS) [cyclo(-Val-Orn-Leu-D-Phe-Pro-)$_2$], many GS analogues consisting of six [12], eight [13], ten [14,15] and fourteen amino acid residues [16], and with various sequences around the Pro residue were synthesized. These cyclic peptides showed a single peak after chromatographic separation under the conditions described earlier. The results suggest that in addition to the amino acid sequence around the Pro residue, the presence of a flexible ring structure is important for the separation of peaks.

In studies of the HPLC separation of peptides related to GS and GR, it was reported that the hydrophobicity plays a predominant role in their antibiotic activity; antibiotics with a stronger activity show a higher degree of effective hydrophobicity [17]. The presence of two peaks in the HPLC separation indicates that each conformer has a different affinity for the reversed-phase column. Also, each conformer has a different hydrophobic interaction with the surface of the cell membrane. The measurement of the antibiotic activity of these conformers may be impossible because they are rapidly equilibrated under assay conditions. However, it is suggested that the conformer eluted more slowly from the reversed-phase column shows a stronger activity. The peptides discussed here, except GR-1, -3, -7, -11, -12 and -17, have antibiotic activities, although of various strengths. Thus the peak-splitting phenomenon is not related to the biological activity.

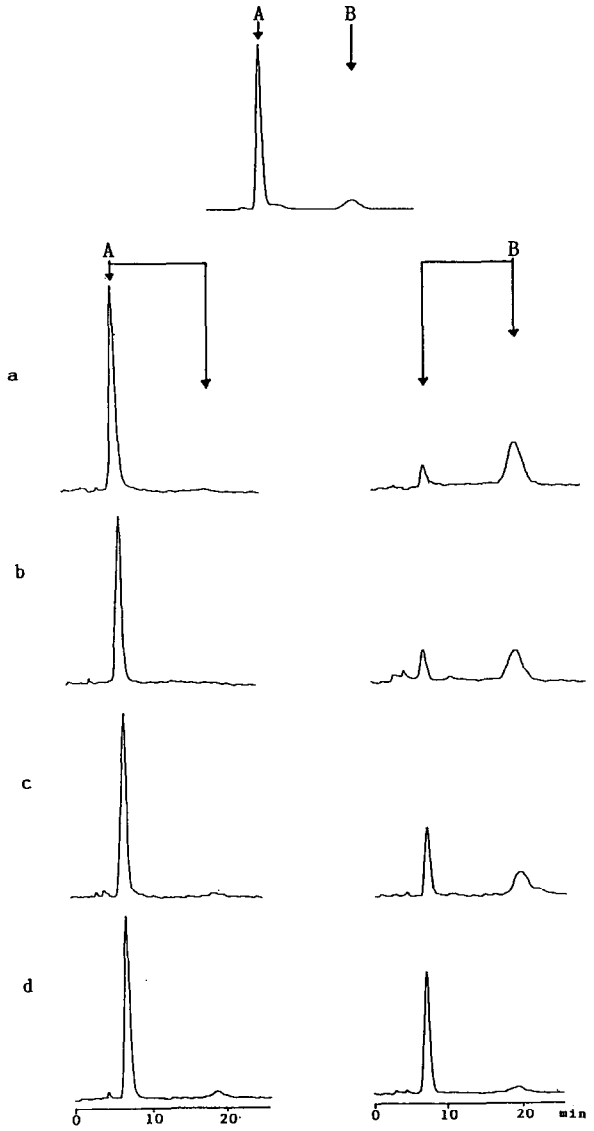


Fig. 6. Chromatograms of the isolated conformers (A, B) of GR-10 after storage at 0°C for (a) 1, (b) 2, (c) 5 and (d) 24 h.

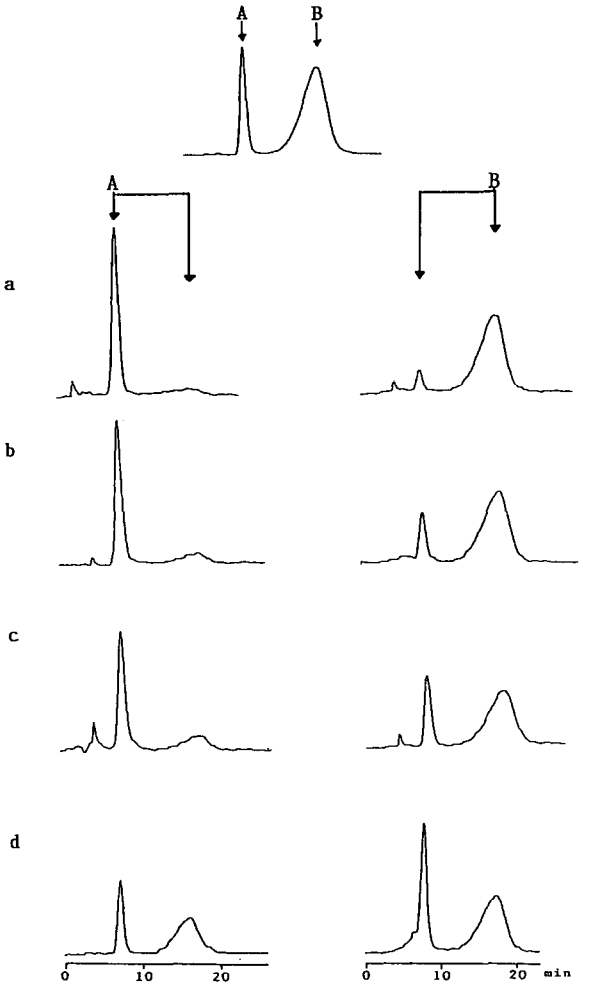


Fig. 7. Chromatograms of the isolated conformers (A, B) of GR-13 after storage at 0°C for (a) 1, (b) 2, (c) 5 and (d) 24 h.

CONCLUSIONS

This paper has shown that some synthetic peptides related to GR, which contain D-X-D-Y-L-Pro and L-Pro-D-X-D-Y sequences, give double peaks on HPLC with a reversed-phase column. The influence of the concentration of peptides injected, the column temperature and flow-rate on these phenomena was examined. The results can be understood by assuming the presence of two slowly interconvertible conformers. In addition, it was found that the ring structure of peptides related to GR consisting of twelve amino acid residues is important for showing this phenomenon. Further investigations by other techniques are necessary to clarify the conformational isomerization of the antibiotics with respect to their secondary structures.

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