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DIRECT RESOLUTION OF α-AMINO ACID ENANTIOMERS BY LIGAND EXCHANGE: STEREOSELECTION MECHANISM ON SILICA PACKINGS COATED WITH A CHIRAL POLYMER

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SUMMARY

A chiral polymer easily obtained by treatment of a linear polyacrylamide with L-proline was adsorbed on a silica support treatment of a linear polyacrylamide with L-proline. After complexation with copper(II), and in spite of a rather low efficiency due to slow ligand exchange, such packings are very convenient for the resolution of amino acid enantiomers elute[1] in water (relative retentions, k'_L/k'_D, higher than 2 may be observed). Other eluents may also be used. The chiral polymer was studied in solution by potentiometry and nuclear magnetic resonance spectroscopy (^{13}C relaxation measurements). A model of the complex involved in the stereoselection is proposed which accounts for the chromatographic results obtained.

INTRODUCTION

Direct resolution of α-amino acids by column liquid chromatography has been progressively improved during the last twelve years. Some experiments have recently been performed with a chiral eluent, but most studies employ a chiral stationary phase, generally a cross-linked macromolecular compound or sometimes a grafted phase (see reviews[1,2]). The few packings which are efficient in high-performance liquid chromatography (HPLC) (see refs. 1 and 2) are difficult to prepare and are expensive. Moreover organic gels, because of their swelling ability, may be damaged under high pressure and are not compatible with every type of eluent.

We describe in this paper an inexpensive, versatile silica-modified phase which shows good selectivity for α-amino acids. We have used this packing to explain the separation mechanism of enantiomers by ligand-exchange chromatography.

Previously[3–6] we showed that an acrylamide type gel, when first treated with L-proline and then complexed by cupric ions, can give a complete resolution of about ten α-amino acids in less than 1 h (for a column length of 25 cm or less). We then prepared another stationary phase from linear polyacrylamide and L-proline. The packing is obtained by simple coating of silica beads with this chiral polymer.

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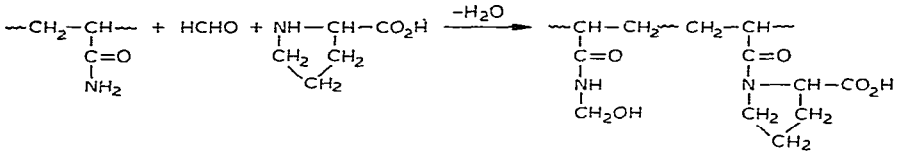
The reaction of polyacrylamide and L-proline is carried out in the presence of formaldehyde:



The polymer is characterized by the mean number, p, of proline residues per monomer unit or by its capacity, x, in amino acid (expressed in mequiv./g of dry polymer). The chiral macromolecule is used after complexation by cupric ions and the extent of uptake, r, of cupric ion by the proline graft in the stationary phase ($0 < r < 0.5$) has to be taken in account.

EXPERIMENTAL

Stationary phase

To 7 g of polyacrylamide ($\bar{M}_n = 23{,}000$) dissolved in 50 ml of water, were added successively the following reagents: 0.68 g of potassium hydroxide (in 10 ml of water); 4.9 g of formaldehyde (in the form of polyoxymethylene) and 2.38 g of L-proline. The mixture was stirred for 1.5 h at room temperature and finally neutralized by hydrochloric acid. The capacity in proline was obtained by titration of this purified polymer. We think that the crude reaction mixture may be used directly (after neutralization) for the coating of the silica beads.

These conditions yield a capacity for proline of $x = 0.86$ mequiv./g. By increasing the concentration of the reactants and the reaction time, polymers with capacities in the range of 0–4.2 mequiv./g may be obtained (*i.e.*, p values between 0 and 0.90)[6].

Packing material

The polymers obtained were adsorbed on mineral beads. LiChrosorb (E. Marck, Darmstadt, G.F.R.) and Spherosil (Rhône Poulenc, Paris, France) were tested. The best results were obtained with silica Spherosil XOA 600 (particle diameter 5–7 μm).

For a 15-cm column, 400 mg of polymer (dissolved in 15 ml of water) were used. To this solution were added 2 g of silica beads (in suspension in 10 ml of water) and the mixture was stirred for 15 min. The unadsorbed polymer was removed by filtration of the beads, and by washing them with water on the filter. The packing was then complexed by stirring with a large excess of cupric ions in a buffer (generally acetate). After filtration and washing with water (pH 6–7) the stationary phase is ready to be packed.

The degree of loading of the beads can be deduced from a titration of the non-adsorbed polymer or from the capacity for cupric ions. Saturation in Cu(II) ($r = 0.5$) was obtained with a buffer of pH > 5[5], typically, after contact with Cu(II) and glutarate buffer (pH 5.4). The beads were washed with water (pH = 6–7), the Cu^{2+} was displaced in acid medium and titrated (spectrophotometric titration with diethyldithiocarbamate at 450 nm gives good results).

Packing and chromatographic apparatus

The columns were packed in water (2 g of packing per 20 cm^3 of water) under pressure (400 bars) with an Haskel MCP 110 pump. A Waters ALC 201 chromatograph with an U6 K injector, R 401 refractometer, a MC 440 spectrophotometer and a Perkin-Elmer 241 MC polarimeter was used.

RESULTS

Extent of loading

The weight of polymer adsorbed per gram of silica (τ, expressed in percent) increases with the polymer concentration in the solution and becomes almost constant (τ = 6–7%) when the concentration is greater than 10% (20% was used for the preparation of our packings).

Column efficiency

Spherosil (5–7 μm) is said to give an efficiency of about 7000–8000 plates for a 10 cm column[7]. After coating with polymer, the efficiency strongly decreases: we found about 1200 plates with naphthalene (eluted in *n*-hexane); for the elution of α-amino acids in water, the efficiency is unfortunately very low (about 100 plates for 10 cm). This behaviour is not due to the packing conditions but is a consequence of the slowness of the ligand-exchange kinetics, as pointed out previously[4,8,9].

Influence of the amount of sample

Although the loading of stationary phase is higher than for classical grafted packings, the k' values are very sensitive to the amount of the injected sample. Typically, for D,L-valine eluted in pure water (1 ml/min) on a 10 × 0.48 cm I.D. column, the capacity factor of the D isomer shifts from 3 to 5.5 when the amount of injected sample decreases from 1 mg to 0.15 mg. On the other hand, the selectivity factor, α (ratio of the capacity factors fro the two isomers, k'_L/k'_D), is practically constant when the column is not overloaded.

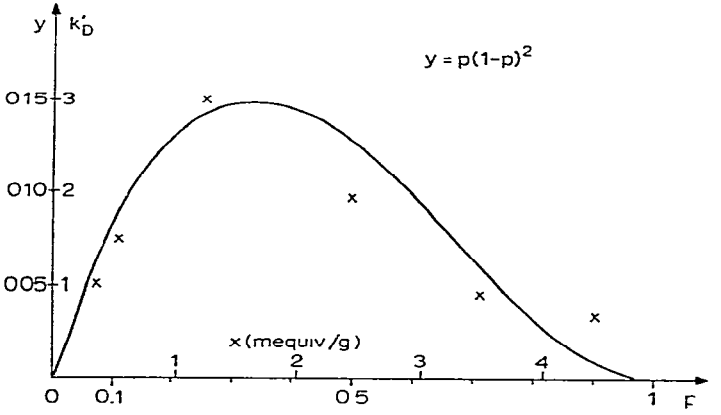


Fig. 1. Dependence of capacity factor, k'_D, of D-valine *versus* the capacity, x, or the extent of substitution, p, of various stationary phases. Eluent: 0.1 *M* KNO_3; flow-rate, 1 ml/min. Column: 10 × 0.48 cm I.D. ×, Experimental points; ——, the curve $y = p(1-p)^2$, arbitrary units on y axis.

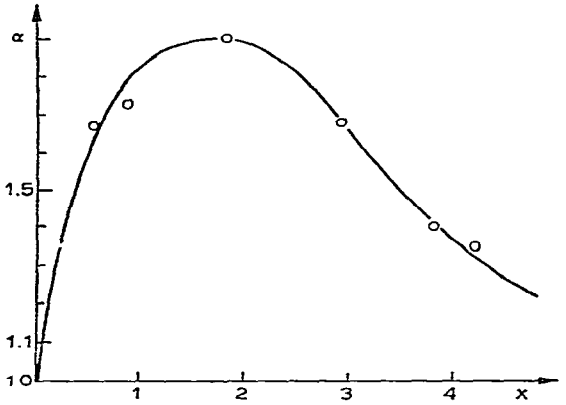


Fig. 2. Dependence of selectivity factor, α, *versus* the graft capacity, x, of the packings. Solute: D,L-valine. Other details as in Fig. 1.

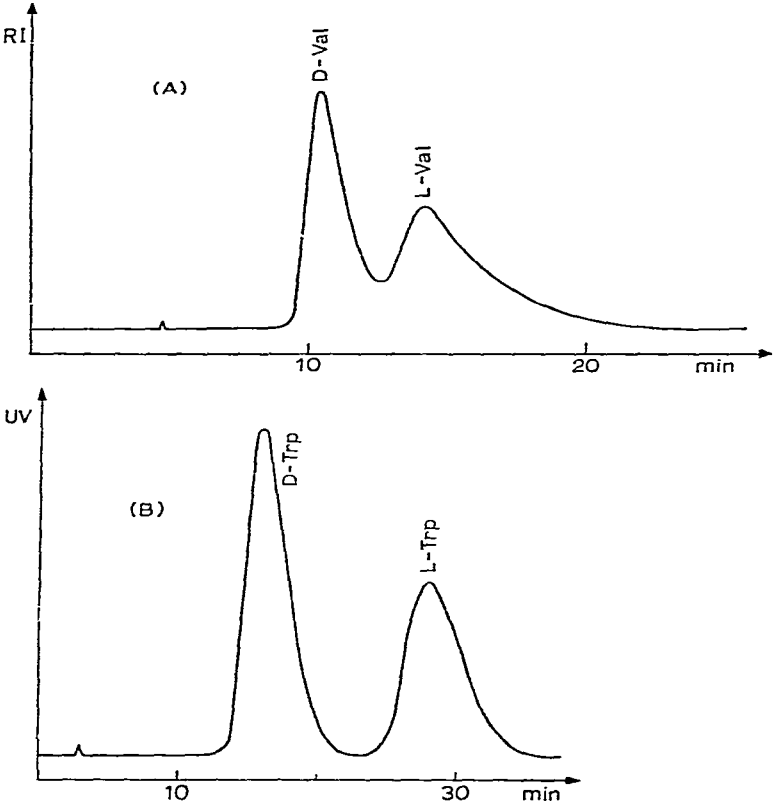


Fig. 3. Chromatograms of amino acids. (A), Column 10 × 0.48 cm I.D., eluent 0.5 ml/min water, $r = 0.3$, $x = 1.8$ mequiv./g, injection of 3 μl of a saturated water solution of D,L-valine, refractive index (RI) detection; (B), column as in (A), eluent 0.1 M KNO_3 (1 ml/min), $r = 0.4$, $x = 0.56$ mequiv./g, injection of 0.1 μl of a saturated water solution of D,L-tryptophan, UV detection.

Influence of the degree of complexation

As previously described[5], the retention of amino acid solutes considerably decreases with the mean number, r, of cupric ions per graft.

Influence of the degree of grafting

Curiously, the capacity factor of a given solute does not continuously increase with the number of sites for complexing in the column, *i.e.*, with the capacity, x, of the polymer. A maximum is observed for $x \approx 2$ mequiv./g (Fig. 1). The same behaviour is observed for α as a function of x (Fig. 2).

Influence of the solute

From our results on the effect of the sample size on capacity factors, it is difficult to compare k' values of different solutes (the amount of the sample is probably not the most important parameter, and the number of injected molecules should also be considered). α values are practically independent of the sample size. The results concerning thirteen amino acids are given in Table I and two examples of chromatograms are shown in Fig. 3.

TABLE I

CAPACITY FACTORS, k', AND SELECTIVITY FACTORS, α, FOR SOME SOLUTES

Column: 25 × 0.48 cm I.D.; $r = 0.4$; $x = 0.86$ mequiv./g. Eluent: 0.1 M KNO_3 with $0.2 \cdot 10^{-4} M$ Cu(II); flow-rate, 0.4 ml/min.

Solute (D,L form)	*Amount injected**	k'_D	k'_L	α
Alanine	$\approx 10^{-4}$g	≈ 1.80	≈ 1.80	≈ 1 (but > 1)
Aminobutyric acid	$\approx 10^{-3}$g	1.49	1.94	1.25
Norvaline	10 µl	1.80	2.34	1.30
Norleucine	5 µl	3.94	4.81	1.20
Valine	10 µl ≈ 10^{-3}g	1.76	3.09	1.75
Isoleucine	5 µl	2.84	4.37	1.55
Proline	≈ 10^{-3}g	3.20	2.06	0.70
Phenylalanine	10 µl ≈ 10^{-4}g	6.10	12.35	2.00
Tyrosine	10 µl	16.31	36.27	2.20
Tryptophan	50 µl	15.73	38.28	2.45
Asparagine	10 µl	3.29	4.16	1.25
Serine	10 µl ≈ $5 \cdot 10^{-4}$g	2.27	4.20	1.85
Threonine	3 µl	2.80	5.31	1.90

* All volumes were of saturated solutions.

DISCUSSION

Stereoselection mechanism

The complexation properties of our grafted linear polymers and of corresponding low-molecular-weight models were studied by potentiometry and NMR spectroscopy[6,10]. In solution, at pH > 5.5, the main complexed species are the GMG, SMS and GMS forms where G is the graft (L-proline), M the metal ion (Cu^{2+}) and S the solute. The equilibrium involved in the stereoselection is the exchange between a graft and a solute molecule:

$$GMG + S \overset{K}{\rightleftharpoons} GMS + G$$

The constant K varies according to the solute form (D or L). For instance with S = valine and a polymer for which $x = 0.86$ mequiv./g, in solution in 0.1 M KNO_3, at 25°C, $K_L/K_D = 1.5$ (error in each pK value is estimated as $\pm$ 0.05 pK unit); with S = proline, $K_L/K_D = 0.8$. Although these differences are small (but significant) they are consistent with the chromatographic results: the more strongly complexed is the isomer, the larger is the k' value[6,10].

Effect of the degree of grafting

It also appears that the stability of the GMG complex increases with the degree of grafting of the polymer; at the same times the difference between K_L and K_D decreases. For instance, regarding the complexation constants, a polymer with $x = 3.83$ forms a complex with copper which is about 1000 times more stable than that formed by a polymer with $x = 0.86$ mequiv./g; at the same time the values of K_L/K_D are 1.35 and 1.5 for valine and 0.9 and 0.8 for proline respectively. This suggests that different types of grafts are formed in the stationary phase (see Fig. 4): if in the GMG complex the two grafts are located on vicinal sites, the complex is very stable, more stable than a complex with no vicinal grafts.

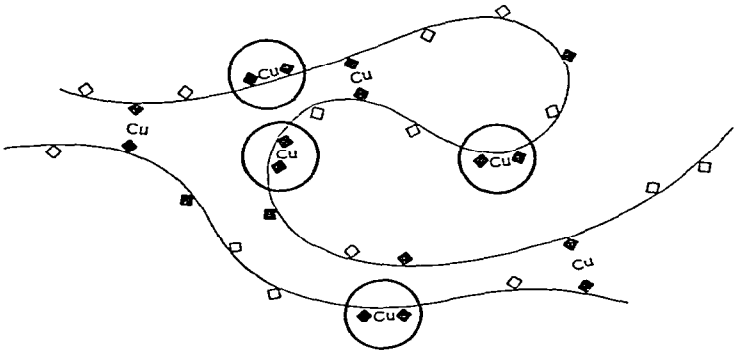


Fig. 4. The different kinds of copper complexes involved in the grafted polymer chains. ◆, L-proline graft; ◇, ungrafted site. The circles represent complexes involving vicinal grafts.

The mean number, p, of grafts per monomer unit is also the probability of finding a graft on a given repeat unit[11], and $(1 - p)$ is the probability of finding an ungrafted unit. Consequently, the probability of finding a grafted unit with free sites in vicinal position (neighbouring units being free) is $p(1 - p)^2$.

If we assume that the "vicinal" GMG complexes are too stable to participate in the ligand exchange, only the complexes involving isolated grafts are efficient for the retention and the separation on the chromatographic column. Hence, the k' values would be described by an equation of the $p\,(1 - p)^2$ form (which can be expressed in terms of x by taking in account the molecular weights of grafted and free sites). This is fairly well verified (see Fig. 1). The maximum value of k' is observed for $p = 0.33$, *i.e.*, $x \approx 2$ mequiv./g.

Calculation of k'

The value of k' was determined[6] by taking in account the various equilibria (dissociation, complexation, partition) involved

$$k'_D = (V_S/V_0)\, k\, (1 + K_D Z) \qquad k'_L = (V_S/V_0)\, k\, (1 + K_L Z)$$

where $Z = [r/(1 - 2r)]p(1 - p)^2$, and V_s and V_0 are respectively the volume of the stationary phase and the column void volume. k is the distribution coefficient by Donnan effect, *i.e.*, the concentration of uncomplexed solute in the stationary phase divided by the concentration of the solute in the mobile phase. This relationship not only describes k' as a function of p but also accounts for the strong increase in retention when r tends to 0.5, and gives a simple expression of α:

$$\alpha = k'_L/k'_D = (1 + K_L Z)/(1 + K_D Z)$$

α is close to 1 (no separation) when $K_L Z$ and $K_D Z < 1$, that is when there is no copper ($r = 0$) or when the extent of grafting is very low ($p \approx 0$) or, on the contrary, almost) complete ($p \approx 1$). On the other hand, if $K \gg 1$, α is virtually constant ($\alpha_{max.} = K_L/K_D$) over a large range of p and r. Such behaviour is observed (see Fig. 2: α tends to zero for extreme values of the extent of grafting and the curve has a flat maximum).

The maximum resolution between two isomers, $R_{max.}$, may easily be predicted by using the basic relationship

$$R = \left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'_2}{1 + k'_2}\right)N^{1/2}$$

and the above expressions for k'_L and α yield:

$$R_{max.} = \frac{N^{1/2}}{4}\left(1 - \frac{1}{\alpha_{max.}}\right)$$

The unusually strong dependence of k' on the amount of injected sample may also be derived from the k' expression:

$$k' = \frac{V_S}{V_0} k \left(1 + K \frac{[GMG]}{[G]}\right)$$

In the ligand-exchange reaction, an increase of the solute concentration leads to a decrease in [GMG] and an increase in [G] that is, as observed, a decrease of k'. Consequently the peaks are not gaussian, but exhibit tailing (see Fig. 3).

Structure of the stereocomplexes

Potentiometric and nuclear magnetic resonance (NMR) spectroscopic studies[10] suggest a structure for the GMG complexes. In particular, the study of the relaxation time, T_1 in ^{13}C NMR gives an indication of the relative distance between each carbon atom and the copper ion.

The structure with S = valine is shown in Fig. 5. The amide function of the

polymer is in an apical position in the complex. Our results are therefore different from those[12–15] for chiral packings obtained with the same graft and the same complexing ion but with a styrene-type polymer. However, they are similar to those published on another kind of hydrophilic stationary phase[16]. As suggested by the NMR results and supported by molecular models, there is a steric hindrance between the amide group and the isopropyl substituent of the D-valine (see Fig. 5A). This effect does not exist with L-valine because these two groups are not on the same side of the main coordination plane (Fig. 5B). Consequently, L-valine is the most strongly complexed and shows the greatest retention volume.

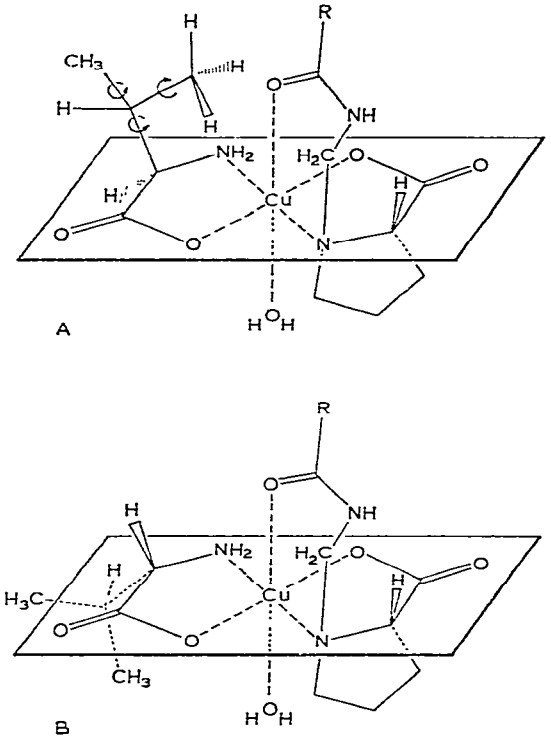


Fig. 5. Structures of the GMG complexes: A = L-proline–Cu^{2+}–D-valine (R is the macromolecular chain); B = L-proline–Cu^{2+}–L-valine.

More generally, this structure may be adopted for all amino acids having low polarity substituents. It may be noted that the larger are the substituents R which may interfere with the carbonyl group the stronger is the hindrance to rotation of R and the higher is the separation factor: for instance (see Table I), better separations are obtained for valine [R = $CH(CH_3)_2$; $\alpha = 1.75$] and serine ($R = CH_2OH$; $\alpha = 1.85$) than for the *n*-alkyl parent compounds norvaline (R = $CH_2CH_2CH_3$, $\alpha = 1.3$) and alanine (R = CH_3, $\alpha \approx 1$). In the same way, threonine [R = $CH(CH_3OH$; $\alpha = 1.90$] may be separated more readely than valine ($\alpha = 1.75$). As a result, the best resolutions are obtained for amino acids with bulky aromatic substituents: phenylalanine ($\alpha = 2.0$); tyrosine ($\alpha = 2.2$) and tryptophan ($\alpha = 2.45$).

In the series of amino acids with R = *n*-alkyl (alanine, $n = 1$, $\alpha \approx 1$; aminobutyric acid, $n = 2$, $\alpha = 1.25$; norvaline, $n = 3$, $\alpha = 1.30$; norleucine, $n = 4$, $\alpha = 1.20$), the selectivity first increases, as expected, with the size of the substituent and then remains constant or slightly decreases: it should be borne in mind that for such complexes the long substituent tends to be in an equatorial position (in the main complexation plane) and cannot greatly interfere with the carbonyl group in the apical position.

For D,L-proline as solute, a hindrance to rotation of R cannot be invoked because in this case the cyclic substituent is blocked. The separation mechanism is probably different and for this solute the L isomer is eluted first.

CONCLUSION

Chromatographic experiments and physico-chemical studies in solution with a chiral linear polymer, used as stationary phase, explain the difficulties generally encountered in separation of optical isomers by ligand-exchange chromatography: the necessity for similar stabilities of the complex formed by the graft and by the solutes; the absence of universality because the stereocomplex is formed only with a family of solutes, and even in this series the effect of the substituents might be predominant.

The proposed packing has some benefits: it is not expensive and is easily prepared. It yields high separation factors and the k' values can readily be adjusted, according to the solute to be separated, by selecting the complexation level of the ion fixed on the packing. Moreover, the use of a mineral support permits variation of the solvent. The last advantage has not yet been explored and may provide a way[17] of improving the low plate number.

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