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Note

Enantiomeric resolution of amino acids by high-performance ligand-exchange chromatography using a chemically modified hydrophilic porous polymer gel

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Resolution of enantiomeric amino acids by high-performance liquid chromatography (HPLC) has attracted much attention in recent years. Although there has been much progress in gas chromatography (GC) after the first success with chiral stationary phases by Gil-Av *et al.*[1], disadvantages such as the necessity for derivatization of the solute and the thermal instability of stationary phase still remain, and have stimulated interest in HPLC. Methods for resolving enantiomers by LC can be classified as follows: (1) pre-column derivatization of a solute to a diastereomer formed by a coupling reaction with another enantiomer and its resolution on an ordinary (achiral) stationary phase; (2) conversion of a solute to a diastereomer *in situ* by reaction with the resolving agent in eluent and its resolution on an ordinary stationary phase; and (3) resolution of a solute directly on a chiral stationary phase. Of course, it may be possible to combine (2) and (3) to improve the resolution.

Typical examples of type (1) are the separation of dipeptides by utilizing an ion exchange[2], a porous polymer[3] and chemically bonded silica gel C_8 (ref. 4) and C_{18} (ref. 5) as stationary phases. These need complicated pre-treatment involving derivatization to a diastereomer such as a dipeptide.

The use of stationary phase–resolving agent pairs in the eluent [class (2)] became widespread, *e.g.*, ion-exchanger[6] or ODS-silica[6–8] and Cu^{2+}–proline complex, ODS-silica and L-aspartyl-L-phenylalanine methyl ester metal complex[9], ODS-silica and Cu^{2+}–L-aspartylcyclohexylamide[10] and C_8-silica and octyldiethylenetriamine metal complex[11]. It was possible to resolve underivatized enantiomers of amino acids directly, except for the last example above, in which amino acids were resolved as dansyl derivatives. Advantageously large resolution factors have been often attained in these methods[6,7,11,12]. However, most resolving agents show intense UV absorption, which prevents conventional detection, the expensive resolving agent has to be involved in the eluent all the time and additionally its optical purity must be strictly controlled.

In methods of type (3), ligand-exchange chromatography has been extensively adopted, using packing materials with chemically bonded L- (or D-)amino acids and further loaded with a metal ion such as copper(II) on its surface as the chiral stationary phase. Chloromethylated polystyrene gel[13,14], polyamide gel[15] and silica

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gel[16,17] have been used as supporting matrices and L-proline has been successfully grafted in most instances. Although enantiomers of amino acids can be resolved directly without any derivatization of the solutes, the inclusion of copper(II) ions in the eluent is necessary in order to stabilize the elution and it is not necessarily easy to attain a sufficient column efficiency owing to the slow mass transfer, limited by ligand exchange. There have also been some direct applications that were classified as type (3), but they did not use the help of ligand exchange. Enantiomers of N-acylamino acid esters were resolved by normal-phase chromatography using silica gel chemically modified with N-acylvaline[18], and 3,5-dinitrobenzoyl derivatives of amino acid enantiomers were resolved by normal-phase chromatography using silica gel grafted with anthryltrifluoromethyl alcohol[19]. However, these resolutions require elaborate pre-treatment for derivatization of the amino acids.

In this paper, we describe ligand-exchange chromatography using a hydrophilic gel for high-performance gel filtration. TSK2000PW (Toyo Soda, Tokyo, Japan) was employed as a base matrix. Although full information about the composition and structure of the gel is not available, it is known to be a polyethylene glycol and has primary alcohol groups on its surface[20], which can readily be activated and chemically modified. Other features of this gel are high porosity, hydrophilicity, low specificity in adsorption, high mechanical strength and small spherical diameter, desirable for high column efficiency.

EXPERIMENTAL

Typical procedure for chemical modification of the gel

Activation of gel. A 10-volume of TSK2000PW gel (diameter 10 μm) was washed successively with distilled water, 0.01 *M* hydrochloric acid, distilled water and 0.2 *M* sodium hydroxide solution, then suspended in 100 ml of 0.2 *M* sodium hydroxide solution. To the suspension were added 4 ml of epichlorohydrin and the mixture was stirred for 24 h at room temperature.

Bonding of amino acid. The activated gel was washed thoroughly with 1 l of 0.2 *M* sodium carbonate buffer solution (pH 10.8). Then 5 mmole of L-amino acid were dissolved in 100 ml of the same buffer solution and to this solution was suspended the washed, activated gel and the mixture was stirred for 72 h at room temperature. After the reaction, the gel was washed successively with distilled water, 0.01 *M* hydrochloric acid and distilled water.

Analysis of bonded phase. Nitrogen analysis by the Kjeldahl method gave the following amounts of bonded amino acid: 140 μmole/g of dried gel for L-phenylalanine and 220 μmole/g for L-tryptophan and L-histidine. The amino acid-bonded gel turned blue on treatment with a copper(II) solution.

Column and high-performance liquid chromatography

The stainless-steel column (15 × 0.4 cm I.D.) was packed with copper-loaded amino acid-bonded gel using a slurry in 0.1 *M* sodium chloride solution. The high-performance liquid chromatograph was equipped with a Milton Roy Constametric IIG constant-flow pump using a flow-rate of 0.2–0.8 ml/min, a Uvidec-100 variable-wavelength UV detector (Japan Spectroscopic, Tokyo, Japan) monitoring at 210 nm, and a Rheodyne Model 7120 with syringe-loading sample injector. The eluent was

1/15 *M* phosphate buffer prepared with distilled water, adjusted to the appropriate pH and containing copper(II) ion.

RESULTS AND DISCUSSION

Enantiomeric resolution was accomplished with the gels bonded with L-phenylalanine, L-tryptophan and L-histidine (the following abbreviations are employed for copper(II)-loaded gels: Cu/L-Phe2000PW, Cu/L-Trp2000PW and Cu/L-His2000PW, respectively). The chemically modified gels without copper(II) were unable to resolve enantiomers of amino acids.

As the amount of copper(II) loaded on the modified gel depends on pH, the elution of amino acids strongly depends on pH. The pH dependences of the capacity ratio, k', and resolution factor, $\alpha_{D/L}$ ($\alpha_{D/L}$ is defined by ratio of k'_D to k'_L), are shown in Fig. 1. Whereas k' increases rapidly with increase of pH, $\alpha_{D/L}$ remains fairly constant. The adequate pH range for the k' of each amino acid was narrow. An appropriate pH should be chosen for each kind of amino acid as a compromise of speed and separation within the limitations of column efficiency.

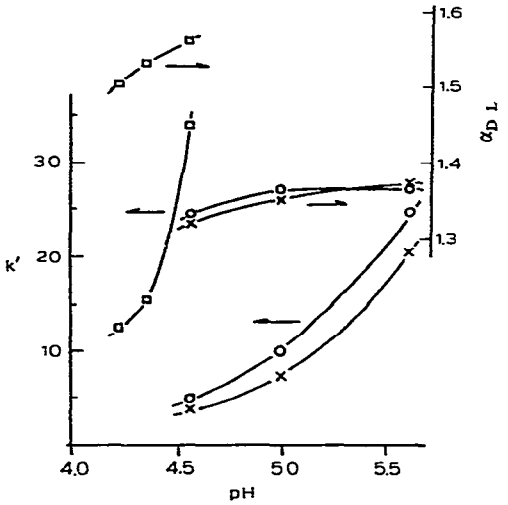

Fig. 1. Dependences of capacity ratio (k') and resolution factor ($\alpha_{D/L}$) on pH. Column, Cu/L-Trp2000PW; eluent, 1/15 *M* phosphate buffer–$5 \cdot 10^{-5}$ *M* Cu^{2+}; flow-rate, 0.53 ml/min. ○, Asparagine (L- for k'); ×, threonine (L- for k'); □, histidine (D- for k').

It is difficult to keep the column stable without copper(II) in the eluent, because copper is gradually eliminated from the column, particularly at low pH. The dependences of k' and $\alpha_{D/L}$ on the concentration of copper(II) are shown in Fig. 2. $\alpha_{D/L}$ was constant over the range studied and only a slight variation in k' was observed. As high concentration of copper(II) prevent the satisfactory use of UV detection, an eluent containing $5 \cdot 10^{-5}$ *M* copper(II) was employed in this work.

The dependences of k' and $\alpha_{D/L}$ on flow-rate were negligibly small over the range studied (0.2–0.8 ml/min).

The data given in Table I were thus obtained with the three kinds of modified

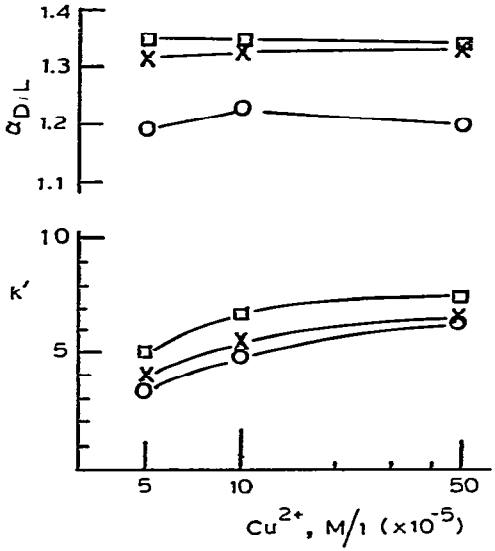

Fig. 2. Dependence of k' and $\alpha_{D/L}$ on the concentration of Cu^{2+} in the eluent. Column, Cu/L-Trp2000PW; eluent, 1/15 *M* phosphate buffer (pH 4.56); flow-rate, 0.53 ml/min. O, Glutamine; ×, threonine; □, asparagine.

gels. The modified gels showed individual characteristics depending on the kind of amino acids bonded. Neutral, acidic and aromatic amino acids could be resolved but basic amino acids could not. The order of elution of enantiomers for aromatic amino acids was the opposite of those for neutral and acidic amino acids obtained with Cu/L-Phe2000PW or Cu/L-Trp2000PW. On the other hand, Cu/L-His2000PW provided the opposite elution order to those obtained with the other two gels. D,L-Histidine could not be resolved with Cu/L-Phe2000PW but could be with the other two gels. D,L-Aspartic acid, which normally is difficult to resolve, could be well resolved with Cu/L-Phe2000PW.

Examples of resolution for acidic amino acids are shown in Fig. 3. The variation of the chromatogram with column temperature is shown in Fig. 4a. Increasing the column temperature improved the column efficiency, but the separation could not be always improved. The relationship between $\alpha_{D/L}$ and column temperature is shown in Fig. 4b. The lower the temperature, the better was the resolution factor.

The enthalpies of formation of the complexes formed on the gel were evaluated as -1.10 and -1.63 kcal/mole for L- and D-asparagine, respectively, from the plot of ln k' *versus* $1/T$, and the entropies of formation were calculated to be -1.23 and -1.24 cal/°K·mole, respectively. Hence the enthalpy term is assumed to be the determining factor for enantiomeric resolution in this work.

The column efficiency was fairly good. The HETP value was calculated as 0.25 mm for L-asparagine at room temperature, and well shaped symmetrical peaks were obtained. Most amino acids were resolved within several minutes. Basic amino acids such as lysine and arginine were not retained on these columns. On the other hand, acidic amino acids were strongly retained. This behaviour was contrary to the expectation that the predominant surface functionality of the chemically modified gels

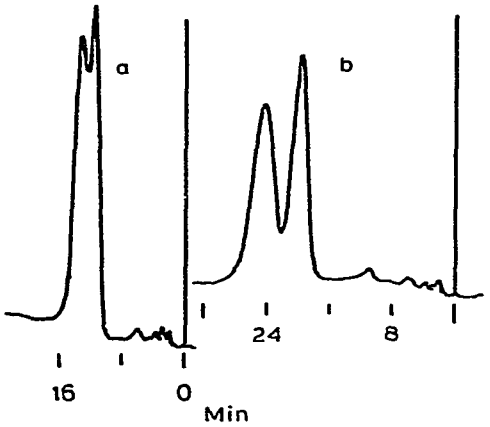

Fig. 3. Examples of enantiomeric resolution for acidic amino acids. Column, Cu/L-Phe2000PW (10 μm), 15 × 0.4 cm I.D.; eluent, 1/15 *M* phosphate buffer (pH 4.17)–$5 \cdot 10^{-5}$ *M* Cu^{2+}; flow-rate, 0.53 ml/min; UV detection, 210 nm; 0.02 a.u.f.s. (a) D,L-Glutamic acid; (b) D,L-aspartic acid.

TABLE I

CAPACITY RATIO (*k'*) AND RESOLUTION FACTOR ($\alpha_{D/L}$) FOR AMINO ACIDS RESOLVED BY THREE KIND OF COLUMNS

Eluent: 1/15 *M* phosphate buffer (pH 4.56)–$5 \cdot 10^{-5}$ *M* Cu^{2+}.

Amino acid	*Enantiomer*	*Cu/L-Phe2000PW*		*Cu/L-Trp2000PW*		*Cu/L-His2000PW*	
		k'	$\alpha_{D/L}$	*k'*	$\alpha_{D/L}$	*k'*	$\alpha_{D/L}$
Alanine	L	1.23	1.12				
	D	1.38					
Valine	L	1.62	1.15	1.30	1.15		
	D	1.86		1.49			
Glutamine	L	2.35	1.19	3.39	1.22		
	D	2.79		4.12			
Leucine	L	2.40	1.17	1.69	1.14		
	D	2.80		1.93			
Serine	L	2.59	1.23	3.24	1.19		
	D	3.19		3.86			
Threonine	L	3.36	1.36	4.08	1.32		
	D	4.55		5.40			
Asparagine	L	3.68	1.34	5.04	1.33		
	D	4.95		6.68			
Phenylalanine	L	2.66*	0.89	12.7	0.92	4.78	1.14
	D	2.39*		11.6		5.47	
Tyrosine	L	4.96*	0.89	22.4	0.75	8.26	1.31
	D	4.43*		16.9		10.9	
Tryptophan	L	17.5*	0.76				
	D	13.3*					
Glutamic acid	L	4.49*	1.17				
	D	5.25*					
Aspartic acid	L	8.26*	1.26				
	D	10.4*					
Histidine	L			20.6	1.53	3.41	0.71
	D			31.7		2.43	

* pH 4.17.

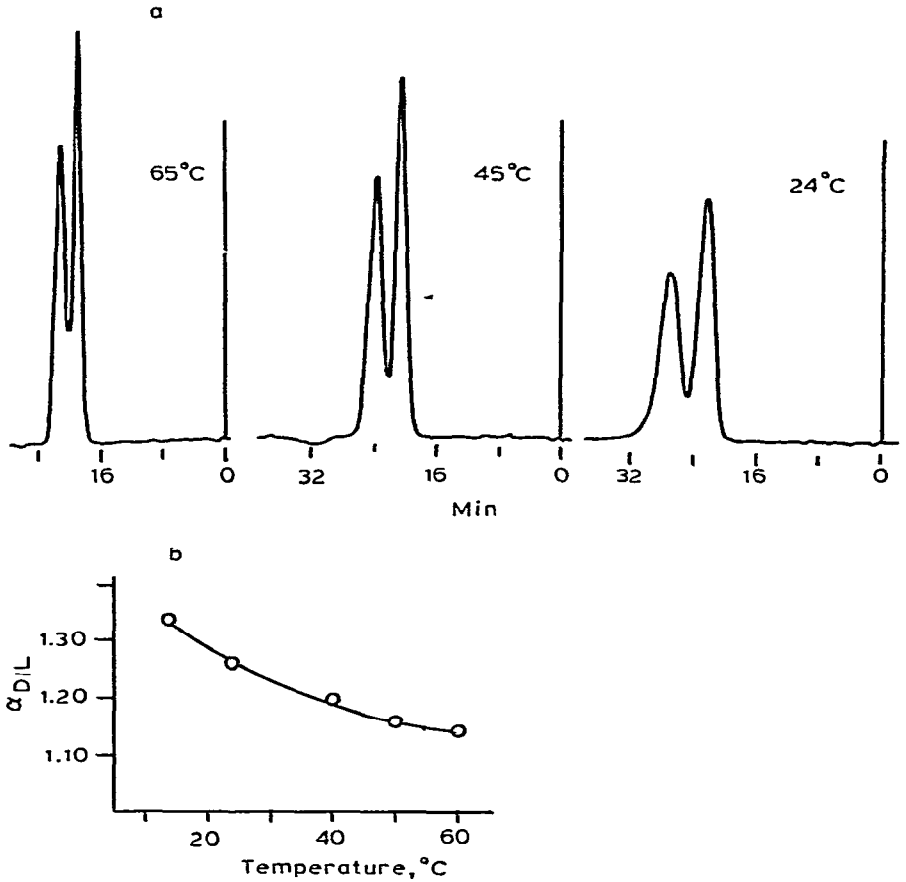

Fig. 4. Temperature dependence of resolution. (a) Variation of chromatogram with temperature; (b) resolution factor *versus* temperature. Column, Cu/L-Phe2000PW (10 μm, 15 × 0.4 cm I.D.); sample, D,L-asparagine; eluent, 1/15 *M* phosphate buffer (pH 4.56)–$5 \cdot 10^{-5}$ *M* Cu^{2+}; flow-rate, 0.27 ml/min.

should be the carboxylic acid of the attached amino acid. These results can presumably be ascribed to an unknown basicity remaining on the original gel. This is not an obstacle in practice. However, detailed information about the gel structure should be elucidated as the base matrix is no less important for enantiomeric resolution than an attached functionality. This aspect requires further work.

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