ELSEVIER

Journal of Chromatography A, 771 (1997) 81–88

JOURNAL OF CHROMATOGRAPHY A

Peptide separation by pH-zone-refining countercurrent chromatography

Ying Ma, Yoichiro Ito*

Laboratory of Biophysical Chemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bldg. 10, Rm. 7N322, 10 Center Drive MSC 1676, Bethesda, MD 20892 1676, USA

Received 25 September 1996; revised 24 December 1996; accepted 3 January 1997

Abstract

Peptides without protecting groups have been successfully separated by pH-zone-refining countercurrent chromatography (CCC) using an ion-pair reagent, di-(2-ethylhexyl)phosphoric acid (DEHPA), as a modifier in the stationary phase. Preliminary studies indicated that two parameters, i.e., the DEHPA concentration in the stationary phase and hydrophobicity of the solvent system should be adjusted according to the hydrophobicity of the analytes. Hydrophobic and hydrophilic groups of dipeptides were each separated under optimized conditions. The method was successfully applied to gram-quantity separations of bacitracin complex and bovine insulin.

Keywords: pH-zone-refining countercurrent chromatography; Peptides

1. Introduction

Countercurrent chromatography (CCC) is a generic term for a form of support-free liquid–liquid partition chromatography. Being free of solid support in the separation column, it can provide some important advantages over other chromatographic methods such as high sample recovery and minimum denaturation. In the past, the method has been used for the separation and purification of various peptides [1].

pH-Zone-refining CCC is a new preparative separation method recently developed in our laboratory [2–5]. The method uses two reagents, a retainer in the stationary phase and an eluter in the mobile phase. It shares many features with displacement chromatography and provides various advantages over the conventional CCC technique such as an order of magnitude or in specific cases even more (see below) increase in sample loading capacity, elution of highly concentrated fractions, and detection and concentration of minor components. The method has been applied to the separation of a variety of compounds including both acidic and basic derivatives of amino acids [5–7] and peptides [5,8], hydroxyxanthene dyes [2,3,5,9–13], alkaloids [3–5,14], indole auxins [3,5], and structural [4,5,15], geometrical [3,5,16] and optical [3,5,17] isomers.

In this paper, pH-zone-refining CCC is applied for the first time to the separation of underivatized peptides by introducing a selectivity modifier or ion-pair reagent, di-(2-ethylhexyl) phosphoric acid (DEHPA) in the stationary phase in addition to a retainer in the stationary phase and an eluter in the mobile phase. Although our aim is to purify biologically active polypeptides, the optimization of the

*Corresponding author.

PII S0021-9673(97)00065-4

experimental conditions such as the DEHPA concentration and polarity of the solvent system are carried out using a set of dipeptides with a broad range in hydrophobicity. Preliminary data on gram-quantity separations of bacitracin complex and bovine insulin are presented.

2. Experimental

2.1. CCC apparatus

A commercial model (Ito Multilayer coil separator/extractor, P.C., Potomac, MD, USA) of the high-speed CCC centrifuge was used throughout the present studies. The basic design of the apparatus was given elsewhere [18].

The separation column was prepared in our laboratory by winding a single piece of 160 m×1.6 mm I.D. Tefzel tubing (Zeus Industrial Products, Orangeburg, SC) around the column holder hub making 16 layers with a 315-ml capacity.

The rotational speed of the apparatus was regulated with a speed controller (Bodine Electric Company, North Chicago, IL, USA). In the present studies an optimum speed of 800 rpm was used throughout.

2.2. Reagents

Methyl *tert.*-butyl ether, butanol, acetonitrile (HPLC grade), triethylamine (reagent grade) and hydrochloric acid (reagent grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Di-(2-ethylhexyl)phosphoric acid (DEHPA), bovine insulin and bacitracin were obtained from Sigma (St. Louis, MO, USA). Peptides including glycyl–tyrosine (gly–tyr), tyrosyl–glycine (tyr–gly), alanyl–tyrosine (ala–tyr), tyrosyl–alanine (tyr–ala), valyl–tyrosine (val–tyr), tyrosyl–valine (tyr–val), leucyl–tyrosine (leu–tyr), and tyrosyl–leucine (tyr–leu) were all from Aldrich (Milwaukee, WI, USA).

2.3. Preparation of solvent phases and sample solutions

A pair of solvent phases, one for organic stationary phase and the other for aqueous mobile phase are prepared from a basic solvent system composed of methyl *tert.*-butyl ether–*n*-butanol–acetonitrile–water (1:0:0:1, 4:0:1:5 or 2:2:1:5, v/v). For pH-zone-refining CCC, a pair of reagents, retainer base (triethylamine) is added to the organic stationary phase and an eluter acid (HCl) to the aqueous mobile phase. Separation of underivatized peptides further requires DEHPA in the stationary phase.

In order to prevent emulsification of the solvents during separation, the pH of the organic stationary phase should be adjusted towards the acidic side (pH 5–6) with HCl or TFA before introduction into the column. This may be done by acidifying the solvent either before or after the equilibration of the two-phase solvent system. In the former case a pair of two-phase solvent systems may be necessary, one for the stationary phase and the other for the mobile phase. Detailed compositions of the solvent systems used for each separation are described in the figure captions and Section 3.

The sample solutions of dipeptides were prepared by dissolving a sample mixture containing 100–500 mg of each component in 5 ml of the solvent consisting of equal volumes of the organic phase and unacidified aqueous phase. For bacitracin separation, a sample of 5 g was similarly dissolved in 60 ml (30 ml of each phase) of the solvent.

2.4. Separation procedure

The column was first partially filled with the organic stationary phase (~200 ml) free of DEHPA. This was followed by pumping 150 ml of the stationary phase containing DEHPA and triethylamine (retainer base), discharging an excess amount of the DEHPA-free stationary phase from the outlet of the column. After injection of the sample solution through the sample port, the acidified aqueous phase containing HCl (eluter acid) at 20 m*M* was eluted through the column at a flow-rate of 3.0 ml min^{-1} in the head-to-tail elution mode, while the column was rotated at 800 rpm. The effluent from the outlet of the column was continuously monitored with a UV monitor (Uvicord S, LKB Instruments, Bromma/Stockholm, Sweden) at 280 nm, then through a pH monitor cell (Orion, Boston, MA, USA) [19] and finally collected into test tubes at 2-min intervals (6.0 ml/tube) with a fraction collector (Ultrorac, LKB

Instruments). After the desired peaks were eluted, the apparatus was stopped and the column contents were collected into a graduated cylinder by connecting the inlet of the column to a nitrogen line at 80 p.s.i. The retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column.

2.5. Analysis of CCC fractions

The dipeptides were identified by TLC (AL SIL G/UV, Whittman, Kent, UK) using a solvent system of $CHCl_3$–MeOH–H_2O (95:5:0.1, v/v) and also by their standard partition coefficients, K_{std}, in a two-phase system composed of methyl *tert.*-butyl ether–DEHPA–aqueous solution containing ammonium acetate (0.1 *M*) and HCl (0.05 *M*) (9:1:10). The standard partition coefficients were determined as follows: An aliquot of each fraction (0.1 ml) was delivered into a test tube. Then 2 ml of the standard solvent system (1 ml of each phase) was added to each tube which was then vigorously shaken to equilibrate the contents. After the two layers were formed, 0.1 ml of each phase was diluted with 2 ml of methanol and the absorbance determined at 280 nm. The standard partition coefficient (K_{std}) was obtained by dividing the absorbance value of the upper phase by that of the lower phase.

The bacitracin fractions were analyzed by analytical HPLC (Shimadzu Scientific, Columbia, MD,

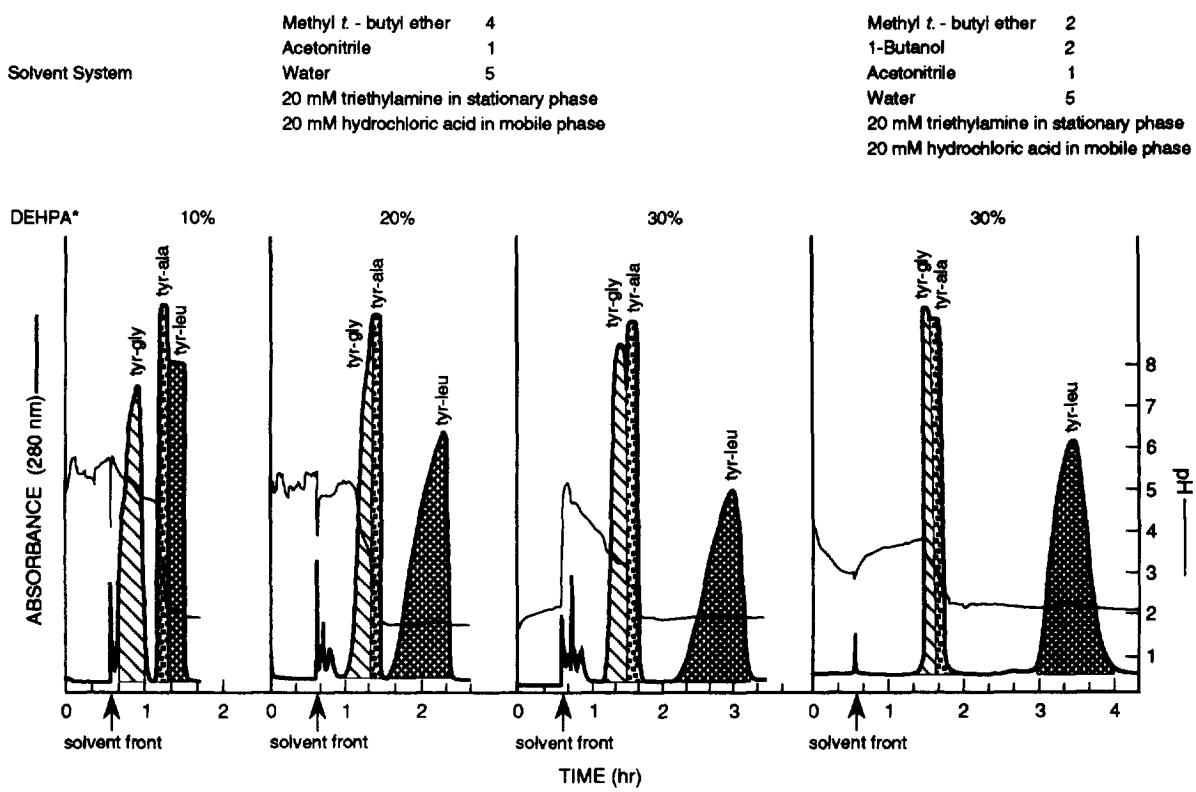

Fig. 1. Elution profile of three dipeptides with a broad range of hydrophobicity under 4 sets of experimental conditions. Experimental conditions are as follows: apparatus: commercial high-speed CCC centrifuge with 10-cm revolution radius; column: 1.6 mm I.D. Tefzel multilayer coil with 330-ml capacity; sample: tyr–gly, tyr–ala and tyr–leu each 100 mg in 5 ml solvent; stationary phase: upper organic phase of methyl *tert.*-butyl ether–*n*-butanol–acetonitrile–50 m*M* HCl (4:0:1:5 or 2:2:1:5) to which DEHPA (10–30%) and triethylamine (20 m*M*) were added; mobile phase: lower aqueous phase of methyl *tert.*-butyl ether–*n*-butanol–acetonitrile–water at the respective volume ratio to which 20 m*M* HCl was added; flow-rate: 3 ml min^{-1}; mobile phase: lower aqueous phase; revolution: 800 rpm; retention of the stationary phase: 63–67%.

USA) with a C_{18} column (3.9×200 mm) (Capcell Pak, Shiseido, Tokyo, Japan) eluted isocratically with 5% TFA and 40% acetonitrile aqueous solution.

3. Results and discussion

3.1. Separation of dipeptides

A series of studies was performed to optimize the separation of dipeptides using DEHPA [di-(2-ethylhexyl)phosphoric acid] in the organic stationary phase. The separations were performed with a two-phase solvent system composed of methyl *tert.*-butyl ether, *n*-butanol, acetonitrile and dilute HCl or water (4:0:1:5 or 2:2:1:5, v/v). Various amounts of DEHPA and triethylamine (retainer base) were added to the organic stationary phase and HCl (eluter acid) to the aqueous mobile phase. Two parameters – (1) DEHPA concentration in the organic stationary phase and (2) hydrophobicity of solvent system – were examined for the separation of both hydrophobic and hydrophilic groups of dipeptides. Effects of the DEHPA concentration were investigated using the solvent ratio of 4:0:1:5 by varying the amount of DEHPA from 10% to 30% in the stationary phase while the concentration of triethylamine (20 m*M*) in the stationary phase and HCl (20 m*M*) in the mobile phase were unaltered.

The results are shown in Fig. 1. At 10% DEHPA concentration, the trailing edge of the second and the leading edge of the third peaks were joined whereas the polar tyr–gly peak was completely isolated and eluted earlier. Increasing the DEHPA concentration to 20–30% resulted in a similar joining of the edges of the first and the second peaks while the hydrophobic tyr–leu peak was isolated and eluted much later. Increasing the polarity of the solvent system by modifying the phase composition improved the sharpness of the fused first and second peaks as shown in the right chromatogram (Fig. 1). The overall results of these preliminary studies indicate that both DEHPA concentration and solvent phase composition affect the elution of these analytes according to their hydrophobicities.

The hydrophobic dipeptides can be successfully separated at a 10% DEHPA concentration with 20 m*M* each of triethylamine and hydrochloric acid (experimental conditions shown in Fig. 1, left). Two pairs of hydrophobic dipeptides, each component 250 mg, were readily resolved as shown in Fig. 2.

Optimization of the parameters for separation of hydrophilic dipeptides required more challenge. In Fig. 3, two isomeric pairs of polar dipeptides were separated with a similar solvent system by varying both DEHPA concentration and solvent volume ratio while other experimental conditions (indicated above the chromatogram) were unaltered. At a 30% DEHPA concentration and the 4:0:1:5 volume ratio, the first and the second peaks formed a wide mixing zone as shown in the left chromatogram. Increasing the DEHPA concentration to 40% substantially nar-

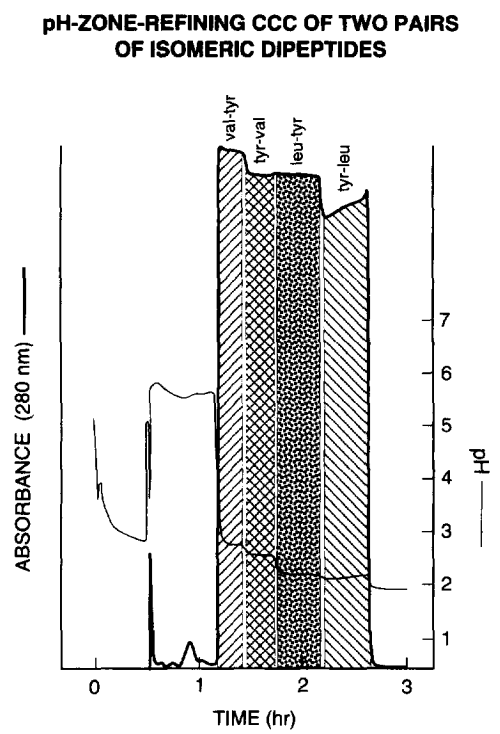

Fig. 2. Separation of 2 isomeric pairs of hydrophobic dipeptides by pH-zone-refining CCC. Experimental conditions are as follows: apparatus and column: see Fig. 1; sample: val–tyr, tyr–val, tyr–leu and leu–tyr each 500 mg in 30 ml solvent; stationary phase: upper organic phase of methyl *tert.*-butyl ether–acetonitrile–50 m*M* HCl (4:1:5) to which 20 m*M* triethylamine was added; mobile phase: lower aqueous phase of methyl *tert.*-butyl ether–acetonitrile–water (4:1:5) to which 20 m*M* HCl was added; flow-rate: 3 ml min^{-1}; mobile phase: lower aqueous phase; revolution: 800 rpm; retention of the stationary phase: 68%.

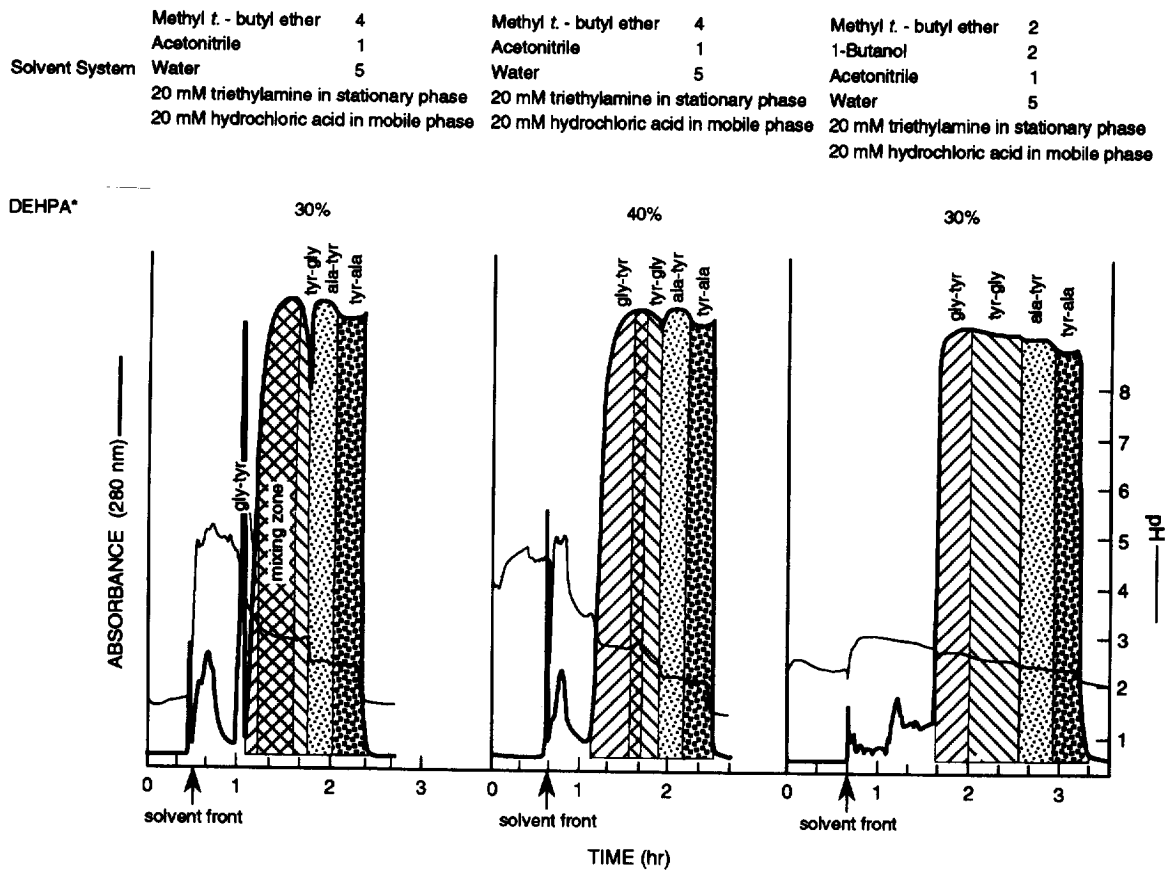

Fig. 3. Effects of DEHPA concentration and hydrophobicity of solvent system on separations of two isomeric pairs of polar dipeptides. Experimental conditions are as follows: apparatus and column: see Fig. 1; sample: gly–tyr, tyr–gly, tyr–ala and ala–tyr each 500 mg in 30 ml solvent; stationary phase: upper organic phase of methyl *tert.*-butyl ether–*n*-butanol–acetonitrile–50 m*M* HCl (4:0:1:5 or 2:2:1:5) to which DEHPA (30–40%) and 20 m*M* triethylamine were added; mobile phase: lower aqueous phase of methyl *tert.*-butyl ether–*n*-butanol–acetonitrile–water at the respective volume ratio to which 20 m*M* HCl was added; flow-rate: 3 ml min^{-1}; mobile phase: lower aqueous phase; revolution: 800 rpm; retention of the stationary phase: 67, 63 and 54% from left to right.

rowed the mixing zone as shown in the middle chromatogram. Finally, increasing the polarity of the solvent system by changing the volume ratio from 4:0:1:5 to 2:2:1:5 at a 30% DEHPA concentration resulted in a complete resolution of all components with minimum overlap as shown in the right chromatogram. Increased polarity of the solvent system caused a considerable increase in retention time of the analytes which may have largely contributed to the improved resolution between the polar components.

In the past, the same apparatus was used for the separation of similar isomeric sets of dipeptides using a similar polar solvent system but without a modifier in the stationary phase [20]. Comparison between the results obtained by the two CCC techniques clearly shows an important advantage of pH-zone-refining CCC over the conventional high-speed CCC technique especially for the preparative-scale separation of polar peptides. In the conventional high-speed CCC technique, tyr–gly and tyr–ala, each 50 mg, could be resolved but the separation of each isomer was not attainable even in a polar solvent system composed of methyl-*tert.*-butyl ether, *n*-butanol, acetonitrile and 1% TFA (2:2:1:5). With the present technique using DEHPA in the stationary phase, a 10-fold sample size (500 mg of each isomer) was well resolved in slightly over 3 h.

3.2. pH-zone-refining CCC of bacitracin complex

pH-Zone-refining CCC of bacitracin complex can be successfully carried out by the use of DEHPA (Fig. 4). Five grams of bacitracin complex consisting of multiple components were subjected to pH-zone-

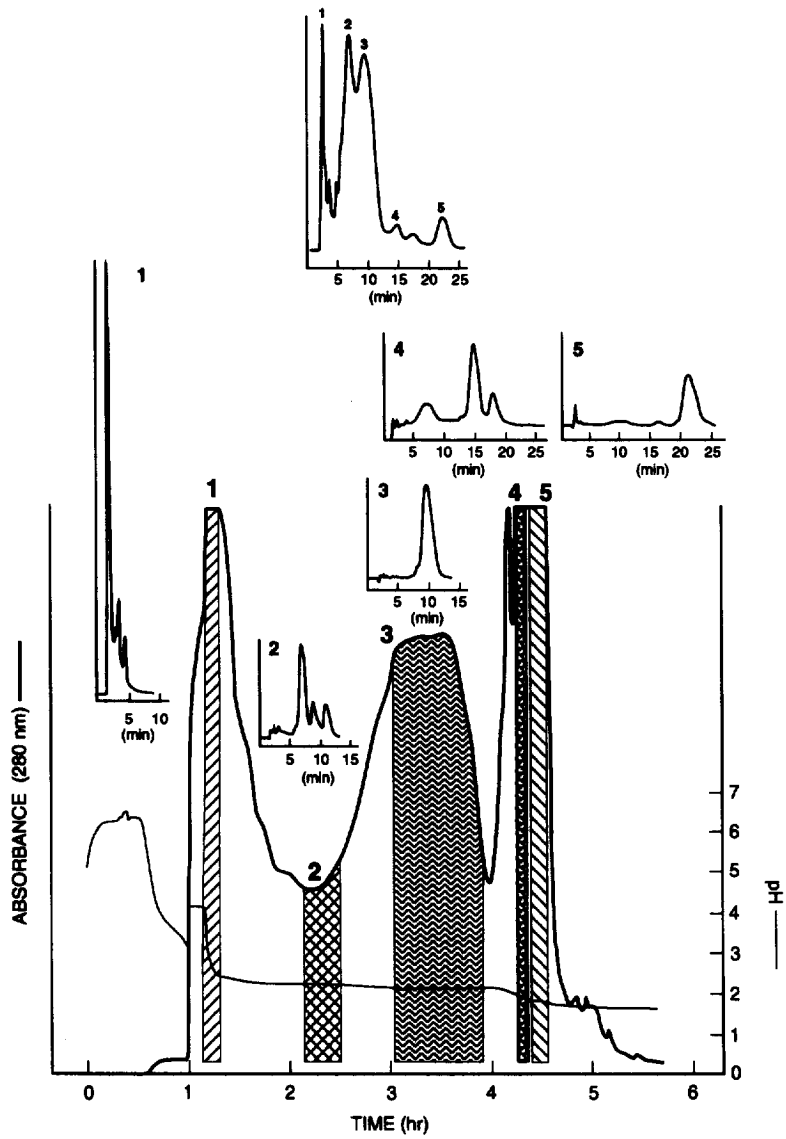

Fig. 4. Separation of bacitracin complex by pH-zone-refining CCC. Two major components, bacitracin A (peak 3) and F (peak 5) are purified from 5 g of the sample as shown by HPLC analyses. The top diagram shows HPLC analysis of the original sample. Experimental conditions are as follows: CCC apparatus and column: see Fig. 1; sample: bacitracin 5 g in 60 ml solvent; stationary phase: upper organic phase of methyl *tert.*-butyl ether–acetonitrile–50 m*M* HCl (4:1:5, v/v) to which 40 m*M* triethylamine was added; mobile phase: lower aqueous phase of methyl *tert.*-butyl ether–acetonitrile–water (4:1:5) to which 20 m*M* HCl was added; flow-rate: 3 ml min^{-1}; revolution: 800 rpm; retention of the stationary phase: 21%. HPLC analysis. instruments: Shimadzu HPLC set including LC-6A pump and SPD-2A detector; column: Capcell Pak C_{18}, 5 μm, 3.9×200 mm; mobile phase: 5% TFA+40% acetonitrile; flow-rate: 1 ml min^{-1}; elution mode: isocratic; detection: 234 nm.

refining CCC under the optimized experimental conditions. The UV tracing at 280 nm produced multiple peaks while the pH curve revealed a flat zone around pH 2. As shown by HPLC analysis, the two major components, bacitracins A and F, were isolated in peaks 3 and 5, respectively.

Bacitracin complex has been separated by high-speed CCC using a comparable multilayer coil centrifuge (Shimadzu Corporation, Kyoto, Japan). A sample size of 50 mg was resolved in 2–3 h with a solvent system composed of chloroform–ethanol–methanol–water at a volume ratio of 5:4:0:3 or 5:3:3:4 using the lower organic phase as the mobile phase [21]. The present method can increase sample loading capacity by 100 fold for isolation of two major components, bacitracins A and F.

3.3. pH-zone-refining CCC of bovine insulin

Using the present pH-zone-refining CCC technique, bovine insulin of 500 mg sample size has been successfully separated with a polar solvent system composed of methyl *tert.*-butyl ether–*n*-butanol–acetonitrile–10 m*M* HCl (2:2:1:5, v/v) by adding DEHPA to the organic stationary phase (Fig. 5). Mass spectrometric analysis indicated that the components eluted in the second (main peak) and the third peaks are only one mass unit different to each other, i.e. 5733.5 and 5734.5, respectively, suggesting that the deaminated form of insulin (the third peak) was completely resolved. The first peak eluting at the solvent front was found to be myoglobin, probably from contamination of the sample injection syringe. An attempt to reproduce the above results was unsuccessful because a newly acquired sample from the same source did not contain detectable amounts of deaminated insulin.

In the past, a small amount (1 mg) of bovine insulin was efficiently separated by the angle rotor coil planet centrifuge using a two-phase solvent system composed of butanol–2–3% dichloroacetic acid (1:1, v/v) [22,23]. Because of its characteristic physical properties such as interfacial tension and high viscosity, the above solvent system tends to produce emulsification and carryover of the stationary phase in the multilayer coil of the high-speed CCC instrument. Consequently, the high-speed CCC system failed to resolve the deaminated form of insulin from the main peak in 100 mg sample [23].

In addition to the above polypeptides, several synthetic polypeptides have been successfully purified using a similar solvent system containing DEHPA. A suitable partition coefficient for polar peptides can be obtained by increasing the DEHPA

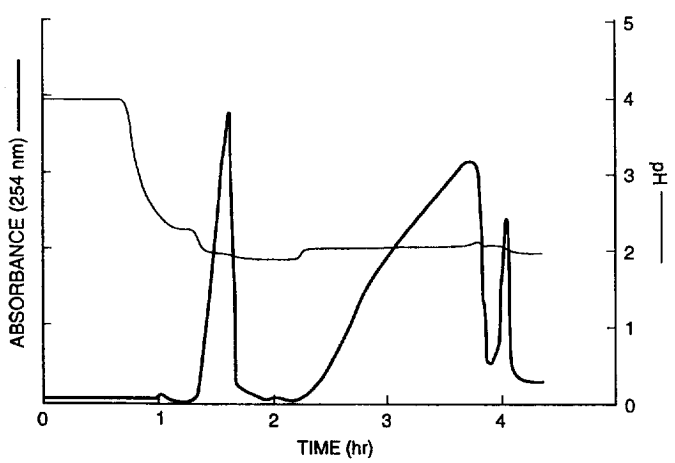

Fig. 5. Separation of bovine insulin by pH-zone-refining CCC. Experimental conditions are as follows: apparatus and column: described in the Fig. 1 caption; sample: 500 mg bovine insulin dissolved in a mixture of 12 ml upper phase and 3 ml lower phase; stationary phase: upper organic phase of methyl *tert.*-butyl ether–*n*-butanol–acetonitrile–10 m*M* HCl (2:2:1:5), to which 10% DEHPA and 20 m*M* triethylamine and 20 m*M* TFA were added; mobile phase: lower aqueous phase of the above solvent systems; flow-rate: 3 ml min^{-1}; revolution: 800 rpm; retention of the stationary phase: 28%.

concentration in the stationary phase according to the polarity of the analyte.

4. Conclusion

The result of the above studies indicates that pH-zone-refining CCC is very useful for the separation and purification of dipeptides once the polarity of the solvent system and/or the concentration of DEHPA are optimized. The present method can separate mg to gram quantities of dipeptides with minimum sample loss and decomposition, but of course the aim is to separate biologically important classes of peptides! We are confident that adjustment in the discussed parameters will allow useful separation of much larger peptides as well.

Acknowledgments

The authors wish to thank Dr. Henry M. Fales for his help in mass spectrometric analysis of insulin and also in editing the manuscript.

References

[1] M. Knight, in N.B. Mandava and Y. Ito (Editors), Countercurrent chromatography: Theory and Practice, Marcel Dekker, New York, 1988, pp. 583–616.
[2] A. Weisz, A.L. Scher, K. Shinomiya, H.M. Fales and Y. Ito, J. Am. Chem. Soc., 116 (1994) 704.
[3] Y. Ito, K. Shinomiya, H.M. Fales, A. Weisz and A.L. Scher, in W.D. Conway and R.J. Petroski (Editors), ACS Monograph on Modern Countercurrent Chromatography, 1995, Ch. 14, pp. 154–183.
[4] Y. Ito, in Y. Ito and W.D. Conway (Editors), High-Speed Countercurrent Chromatography, John Wiley and Sons, New York, 1996, Ch. 6, pp. 121–175.
[5] Y. Ito and Y. Ma, J. Chromatogr. A, 753 (1996) 1.
[6] Y. Ito and Y. Ma, J. Chromatogr. A, 672 (1994) 101.
[7] Y. Ma and Y. Ito, J. Chromatogr., 648 (1994) 233.
[8] Y. Ma and Y. Ito, J. Chromatogr. A, 702 (1995) 197.
[9] A. Weisz, D. Andrzejewski and Y. Ito, J. Chromatogr. A, 678 (1994) 77.
[10] A. Weisz, D. Andrzejewski, R.J. Highet and Y. Ito, J. Chromatogr. A, 658 (1994) 505.
[11] A. Weisz, D. Andrzejewski, K. Shinomiya and Y. Ito, in W.D. Conway and R.J. Petroski (Editors), ACS Monograph on Modern Countercurrent Chromatography, 1996, Ch. 16, pp. 203–217.
[12] K. Shinomiya, A. Weisz and Y. Ito, in W.D. Conway and R.J. Petroski (Editors), ACS Monograph on Modern Countercurrent Chromatography, 1996, Ch. 17, pp. 218–230.
[13] A. Weisz, in Y. Ito and W.D. Conway (Editors), High-Speed Countercurrent Chromatography, John Wiley and Sons, 1996, Ch. 12, pp. 337–384.
[14] Y. Ma, Y. Ito, E. Sokoloski and H.M. Fales, J. Chromatogr. A, 685 (1994) 259.
[15] Y. Ma, Y. Ito, D. Torok and H. Ziffer, J. Liq. Chromatogr., 17(16) (1994) 3507.
[16] C. Denekamp, A. Mandelbaum, A. Weisz and Y. Ito, J. Chromatogr. A, 685 (1994) 253.
[17] Y. Ma, Y. Ito and A. Foucault, J. Chromatogr. A, 704 (1995) 75.
[18] Y. Ito, CRC Crit. Rev. Anal. Chem., 17 (1986) 65.
[19] A. Weisz, A.L. Scher and Y. Ito, J. Chromatogr. A, 732 (1996) 283.
[20] M. Knight, M.O. Fagarasan, K. Takahashi, A.Z. Geblaoui, Y. Ma and Y. Ito, J. Chromatogr. A, 702 (1995) 207.
[21] K.-I. Harada, Y. Ikai, Y. Yamazaki, H. Oka, M. Suzuki, H. Nakazawa and Y. Ito, J. Chromatogr., 538 (1991) 203.
[22] Y. Ito and R.L. Bowman, Anal. Biochem., 65 (1975) 310.
[23] M. Knight, Y. Ito, J.L. Sandlin and A.M. Kask, J. Liq. Chromatogr., 9 (1986) 791.