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Note

Separation of mono-, di- and tri-L-leucylglycine by droplet countercurrent chromatography

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A new technique of countercurrent chromatography, namely droplet countercurrent chromatography (DCCC), was introduced by Tanimura *et al.*[1], who demonstrated that a mixture of dinitrophenylamino acids was separated efficiently into its components by this technique. Okamoto *et al.*[2] demonstrated further that pure constituent peptides were obtained from a mixture of gramicidins and of tyrocidines by DCCC. In this paper we report the separation of a mixture of the repeated sequential peptides such as H(L-Leu–Gly)$_n$OH (n = 1, 2 and 3) and discuss the effectiveness of the two techniques DCCC and gel chromatography with Sephadex G-10 for the separation of these peptide mixtures.

EXPERIMENTAL

Three model peptides, H(L-Leu–Gly)$_n$OH (n = 1, 2 and 3), were synthesized by conventional methods. *tert.*-Butyloxycarbonyl-L-leucylglycine benzyl ester was deacylated with hydrogen chloride in dioxane and coupled with benzyloxycarbonyl-L-leucylglycine azide, which was derived from the corresponding hydrazide. A portion of the protected tetrapeptide ester obtained was catalytically hydrogenolyzed to give H(L-Leu–Gly)$_2$OH. Another portion of the tetrapeptide ester was converted into the tetrapeptide hydrazide and the azide derived from this hydrazide was coupled with L-leucylglycine benzyl ester to give the protected hexapeptide ester, which was finally hydrogenolyzed to afford H(L-Leu–Gly)$_3$OH. H(L-Leu–Gly)OH was obtained by hydrogenation of benzyloxycarbonyl-L-leucylglycine benzyl ester, which had been prepared by a known procedure. Details of the synthetic procedure and physical constants of the prepared peptide derivatives will be reported elswhere[3]. All of the peptides that are used for the separation by DCCC and by Sephadex G-10 gel chromatography were obtained as analytically pure compounds. The purity of each compound was verified by the results of elemental analyses and paper and thin-layer chromatography. The R_F values of the synthesized peptides and solvent systems used are summarized in Table I.

DCCC was carried out with an apparatus made by Seikagaku Kogyo Co. (Tokyo, Japan). It consists of 150 column units (mounted perpendicularly) of glass tubing (0.6 mm wall thickness and 2.4 mm I.D.), 60 cm long and connected by PTFE tubing (0.5 mm I.D.). All experiments were carried out at room temperature. The

TABLE I

R_F VALUES OF H(L-LEU-GLY)$_n$OH (n = 1, 2 AND 3)

Solvent system used in thin layer chromatography (TLC), n-butanol–acetic acid–water (4:1:5, upper phase); in paper chromatography (PC), n-butanol–acetic acid–pyridine–water (15:3:10:12).

Method	*H(L-Leu-Gly)OH*	*H(L-Leu-Gly)$_2$OH*	*H(L-Leu-Gly)$_3$OH*
TLC	0.46	0.53	0.86
PC	0.11	0.47	0.53

solvent mixture, n-butanol–acetic acid–water (4:1:5), was allowed to equilibrate in a separating funnel and the upper phase was then loaded into the required units of the glass columns as the stationary phase. A sample dissolved in the lower phase (2 ml) was placed at the top of the first column, and the lower phase was pumped as the mobile phase by nitrogen pressure (2 atm) through the top of this column at a flow-rate of 7 ml/h. Fractions from the last column were collected in fractions of 4 ml each, and the peptide content in each fraction was determined by the method described by Yemm and Cocking[4].

In Sephadex G-10 gel chromatography, a sample dissolved in water (0.5 ml) was applied to a column (100 × 1.3 cm) and development was continued with water. Elution was carried out at room temperature at a flow-rate of 13 ml/h, and 2-ml fractions were collected. The peptide content in each fraction was determined by the method described above. Fractionated peptides were identified by comparison of their R_F values with those of authentic samples.

RESULTS

A mixture of H(L-Leu–Gly)$_n$OH (n = 1, 2 and 3) (5 mg each) was used for the separation experiment by DCCC as described above. The mobile phase was collected up to fraction number 177, and the stationary phase was then ejected from the last column by nitrogen pressure (2 atm). As shown in Fig. 1, the mixture of the model peptides was completely fractionated into three components. The components of peaks

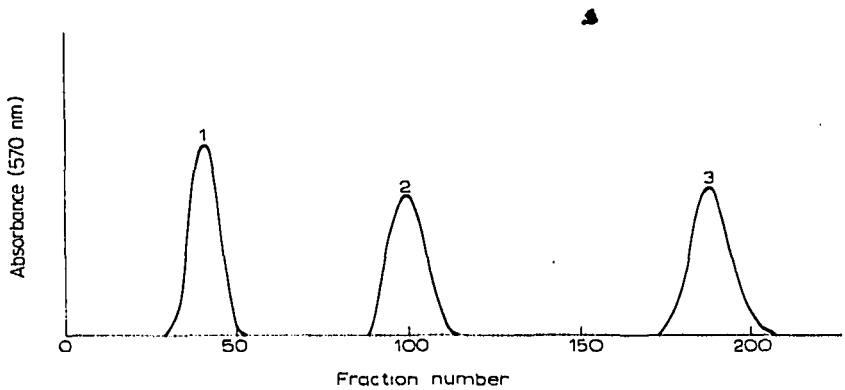

Fig. 1. Droplet countercurrent chromatogram of mono-, di- and tri-L-leucylglycine. Solvent, n-butanol–acetic acid–water (4:1:5). Each fraction is 4 ml. 1 = Mono-L-leucylglycine; 2 = di-L-leucyl glycine; 3 = tri-L-leucylglycine.

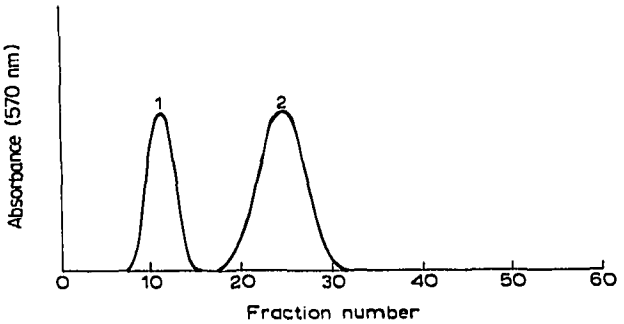

Fig. 2. Chromatography on a 100 × 1.3 cm column of Sephadex G-10 of mono-, di- and tri-L-leucylglycine. Solvent, water; flow-rate, 13 ml/h. Each fraction is 2 ml. 1 = Tri-L-leucylglycine; 2 = mixture of mono- and di-L-leucylglycine.

1, 2 and 3 were identified as H(L-Leu–Gly)OH, H(L-Leu–Gly)$_2$OH and H(L-Leu–Gly)$_3$OH, respectively.

In a separate experiment, a mixture of the three model peptides (1 mg each) was chromatographed on a Sephadex G-10 column. As shown in Fig. 2, two peaks were obtained. Peak 1 was identified as H(L-Leu–Gly)$_3$OH, but peak 2 was found to be a mixture of H(L-Leu–Gly)$_2$OH and H(L-Leu–Gly)OH. This result showed that the complete separation of the oligopeptides with a repeated sequence could not be obtained under these conditions.

It was concluded that, in comparison with gel chromatography with Sephadex G-10, DCCC was a more effective technique for the separation of these similar peptides. It appears to be suitable for the separation of synthetic oligopeptides.

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