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LIGAND-EXCHANGE CHROMATOGRAPHY OF RACEMATES

VII. SEPARATION OF OPTICAL ISOMERS OF AMINO ACIDS ON A POLYSTYRENE RESIN CONTAINING L-ALLO-HYDROXYPROLINE AS THE FIXED LIGAND

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SUMMARY

An asymmetric sorbent has been synthesized containing L-allo-hydroxyproline groupings in a macronet polystyrene matrix. The γ-hydroxy group in allo-hydroxyproline, like the carboxylic and the amino group, is capable of coordinating copper(II) ions. The sorbent thus forms more stable bis-chelate complexes with copper(II) than the analogous sorbent containing L-hydroxyproline residues.

The sorption selectivity of proline enantiomers on the copper(II) forms of the two resins has been studied as a function of copper ion content. Enantioselectivity and the retention parameters for various amino acids on the sorbent containing allo-hydroxyproline have been evaluated using ligand-exchange chromatography. The resolving power of this sorbent with respect to racemates of acidic and basic amino acids, as well as methionine and phenylalanine, is higher than that of similar resins described earlier.

INTRODUCTION

Ligand-exchange chromatography (LEC), which has recently been reviewed[1] makes the efficient separation of amino acid enantiomers possible on both analytical and preparative scales[1–4] without the preliminary modification of their amino and carboxy groups, that is necessary in gas chromatography.

The two previous papers in this series[3,4] were concerned with LEC of amino acid racemates on sorbents containing residues of L-hydroxyproline (I), L-proline (II) and L-azetidine carboxylic acid (III), and loaded with copper(II) ions. We determined the enantioselectivity of the resins and the efficiency of the chromatographic columns and discussed the dependence of these parameters on conditions of the process.

Influence of the fixed-ligand structure on LEC of amino acid racemates is a particularly interesting subject.

It was shown on low-molecular-weight model species[5] that in copper(II) complexes with N-benzyl-L-allo-hydroxyproline the γ-hydroxy group of the amino acid

takes part in complex formation. On the contrary, N-benzylhydroxyproline cannot act as a tridentate ligand. In order to reveal the influence of the configuration of the fixed-ligand γ-carbon atom on the sorbent properties we have compared statics and dynamics of the sorption of enantiomeric amino acids on resins with hydroxy-proline (I) and allo-hydroxyproline residues (IV).
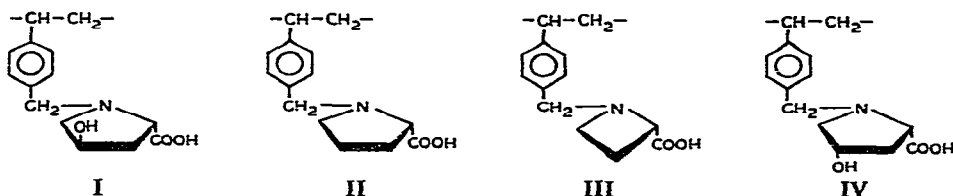
EXPERIMENTAL

The asymmetric sorbent IV was prepared by aminating chloromethylated polystyrene containing 11 mol% cross-links of diphenylmethane structure[6] using methyl-L-allo-hydroxyprolinate hydrochloride, according to a procedure described in ref. 6 for methyl-L-prolinate hydrochloride. The initial L-allo-Hyp was synthesized from L-Hyp according to a scheme[7] involving inversion of configuration at the γ-carbon atom:
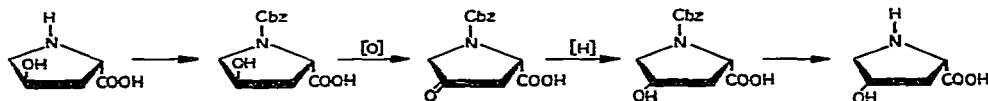
According to elemental analysis and potentiometric titration, the sorbent IV contains 2.82 mmol of fixed ligands per gram of dry resin in its zwitterionic form. The resin particles have an irregular shape, with an average size of *ca.* 100 μm when swollen. The resin was charged with copper ions from a copper–ammonia solution, resulting in quantitative formation of complexes containing two fixed ligands per copper ion. The equilibrium water content in the copper-saturated resin was 140%.

The chromatographic technique is described in detail in ref. 3. The column was 14 cm × 7.8 mm I.D. and contained 6.3 ml of resin. The eluents used were am-monia solutions of concentration 0.1, 0.3 and 1.5 M, containing $1.2 \cdot 10^{-5}$, $3.8 \cdot 10^{-5}$ and $2.0 \cdot 10^{-4}$ M CuSO$_4$, respectively, as well as a 0.017 M ammonium phosphate solution (pH 8.8) containing 2.0×10^{-5} M CuSO$_4$. Aliquots of 1.5–2.0 mg of the amino acid enantiomers introduced into the column were eluted at a flow-rate of 10 ml/h. The detector used was Uvicord-III with a 206-nm light filter.

The complex-formation properties of the resins were estimated by potentio-metric titration[6], by studying the sorption of copper from copper–ammonia solutions[8] and by measuring the pH decomplexation values (DpH) of copper(II) ions, as de-scribed by Hering[9]. The sorption constant of L-proline on resins I and IV was studied and calculated according to the procedure given in ref. 8.

The enantioselectivity of the sorption of proline isomers under static condi-tions was estimated from the equilibrium distribution of the amino acid enantiomers

between the aqueous phase and the asymmetric sorbent in batch experiments. Equilibration was carried out in 15-ml vials equipped with a porous glass filter and capable of being stoppered at both ends[6]. Into the vials were placed 0.300 g of air-dried resin containing 0.540 mmol of functional groups, 1 ml of a 0.5 M solution of D,L-Pro, differing amounts of copper nitrate (0.05–0.5 mmol) and 2 ml of 0.520 M KOH. After the volume was made up to 10 ml with water, the vials were closed and shaken for 72 h at 25°.

The resin phase was then separated by centrifugation at 1600 g for 15 min. To analyse the resin phase copper ions and L-proline were desorbed by washing the sorbent in the same vial with 25 ml of 5 M HCl.

Copper in both phases was determined spectrocolorimetrically with sodium N,N-diethyldithiocarbamate using a Specol spectrocolorimeter at $\lambda = 440$ nm.

Proline was determined by the technique of Pope and Stevens[10], modified by Woiwod[11], $i.e.$ the pH of the solution was increased to 8.0 and then a four-fold volume of a colloidal suspension of copper hydroxy phosphate in 0.2 M Na$_2$HPO$_4$ was added. The resulting mixture was kept at 80° for 30 min. The residual copper phosphate was then filtered off and the copper content in the filtrate, which is equivalent to the proline content, was determined by the method cited above. The predominance of one proline enantiomer over the other (in both phases) was determined polarimetrically, the specific rotation of L-Pro in 5.0 M HCl being assumed to be $[\alpha]_{436}^{20} = -123°$.

The quantitative analysis of both phases makes it possible to calculate the difference between the standard free energies of the two diastereomeric mixed-ligand sorption complexes, R–Cu–D-Pro and R–Cu–L-Pro, according to the equation

$$\delta\Delta G^0 = -RT \ln \frac{[\text{R–Cu–D-Pro}]}{[\text{R–Cu–L-Pro}]} \cdot \frac{[\text{L-Pro}]}{[\text{D-Pro}]}$$

RESULTS AND DISCUSSION

Thermodynamics of sorption of copper(II) ions and proline molecules

Owing to the additional interaction of the γ-hydroxy group of allo-hydroxyproline with the axial position of the copper(II) chelated ion, the complexes of both the amino acid itself and its N-benzyl derivative have higher stability constants than those of hydroxyproline and its derivative (Table I).

The same is true for polymer complexing agents. The sorption isotherms for copper(II) ions from ammonia solutions by the asymmetric resins I and IV (Fig. 1) indicate that the sorbent containing L-allo-hydroxyproline residues exhibits stronger complexing capacity. The stability constants of R–Cu and R–Cu–R complexes, found from potentiometric titration, are also higher for R = allo-Hyp than for R = Hyp (Table I).

The complexing properties of the resins were estimated, also according to Hering[9]. As we have shown earlier[6], his method for calculating stability constants of complexes of the Dowex A-1 type resins, based on measuring the pH values of metal decomplexation (DpH), is inapplicable to chelating sorbents that form 2:1 complexes. However, for a series of sorbents having similar fixed ligands the experimental DpH values are in a good qualitative agreement with the complex-forming

TABLE I

POTENTIOMETRICALLY DETERMINED STABILITY CONSTANTS OF COPPER(II) COMPLEXES WITH LOW MOLECULAR WEIGHT LIGANDS AND POLYMERIC LIGANDS

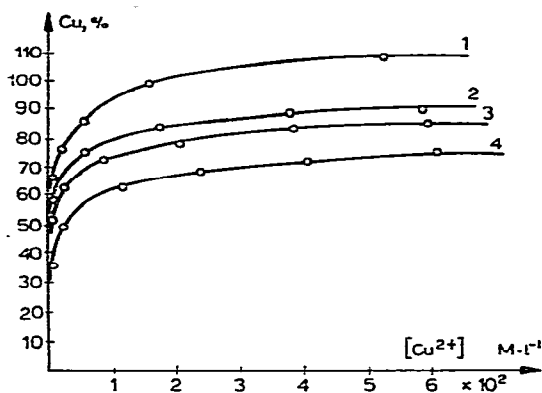| Ligand	$lg\ \beta_1$	$lg\ \beta_2$	DpH	Capacity (mmol/g)
L-Hyp	8.22	15.40	—	—
L-allo-Hyp	8.72	16.81	—	—
N-Bzl-L-Hyp	6.53	11.54	—	—
N-Bzl-L-allo-Hyp	7.97	14.92	—	—
	7.5	12.3	2.46	3.44
L-Hyp resin I	—	—	2.80	2.46
	6.2	10.5	3.41	1.48
L-allo-Hyp Resin IV	7.8	13.3	1.98	2.82

Fig. 1. Sorption isotherms of copper(II) ions from 2 M NH$_4$OH by the asymmetric sorbent IV containing L-allo-hydroxyproline (2.82 mmol/g) groups (1) and the asymmetric sorbent I containing L-hydroxyproline groups in amounts of 3.44 mmol/g (2), 2.46 mmol/g (3) and 1.48 mmol/g (4).

properties of the resins. As seen from the results of DpH measurements (Table I), desorption of copper(II) ions from resin IV requires more acidic eluents than in the case of sorbent I.

Comparison of three samples of hydroxyproline-containing resin shows (Table I and Fig. 1) that the stability constants of 2:1 polymer complexes, estimated by various methods, definitely depend on the exchange capacity of the resins. Therefore, it is correct to compare the complexing properties of different fixed ligands only for resins having an identical matrix structure and an equal content of functional groups. In this case, the exchange capacity of sorbent IV is inside the limits of capacity of the sorbent I samples studied. We can thus unambiguously conclude that hydroxyproline fixed ligands form weaker complexes with copper ions than allo-hydroxyproline ligands, so that the latter appear to be tridentate.

The high affinity of copper(II) ions for allo-hydroxyproline fixed ligands results in a decrease in the affinity of the sorbent chelate for mobile ligands, e.g. L-proline. This affinity is characterized by the sorption constant of the mobile ligand,

which can be determined by the difference between the stability constant of the ultimate sorption complex R–Cu–L-Pro and the constant of the initial fixed complex R–Cu–R^s. Fig. 2 indicates that the affinity of L-proline for sorbent IV is lower than that for the sorbent I, the degree of saturation of both resins with copper ions being taken to be the same.
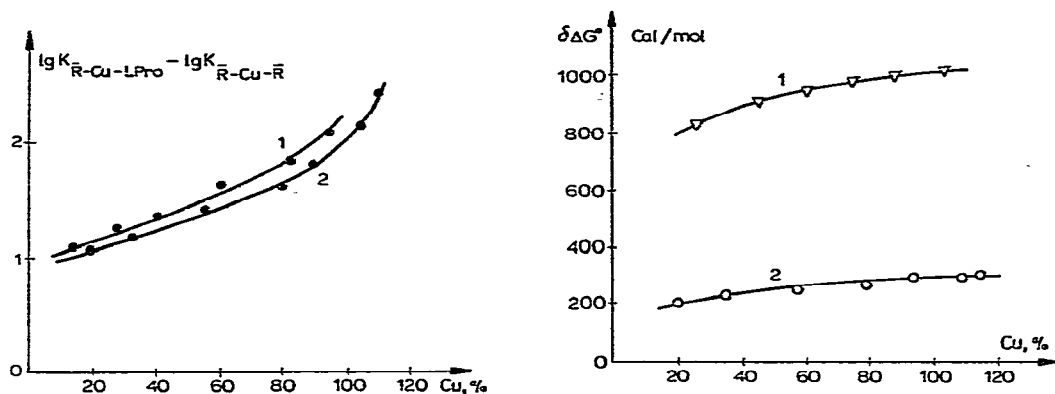

Fig. 2. Sorption constants of L-Pro on the copper forms of sorbent I (1) (capacity 3.44 mmol/g) and sorbent IV (2) as a function of the degree of saturation of the resins by copper(II) ions.

Fig. 3. Enantioselectivity of the sorption of proline enantiomers on sorbent I (1) and sorbent IV (2) as a function of the degree of saturation of the resins by copper(II) ions.

It is interesting to compare the selectivity of sorption of amino acid enantiomers on resins I and IV. It appears from Fig. 3 that coordination of the γ-hydroxy group of allo-hydroxyproline in the axial position of the copper ion results in racemic proline being resolved less well on sorbent IV.

Chromatography of racemates

The results of chromatographic studies of the affinity of sorbent IV for various amino acids and its enantioselectivity are presented in Table II. In general, the properties of sorbent IV have much in common with those of sorbents I, II and III, which may be due to the similar factors underlying the interaction between fixed complexes of these resins with amino acid enantiomers.

In the case of aliphatic amino acids those with larger *n*-alkyl substituents on the α-carbon atom exhibit longer retention times and higher enantioselectivity. However, when passing from Nva to Nle the increase in the value of $\delta \Delta G^0$ is not so great for sorbent IV as for sorbent I.
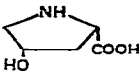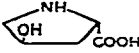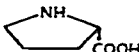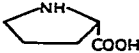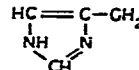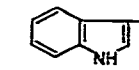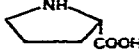
Enantioselectivity and the order of elution of amino acids with branched α-radicals are the same for sorbents I and IV. The resolution factor for Ile which is branched at the β-carbon atom, is higher than that of Leu, which is branched at the γ-carbon atom. The presence of hydroxy groups at the β-carbon atoms of Ser and Thr results in higher enantioselectivity and lower retention times of these amino acids compared with their aliphatic analogues (the same was observed for sorbent I).

Enantioselectivity effects on sorbents I and IV in the case of Asp and Glu were found to be close to each other. However, whereas Asp enantiomers are re-

TABLE II

ELUTION PARAMETERS OF AMINO ACIDS ON THE COPPER(II) FORM OF THE ALLO-HYDROXYPROLINE RESIN

Eluents: 0.1 M NH$_4$OH (N = 1–16); 0.3 M NH$_4$OH (N = 17–23); 1.5 M NH$_4$OH (N = 24–29); 0.017 M (NH$_4$)$_3$PO$_4$, pH 8.8 (N = 30–32).

N	Amino acid	α-Radicals or molecular structure	V (L)	V (D)	α	δΔG° (cal/mol)	HEEP (cm) (L)	HEEP (cm) (D)
1	Glycine	H–	5.55		—	—	1.23	
2	Alanine	CH$_3$–	8.9	9.2	1.04	24	1.16	1.10
3	Aminobutyric acid	CH$_3$CH$_2$–	9.6	11.0	1.14	77	1.36	1.22
4	Norvaline	CH$_3$CH$_2$CH$_2$–	13.4	19.0	1.42	205	1.32	1.3
5	Norleucine	CH$_3$CH$_2$CH$_2$CH$_2$–	22.9	33.4	1.46	220	1.2	1.25
6	Valine	CH$_3$CH(CH$_3$)–	8.6	13.6	1.58	270	1.3	1.25
7	Leucine	CH$_3$CH(CH$_3$)CH$_2$–	21.6	33.7	1.52	245	1.43	1.35
8	Isoleucine	CH$_3$CH$_2$CH(CH$_3$)–	16.2	28.2	1.74	325	1.1	1.0
9	Serine	HOCH$_2$–	4.38	5.25	1.24	125	1.43	1.41
10	Threonine	HOCH(CH$_3$)–	4.82	7.15	1.48	230	1.46	1.35
11	Asparagine	H$_2$NCOCH$_2$–	4.38	5.25	1.20	110	1.48	1.56
12	Glutamine	H$_2$NCOCH$_2$CH$_2$–	3.94	5.52	1.40	200	1.86	1.84
13	Phenylglycine	C$_6$H$_5$–	9.35	16.7	1.78	335	1.85	1.75
14	allo-Hydroxyproline	(ring structure: pyrrolidine, NH, COOH, HO)	18.7	27.8	1.48	230	1.48	1.40
15	Hydroxyproline	(ring structure: pyrrolidine, NH, COOH, OH)	21.3	34.1	1.63	285	1.2	1.3
16	Proline	(ring structure: pyrrolidine, NH, COOH)	52.5	96.0	1.83	355	1.1	1.05
17	Phenylalanine	C$_6$H$_5$CH$_2$–	15.2	47.2	3.10	660	1.98	1.85
18	Tyrosine	HOC$_6$H$_4$CH$_2$–	3.21	7.58	2.36	505	2.6	2.5
19	Methionine	CH$_3$SCH$_2$CH$_2$–	8.62	13.1	1.52	245	1.75	1.70
20	Proline	(ring structure: pyrrolidine, NH, COOH)	20.5	38.1	1.85	360	1.0	0.95
21	Leucine	CH$_3$CH(CH$_3$)CH$_2$–	8.8	13.8	1.56	325	1.40	1.38
22	Isoleucine	CH$_3$CH$_2$CH(CH$_3$)–	7.03	12.2	1.74	325	1.05	1.0
23	Aminobutyric acid	CH$_3$CH$_2$–	4.1	6.0	1.21	112	0.9	0.85
24	Lysine	H$_2$NCH$_2$CH$_2$CH$_2$CH$_2$–	2.25	3.0	1.33	165	1.45	1.0
25	Ornithine	H$_2$NCH$_2$CH$_2$CH$_2$–	1.0	1.2	1.2	110	1.75	1.65
26	Histidine	(imidazole ring, HC=C–CH$_2$–, NH, N, CH)	6.8	9.1	1.32	160	1.3	1.1
27	Tryptophan	(indole ring, CH$_2$–, NH)	63.0	68.8	1.1	57	1.88	1.7
28	Proline	(ring structure: pyrrolidine, NH, COOH)	3.15	5.75	1.82	350	1.0	1.0
29	Leucine	CH$_3$CH(CH$_3$)CH$_2$–	1.3	2.0	1.54	255	1.75	1.75
30	Aspartic acid	HOOCCH$_2$–	9.8	6.8	1.23	120	2.0	1.9
31	Glutamic acid	HOOCCH$_2$CH$_2$–	16.0	11.0	1.45	215	2.2	2.1
32	Iminodiacetic acid	HN(CH$_2$COOH)$_2$	8.0		—	—	2.1	

tained by sorbent I about twice as strongly as Glu, the affinity of both amino acids for sorbent IV is approximately the same.

Sorbent IV exhibits a high affinity for Met; the value of $\delta\Delta G^0$ reaches 245 cal/mol, which is much higher than for sorbents I–III.

Sorbent IV is highly selective with respect to aromatic amino acid enantiomers. Resolution of Phgl was somewhat lower than with sorbent I ($\alpha = 1.78$), and separation of Phe and Tyr isomers was better ($\alpha = 3.10$ and 2.36).

As regards cyclic amino acids, sorbent IV manifests essentially poorer enantioselectivity than its analogues I–III. The values of $\delta\Delta G^0 = 350$–360 cal/mol found on chromatography of racemic proline are consistent with the results of investigations of this system under static conditions (Fig. 3).

There is a specific difference in the order of elution of allo-hydroxyproline isomers. Sorbents I–III have a greater affinity for L-allo-Hyp, but the D-isomer is retained for longer on sorbent IV. The γ-hydroxy group of the fixed L-ligand on sorbent IV apparently blocks the upper axial position of the coordination sphere of the copper ion (Fig. 4), so that tridentate mobile ligands like L-allo-Hyp are capable of interacting with the copper ion only by their two donating groups. Therefore, L-allo-Hyp cannot form sorption complexes of abnormally high stability, which accounts for the strong retention of this isomer and the inverse elution order of allo-Hyp isomers on sorbents I–III.

The same reasons are evidently responsible for the absence of anomalies in the elution order of the isomers of His, Lys and Orn on sorbent IV. Lys and Orn are resolved into their enantiomers by sorbent IV better than by sorbents I–III; for
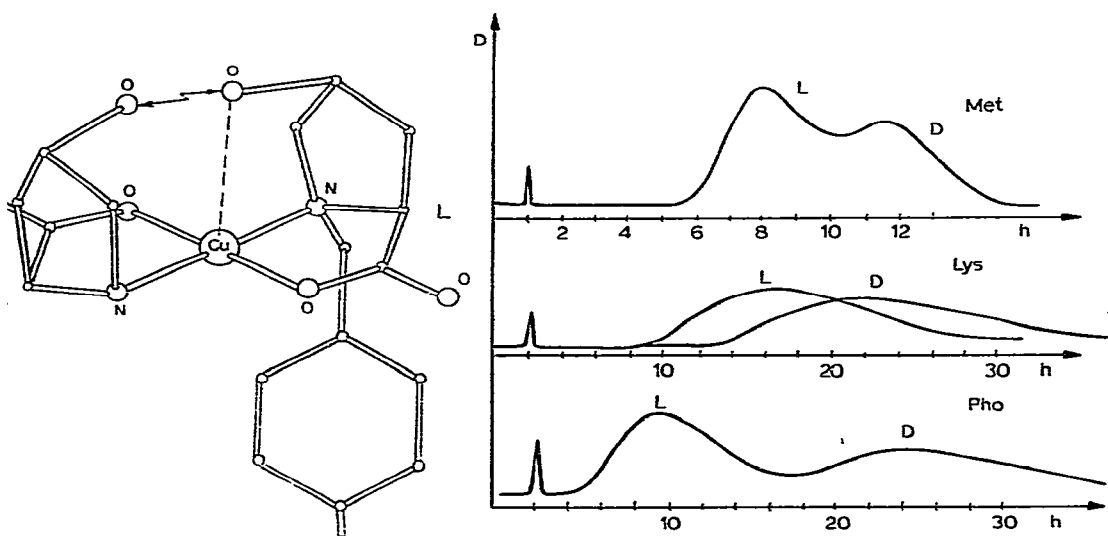

Fig. 4. Repulsion of the two hydroxy groups in the mixed-ligand sorption complex formed by L-allo-hydroxyproline on the asymmetric sorbent IV containing L-allo-hydroxyproline fixed ligands.

Fig. 5. Chromatography of enantiomers of Met, Lys and Phe. The degree of saturation of the L-allo-hydroxyproline resin IV by copper(II) ions was 50%. Column, 5 × 560 mm; 0.1 M NH$_4$OH; 6 ml/h. Particle size, $ca.$ 50 μm.

tryptophan the reverse is true. All the basic amino acids are strongly retained by the column and their chromatography requires higher concentration of ammonia in the eluent.

On the contrary, dicarboxylic amino acids display a low affinity for all the sorbents studied. Their chromatography requires a phosphate buffer with a low concentration of the displacing mobile ligand, *i.e.* NH_3. The partially tridentate nature of sorbent IV unexpectedly results in a considerable increase of enantioselectivity for Glu and Asp, the elution order of their enantiomers being reversed, as for sorbents I–III. The L-isomers of these amino acids can presumably form additional hydrogen bonds between the carboxylate groups of their lateral chains and the hydroxy group of the fixed L-ligand coordinated in the axial position.

CONCLUSION

Unlike sorbents I–III, sorbent IV with fixed L-allo-hydroxyproline residues possesses a γ-hydroxy group capable of interacting with the axial position of the chelated copper(II) ion. Consequently, when the L-isomers of allo-Hyp, His, Lys and Orn form sorption complexes, only two of their three donor groups can interact with the metal ion, and the order of elution of their isomers does not differ from that of other bifunctional amino acids.

Sorbent IV exhibits the highest enantioselectivity among all the resins studied (I–IV) with respect to the following amino acid: Phe, Met, Lys, Orn, Asp and Gln. Compared with its diastereomeric analogue, *i.e.* sorbent I (containing hydroxyproline residues), it provides a better separation of Tyr and Glu.

However, the efficiency of chromatographic columns with sorbent IV was somewhat lower, apparently because sorbent IV has a lower swelling ability than sorbent I.

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