The Selective Sorption of D,L-Amino Acids by Chemically Modified Chitosan Gels and Its Application to Liquid Chromatography

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Synopsis

The sorption of D,L-amino acids, DNP-L-amino acids and dipeptides by *N*-octanoyl- and *N*-benzoyl-chitosan gels was investigated under various conditions. The results indicate that optical resolution of D,L-amino acids by liquid column chromatography has been achieved, using the chemically modified chitosan gels as a stationary phase.

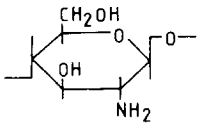
INTRODUCTION

It is well known that the natural polymers, cellulose, starch, and wool have the ability to separate a recemate into enantiomers. Krebs, and Schumacher investigated the chromatographic resolution of D,L-camphorsulphonic acids and their Br derivatives by a starch column.[1] The separation of D,L-tryptophans by a chromatographic technique using bovine serum albumin–succinoylaminoethylagarose as a stationary phase has been reported.[2] Bach and Haas investigated the separation of optical active amino acids by means of thin layer chromatography with cellulose as stationary phase.[3] The effective separation of Tröger's base using fine crystals of acetylated cellulose was reported by Hesse and Hagel.[4] Recently the high efficiency of triacetate supported by silica gel was shown by Ichida et al.[5] and Shibata et al.[6] Cellulose–tribenzoate and –triphenylcarbamate were demonstrated by the same authors to have an excellent ability in recognizing chirality.[5-7]

Recently, with a view to utilizing natural resources, an aminopolysaccharide, chitosan (1) obtained from chitin, was chemically modified, and the products were investigated with the purpose of obtaining polymers of some promise. The supports used in gel and in affinity chromatography are examples of such an approach. Hirano et al. used *N*-acyl and *N*-arylidene–chitosan gels for enzyme immobilization and gel filtration supports.[8,9] Baba et al. carried out the chromatographic purification of maize phosphorylase by *N*-acetylchitosan gels.[10] Noguchi et al. investigated the optical resolution of D,L-mandelic acids by means of benzylchitosan–epichlorohydrin resins.[11,12]

Although the use of chemically modified chitosan gels for separation has been discussed in detail, there has been little fundamental research on the interaction of small molecules with such gels. We therefore investigated the interaction of chitosan and its derivatives containing hydrophobic groups with dyes which have various ionic, hydrophilic, and hydrophobic groups.[13,14]

Journal of Applied Polymer Science, Vol. 38, 997–1009 (1989)
 CCC 0021-8995/89/060997-13$04.00



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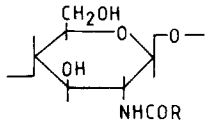

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Sorption of D,L-tryptophan was found to increase by the presence of hydrophobic groups in chitosan.[15]

In this work, hydrophobic chitosan gels (2) were prepared, containing octanoyl and benzoyl group (R), with various degrees of substitution, and these were examined for the selective sorption of D,L-amino acids and the separation of *N*-dinitrophenyl–L-amino acids and dipeptides. Further work was carried out on the separation of various amino acids by liquid chromatography using chemically modified chitosan gels.

EXPERIMENTAL

Materials

Chitosan (degree of substitution of the amino group = 0.77) was supplied by Kyowa Yushi Co. The flakes of chitosan were dissolved in 10% acetic acid in methanol and acylated with octanoic and benzoic acid anhydride. The gels formed were neutralized with 0.1*N* KOH, washed repeatedly, and dried. The particles of the gels used were 50–300 μm of diameter.[13-15] D,L-Amino acids (Special Grade) were purchased from Nakarai Chemicals Co. 2,4-Dinitrophenyl-L-amino acids (DNP-L-amino acids) and dipeptides were obtained from Tokyo Kasei Co.

Equilibrium Sorption

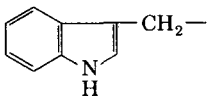
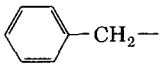
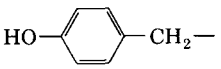
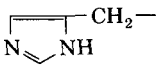
Weighed amounts of gel were soaked in an aqueous solution of amino acid or dipeptide of required concentration and shaken 4 days at 30°C. The attainment of equilibrium sorption was ascertained in preliminary experiments.

The amount of equilibrium sorption was determined by measuring the initial and final concentrations spectrophotometrically. The characteristics and Rekker's hydrophobic fragmental constant, Σf,[16] of amino acids and dipeptides are shown in Tables I–III.

DSC Measurements

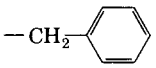
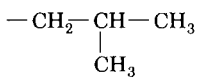
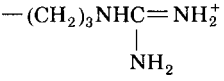
Fifteen milligrams of water-swollen gel was sealed in an aluminum pan, and its thermal properties were determined with a differential scanning calorimeter, SSC575/DSC10, Seiko Instrumental and Electronics Ltd. The temperature was increased at the rate of 2.5 K/min. The amount of freezable water which was assigned as free water was determined from the endothermic peak. The amount of nonfreezable water which was assigned as bound water was calculated by subtracting the weight of free water from the total weight of

TABLE I
Amino Acid Used

Amino acid	Structural formula R—CHCOOH, R / NH_2	MW	Σf [a]	pka	pI [b]	λ (nm)	ε (L mol^{-1} cm^{-1})
Tryptophan (trp)		204.23	2.31	2.38 9.39	5.89	278	5550
Phenylalanine (phe)		165.19	2.24	2.16 9.12	5.48	257	183
Tyrosine (tyr)		181.19	1.70	2.20 9.11 10.07(OH)	5.66	274.5	1340
Histidine (his)		155.16	−0.23	1.78 8.97 5.97(Im)	7.47	210	5798

[a] Rekker's hydrophobic fragmental constant of free amino acids.
[b] Isoelectric point.

TABLE II
DNP-L-Amino Acid Used

DNP-L-amino acid	Structural formula[a] R	MW	Σf [b]	λ (nm)	ε (L mol^{-1} cm^{-1})
DNP-L-phenylalanine (DNP-L-phe)		331	2.24	264	8418
DNP-L-leucine (DNP-L-leu)		297	1.99	264	8969
DNP-L-proline (DNP-L-pro)		281	1.01	260	6396
DNP-L-arginine (DNP-L-arg)		340	0.93	265	7897
DNP-L-alanine (DNP-L-ala)	$-CH_3$	255	0.53	264	8692
DNP-L-glycine (DNP-L-gly)	$-H$	241	0.0	264	8455
DNP-L-serine (DNP-L-ser)	$-CH_2OH$	271	−0.56	264	10388

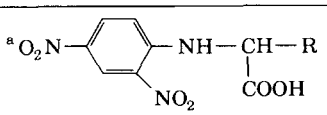
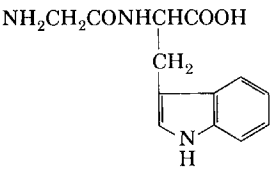
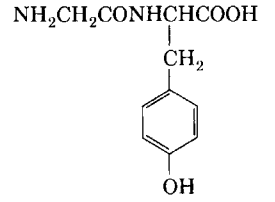
[a] 
[b] Rekker's hydrophobic fragmental constant.

TABLE III
Dipeptide Used

Dipeptide	Structural formula	MW	λ (nm)	ε (L mol^{-1} cm^{-1})
Glycine-L-tryptophan (gly-L-trp)		297	279	5399
Glycine-L-tyrosine (gly-L-tyr)		274	275	1520

water. Dry samples were obtained by heating at 100°C *in vacuo* for more than 2 days.

Chromatography

Water-jacketed glass columns (20 × 1.0 cm ID and 40 × 1.0 cm ID) were packed with chitosan gels, equilibrated with the eluent, and chromatography was carried out at room temperature with an elution rate of 1 mL/min. The eluted solution was scanned with a UV detector (JASCO UVIDEC 100V) connected to a fraction collector (FRAC100, Pharmacia Fine Chemicals) and a recorder (EPR 111A, Tokyo Toa Electrics Ltd.). The partition coefficient K_{av} of solute is given by $(V_e - V_0)/(V_t - V_0)$, where V_t, V_e, and V_0 are the total volume of the gel bed, the elution volume, and the void volume, respectively.[17,18]

RESULTS AND DISCUSSION

Preparation and Properties of Chitosan Gels Containing Hydrophobic Groups

The preparation and properties of *N*-acylchitosan gels are summarized in Table IV. Octanoyl chitosan with various degree of substitution up to approximately 0.7 was prepared by treatment at 25°C for 20 h from chitosan with an amino group content of 0.77. The corresponding benzoyl chitosan was obtained at 50°C for 2 h and further standing at 25°C for 20 h. A DS lower than 0.4 makes the gels highly soluble in acetic acid and a DS higher than 0.5 increases their solubility in LiCl 1 g/*N*-methyl pyrrolidone 10 mL/*N*,*N*-dimethylacetamide 10 mL. The highest water retention by chitosan gels is 76%; for C_B-0.7 it is 43% and for C_8-0.7 it is 28%. The state of water in the gels determined by DSC is given in Table V. The amount of free water is 25.6 per basemol of chitosan, and it decreases to less than 1 by the introduction of an octanoyl group. In the case of benzoyl chitosan the corresponding values are

TABLE IV
Preparation of *N*-Acylchitosan Gels

Sample no.	$[(RCO)_2O]$/[GlcN]	Temp (°C) time (h)	DS[b]	Solubility in[c] 10% AcOH	Solubility in[c] LiCl/NMP/DMAc (1/10/10)	W_p (%)[d]
CS[a]				s	i	76.2
C_8-0.26	0.5	25/20	0.26_3	sw	sw	61.8
0.4	0.5		0.44_4	sw	sw	46.8
0.65	1.0		0.64_7	i	sw	32.0
0.7	1.0		0.67_9	i	s	28.1
C_B-0.3	0.5	50/2 + 25/20	0.31_7	sw	i	76.7
0.5	1.0		0.51_2	i	sw	63.5
0.7	2.0		0.68_3	i	s	43.2

[a]Amino content = 0.77.
[b]Degree of substitution of acyl group.
[c]i = insoluble; s = soluble; sw = swelling.
[d]Water content.

TABLE V
Freezing and Nonfreezing Water of Gels

Sample no.	Freezing water w_f (mol H_2O/base mol)	Nonfreezing water w_{nf} (mol H_2O/base mol)
CS	25.6	4.7
C_8-0.26	8.6	9.7
C_8-0.4	5.2	6.2
C_8-0.65	1.9	4.9
C_8-0.7	0.5	5.3
C_B-0.3	31.5	5.9
C_B-0.5	14.5	7.1
C_B-0.7	15.9	7.2

31.5 for C_B-0.3 and approximately 16 for C_B-0.7, which is more than 30 times larger than for C_8-0.7. An interesting feature is the content of bound water, which is 6.2 and 6.7 molecules for octanoyl and benzoyl chitosan, respectively, and does not depend on the degree of substitution, which suggests that the water is bound to the OH group of the glucosamine residue.

The results suggest that the introduction of a long acyl chain increases hydrophobicity, producing a compact gel structure by strong interchain attraction which reduces the amount of free water. The aromatic acyl compound has a relatively loose structure because of a steric effect and a decrease in crystallinity.[14]

Sorption of D,L-Amino Acids by the Gels

Effects of Substituents

Figure 1 shows the sorption of the hydrophobic amino acids, D,L-trp and D,L-tyr, by octanoyl-chitosan gels. The sorption of the former is higher than L-tyr and reaches a maximum at DS = 0.3. An increase in the DS decreases

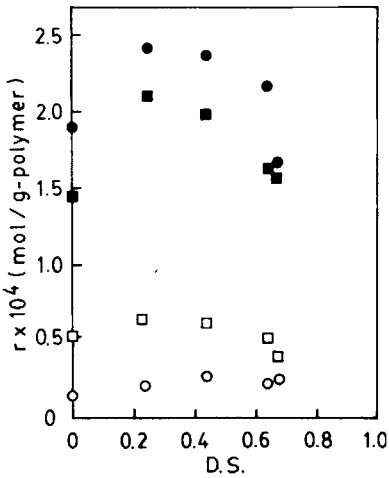


Fig. 1. Effect of DS on the sorption of amino acids. C_8 gels: (●) L-trp; (○) D-trp; (■) L-tyr; (□) D-tyr. Initial concentration of sorption bath: 1×10^{-3} mol L^{-1}.

sorption. If we bear in mind that at pH 5–6 of the bath, the amino acids are near their isoelectric point, the interaction with chitosan gels can be attributed to hydrogen bonding, in addition to hydrophobic interaction with the acyl chain. This argument is consistent with that resulting from our previous work.[15] The decrease of sorption at the higher DS is explained by a more compact gel structure, which is suggested by DSC results discussed in the previous section.

The sorption of the D-isomer is almost independent of the DS. The L-amino acids are sorbed to a greater extent than their D-isomers, which suggests that chemically modified chitosan gels are able to separate D,L-amino acids. The same behavior was noticed with D,L-his · HCl, as shown in Figure 2.

Effects of Inorganic Ions

The effect of inorganic ions that are known to affect the structure of water has been examined. The effects of them on the sorption of L-trp are shown in Figure 3. Ammonium sulfate and sodium chloride, which are known for their

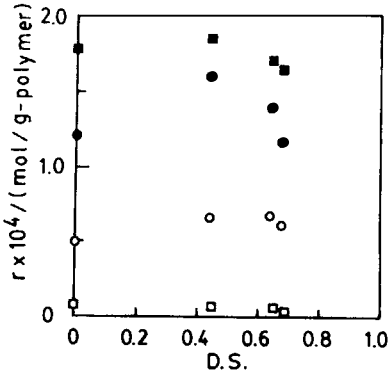


Fig. 2. Effect of DS on the sorption of amino acids. C_8 gels: (●) L-trp; (○) D-trp; (■) L-his · HCl; (□) D-his · HCl. Initial concentration of sorption bath: 2×10^{-4} mol L^{-1}.

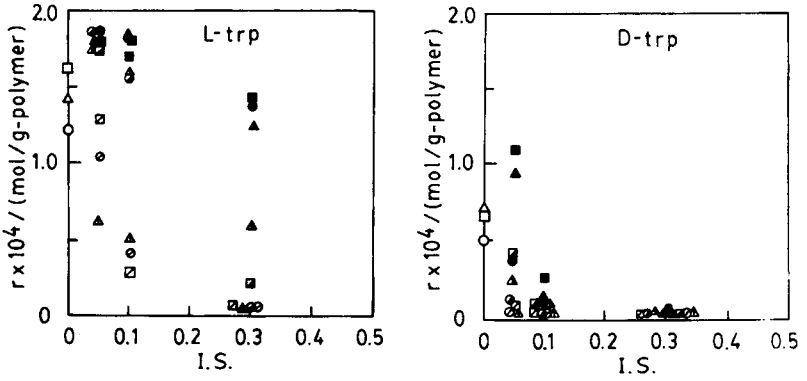


Fig. 3. Effect of IS on the sorption of trp:

	CS	C_8-0.4	C_8-0.65
$(NH_4)_2SO_4$	●	■	▲
NaCl	◕	◪	▲
NaSCN	⊘	□	△

salting out properties, produce a distinct increase in sorption at ionic strengths (IS) up to 0.1. Sodium thiocyanate, a chaotropic salt, reduces sorption of L-trp. At ionic strength 0.3 all three salts cause a decrease in sorption which is attributed to the dehydration of the gels by the higher salt concentration producing a more compact structure. On the other hand, the sorption of D-trp becomes almost zero above an ionic strength of 0.1 for all the salts used (Fig. 3).

Figure 4 shows the effect of the added salts on the sorption of tyr. The sequence of sorption agrees with the salting out effects of these salts, NaSCN

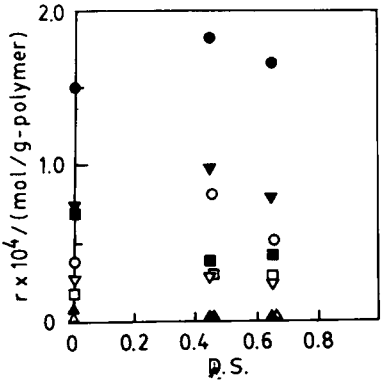


Fig. 4. Effect of salts (IS = 0.1) on the sorption of D,L-tyr. Initial concentration of sorption bath: 8×10^{-4} mol L^{-1}. C_8 gels:

	L-tyr	D-tyr
$(NH_4)_2SO_4$	●	○
NaCl	■	□
NaSCN	▲	△
aq	▼	▽

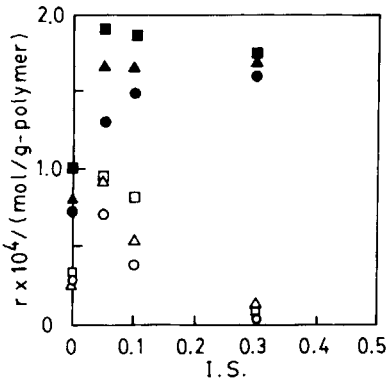


Fig. 5. Effect of ionic strength of $(NH_4)_2SO_4$ on the sorption of D,L-tyr. Initial concentration of sorption bath: 8×10^{-4} mol L^{-1}.

	CS	C_8-0.4	C_8-0.65
L-tyr	(●)	(■)	(▲)
D-tyr	(○)	(□)	(△)

< NaCl < $(NH_4)_2SO_4$. The selective sorption of L-tyr is clearly shown in Figure 4, as it is in Figure 5.

Sorption of DNP-L-Amino Acids and Dipeptides

Zaslavsky et al. reported that the partition coefficients of 20 DNP-L-amino acids between ficoll–dextran and aqueous buffer solution depend linearly on the relative hydrophobicity of the side chain of the amino acids.[19] In our work the relation between the sorption of seven DNP-L-amino acids and Rekker's hydrophobic fragmental constant, Σf,[16] was investigated. Sorption increases with increase in Σf, except DNP-L-pro, -leu, and -arg, as shown in Figure 6. The difference in behavior of DNP-L-arg is attributed to the repulsion of the positive charge. The branched side chain of leu and ring structure of pro could hinder sorption for steric reasons. The role of the hydrophobic effect on sorption now becomes clear.

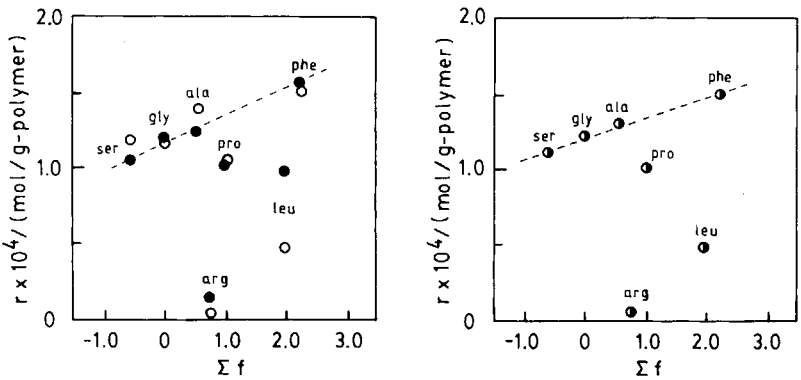


Fig. 6. Σf vs. r: (○) CS; (●) C_8-0.4; (◑) C_B-0.3.

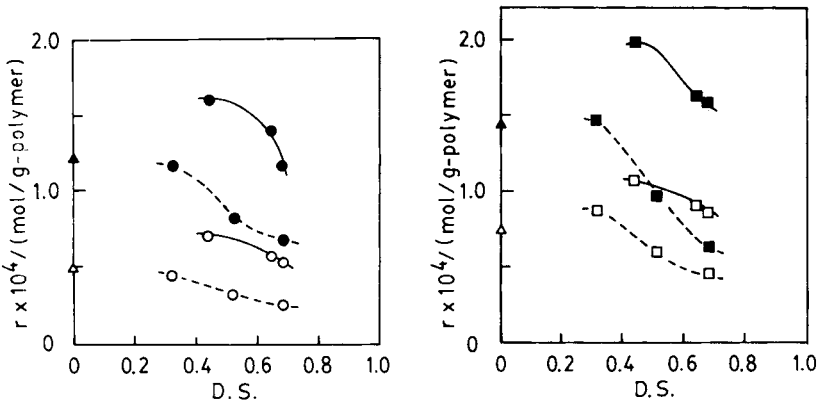


Fig. 7. Effect of DS on the sorption of dipeptides: (—) C_8 gels; (---) C_B gels; (●) L-trp; (■) L-tyr; (○) gly-L-trp; (□) gly-L-tyr; (▲) amino acid (CS); (△) dipeptide (CS).

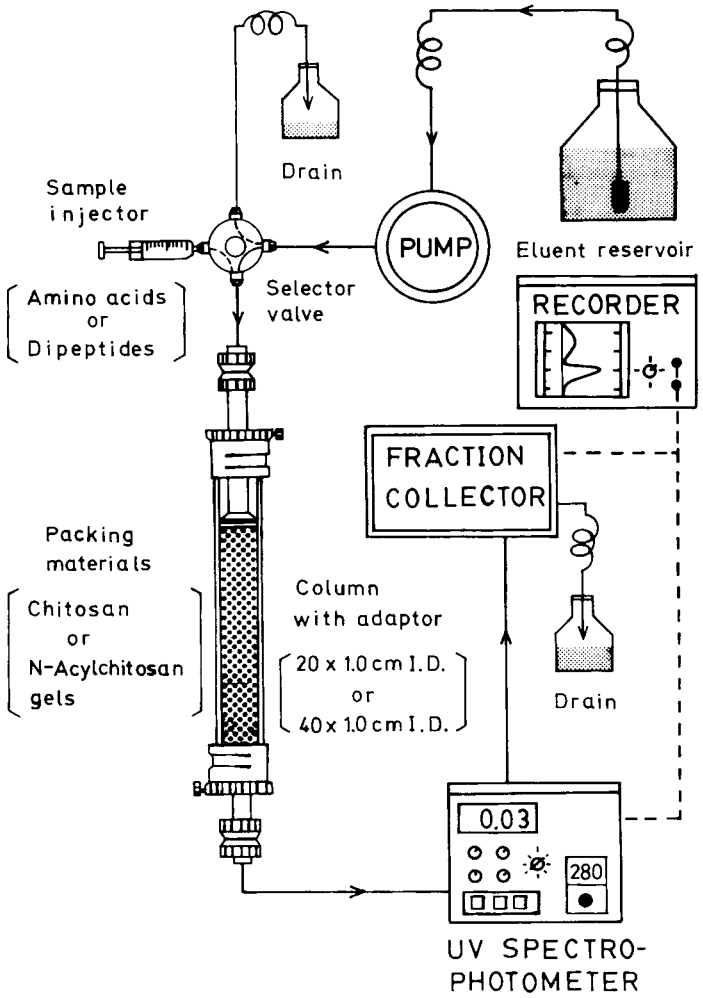


Fig. 8. Liquid column chromatography system for the separation of amino acids and peptides.

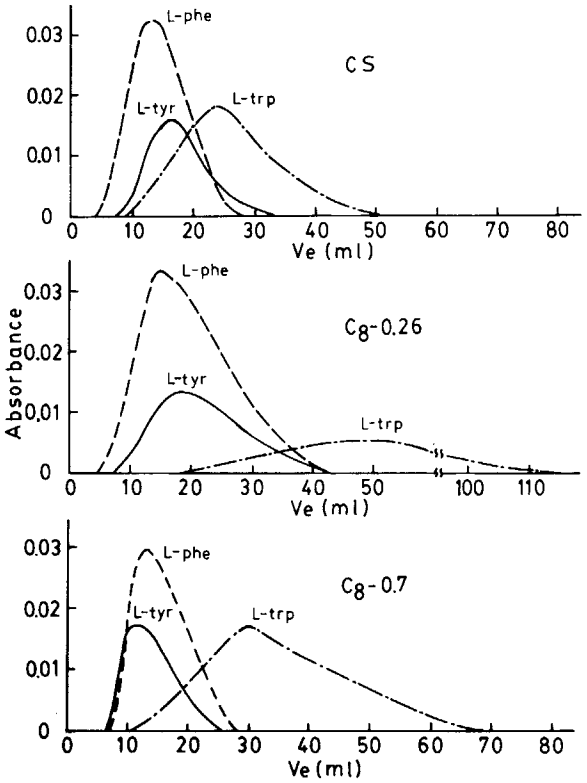


Fig. 9. Chromatogram of various amino acids. Column 20 × 1.0 cm ID. L-trp, L-tyr (λ = 280 nm); L-phe (λ = 265 nm).

The more hydrophobic dipeptide molecules are sorbed by octanoyl and benzoyl chitosan as well as chitosan. The results for gly-L-trp and gly-L-tyr are shown in Figure 7, and indicate an increase in sorption with the introduction of a hydrophobic group. The sorption of dipeptides is approximately half of L-trp or L-tyr.

Liquid Chromatography Performance by Chemically Modified Chitosan Gels Used as Stationary Phase

The information about equilibrium sorption was applied for the separation of D,L-amino acids by liquid chromatography (Fig. 8), using chemically modified chitosan gels as a stationary phase.

A chromatogram of L-trp, L-tyr, and L-phe on a column of 20 × 1.0 cm ID filled with chitosan, C_8-0.26 or C_8-0.7 gels, with water as eluent, is shown in Figure 9. The elution volumes (V_e) of the three amino acids are different, indicating the possibility of the separation. In C_8-0.26, V_e is greater than in chitosan, indicating strong interaction with the amino acids. The more compact gel structure of C_8-0.7 is reflected by a sharper peak in the chromatogram. Thus the separation of phe from trp and tyr from trp is better.

When L-tyr is separated from L-phe in a column containing C_8-0.7 gel, using aqueous $(NH_4)_2SO_4$ solution (IS = 0.1) as eluent, the chromatogram shown in

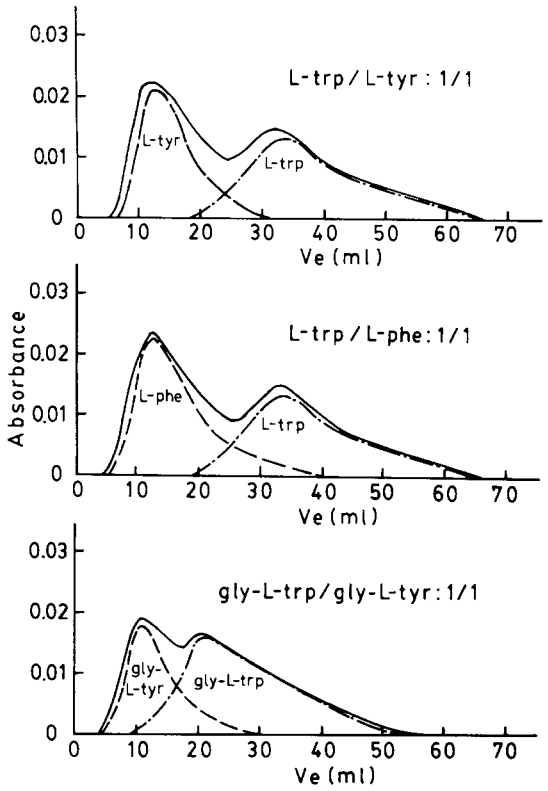


Fig. 10. Chromatogram of various amino acids and dipeptides on C_8-0.7 gel. Column 20 × 1.0 cm ID.

TABLE VI
Partition Coefficient of L-Amino Acids and Dipeptides Using Various Solvents as Eluent on C_8-0.7 Gel

Solvent	L-trp		L-phe		L-tyr		Gly-L-trp		Gly-L-tyr	
	V_e[a](mL)	K_{av}[b]	V_e(mL)	K_{av}	V_e(mL)	K_{av}	V_e(mL)	K_{av}	V_e(mL)	K_{av}
50% EtOH	11.8	0.82	9.4	0.64	7.6	0.50				
20% EG	20.6	1.50	10.8	0.75	9.8	0.67				
10% MeOH	25.0	1.83	11.0	0.84	10.6	0.73				
NaSCN (IS = 0.1)	27.8	1.98	11.2	0.75	10.6	0.73				
H_2O	29.0	2.14	11.4	0.79	10.8	0.75				
$(NH_4)_2SO_4$ (IS = 0.1)	33.2	2.46	11.8	0.90	11.0	0.76	21.0	1.53	9.8	0.67

[a] V_e = elution volume.
[b] Partition coefficient: $K_{av} = (V_e - V_0)/(V_t - V_0)$. V_t = total volume of gel bed, V_0 = void volume.

TABLE VII
Elution Volume and Separation Factor of D- and L-Amino Acids on *N*-Octanoylchitosan Gels

Adsorbent	Solvent	Amino acid	Elution vol (mL) L-	Elution vol (mL) D-	α[a]
CS[b]	NaCl	trp	10.6	10.2	1.06
	(IS = 0.1)	tyr	7.2	7.0	1.06
		phe	6.8	6.6	1.07
	$(NH_4)_2SO_4$	trp	13.0	12.2	1.10
	(IS = 0.1)	tyr	9.3	9.1	1.04
		phe	8.6	8.3	1.07
C_8-0.26[b]	20% MeOH	trp	25.9	20.2	1.33
		tyr	9.0	8.0	1.19
		phe	7.7	7.3	1.09
	$(NH_4)_2SO_4$	trp	23.2	18.8	1.28
	(IS = 0.1)	tyr	7.9	7.3	1.09
		phe	7.1	6.7	1.10
C_8-0.4[c]	20% MeOH	trp	183	148	1.28
		tyr	48.2	21.9	1.20
		phe	29.2	28.1	1.18
C_8-0.7[b]	$(NH_4)_2SO_4$	trp	16.6	18.6	0.89
	(IS = 0.1)	tyr	5.5	6.0	0.90
		phe	5.9	5.9	1.00

[a]Separation factor: $\alpha = K_L/K_D = (V_L - V_0)/(V_D - V_0)$ (V_0 = void volume).
[b]Column 20 × 1.0 cm ID.
[c]Column 40 × 1.0 cm ID.

Figure 10 is produced. Here two peaks occur which coincide with those obtained with the individual amino acids. The elution of dipeptides, of gly-L-trp is slower than of gly-L-tyr, suggesting the possibility of separation in a 1 : 1 mixture.

Table VI, shows the effect of the eluent on the partition coefficient of L-amino acid. An increase in the organic component in the eluent leads to a decrease in the partition coefficient K_{av}, which is explained by the lowering of hydrophobic interaction. $(NH_4)_2SO_4$, on the other hand, increases V_e and K_{av}, which agrees with sorption behavior. The sequence of the increase of V_e and K_{av}, tyr < phe < trp is in agreement with the order of the increase in Σf. For C_8-0.7 gel hydrophobic separation is thought to be responsible for this. The larger value of K_{av} for gly-L-trp than gly-L-tyr supports this view.

Table VII gives the elution volume and α, the separation factor defined as the ratio of the partition coefficients for two isomers (K_L/K_D). The values of α of chitosan gels lie between 1.04 and 1.10, though C_8-0.26 gives a larger value in 20% MeOH, 1.33 for trp, 1.19 for tyr, and 1.09 for phe. Using a 40 × 1.0 cm ID column packed with C_8-0.4 gel, larger values are obtained, 1.18–1.28, which means that the separation of the amino acids is improved (Fig. 11).

We can conclude from the results that the chiral structure of chitosan gels containing hydrophobic groups with a low degree of substitution is effective in the resolution of optical isomers of D,L-amino acids, whereas gels with a high degree of substitution show hydrophobic separation of amino acids.

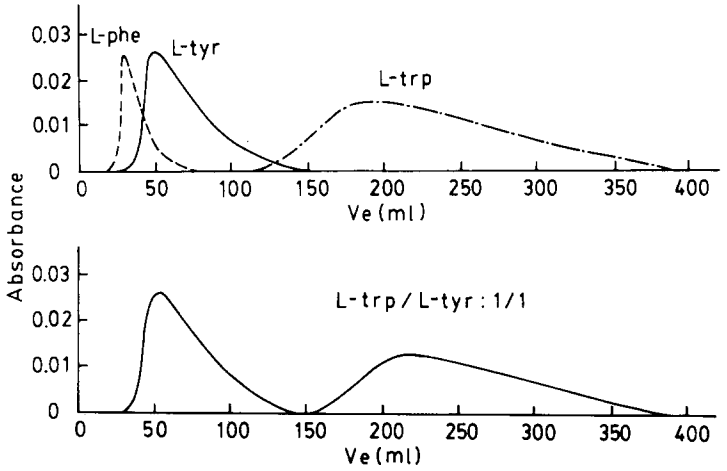


Fig. 11. Chromatogram of various amino acids on C_8-0.4 gel. Column 40 × 1.0 cm ID.

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Received April 27, 1988
Accepted May 4, 1988