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Separation of enantiomers on a lysozyme-bonded silica column

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ABSTRACT

A lysozyme-bonded silica column has been developed for direct separation of enantiomers. Lysozyme was covalently bound to porous silica materials using an N,N'-disuccinimidyl carbonate reaction. By using a mixture of phosphate buffer and organic modifier as an eluent, basic and uncharged enantiomers were resolved, while no resolution of acidic enantiomers was observed.

INTRODUCTION

Many protein-bonded stationary phases have been developed for the resolution of enantiomers [1]. These include albumins such as bovine serum albumin [2] and human serum albumin [3], glycoproteins such as α_1-acid glycoprotein [4], ovomucoid [5], avidin [6] and cellulase [7], and trypsin [8] and α-chymotrypsin [9]. A wide range of compounds have been separated on protein-bonded columns because such columns can provide multiple interactions and/or multiple recognition sites. However, in general, they have the disadvantages of low capacity, low efficiency and lack of column ruggedness. The capacity is low because the surface coverage of proteins on supports is low owing to their large physical size and because a very limited number of chiral recognition sites are present on bonded proteins since proteins include many superfluous regions for chiral recognition. The efficiency is low because it takes a long time to reach dynamic equilibrium between solutes and proteins. The lack of column ruggedness is a result of the intrinsic nature of proteins. In some cases, native protein can be denatured by high temperature, high organic modifier content, high salt concentration and low or high pH. However, the protein bound to supports might be more stable than the native form. Also, for protein-bonded stationary phases, the chiral recognition mechanism is little understood.

To overcome these problems, some trials have been carried out. Previously we modified ovomucoid-bonded stationary phases with glutaraldehyde, glyceraldehyde, formaldehyde and glutaric anhydride [10,11], resulting in long-term column stability or effective chiral separation of basic compounds compared with unmodified ovomucoid-bonded columns. Enzyme-cleaved bovine serum albumin fragments were used as a chiral stationary phase for the resolution of benzoin and oxazepam by Erlandsson and Nilsson [12]. Recently, Pinkerton and Haginaka [13] reported enantioselectivity of 2-arylpropionic acid derivatives and 1,4-benzodiazepines on ovomucoid-bonded phase columns produced with isolated protein domains.

In another experiment, we selected lysozyme as a chiral selector. This protein, whose molecular mass is 14 300 and whose isoelectric point is 10.7, is composed of 129 amino acids and contains four disulphide bonds [14]. Also, lysozyme is a protein that has been thoroughly investigated at the molecular level. X-Ray crystallography of

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the complex between mono-N-acetylglucosamine and lysozyme shows a static structure [15], while examination of the dynamic structure in solution using NMR spectroscopy revealed that three helical regions and two β-sheet regions are identical to those found in the X-ray structure of lysozyme [16]. This report deals with the chiral recognition properties of lysozyme-bonded silica stationary phases.

EXPERIMENTAL

Materials

Benzoin, lormetazepam, temazepam, chlorpheniramine maleate, alprenolol hydrochloride and tolperisone hydrochloride were purchased from Sigma (St. Louis, MO, USA). Lorazepam was kindly donated by Yamanouchi (Tokyo, Japan). Fig. 1 shows the structures of the enantiomers used in this study. Lysozyme from chicken egg white was obtained from Sigma and used without further purification. Other reagents and solvent used were analytical-reagent or HPLC grade. Water purified with Nano-pure II (Barnstead, Boston, MA, USA) was used for the preparation of sample solutions and eluents.

Preparation of lysozyme-bonded silica

An aminopropyl-silica gel (Ultron-NH_2, 5 μm, 300 or 120 Å; Shinwa Chemical Industries, Kyoto, Japan) was activated by N,N′-disuccinimidyl carbonate (DSC) as reported by Miwa *et al.* [5]. A 2-g amount of DSC-activated aminopropyl-silica gel was slurried in 20 ml of 20 m*M* phosphate buffer (pH 6.5). To the slurry, 2 g of lysozyme dissolved in 10 ml of 20 m*M* phosphate buffer (pH 6.5) were gradually added, and the mixture was gently rotated at 30°C for 25 h. The elemental analysis of lysozyme-bonded silica materials was performed using a Model NCH-21 analyser (Sumika Chemical Analysis Service, Osaka, Japan) for nitrogen or ion chromatography combined with the oxygen flask method for sulphur.

The lysozyme-bonded silica materials were packed into a 100 × 4.6 mm I.D. stainless-steel column by conventional high-pressure slurry-packing procedures. The slurry and packing medium was 5% ethanol in water.

Chromatography

The HPLC system used comprised an LC-9A pump, an SPD-6A spectrophotometer and an

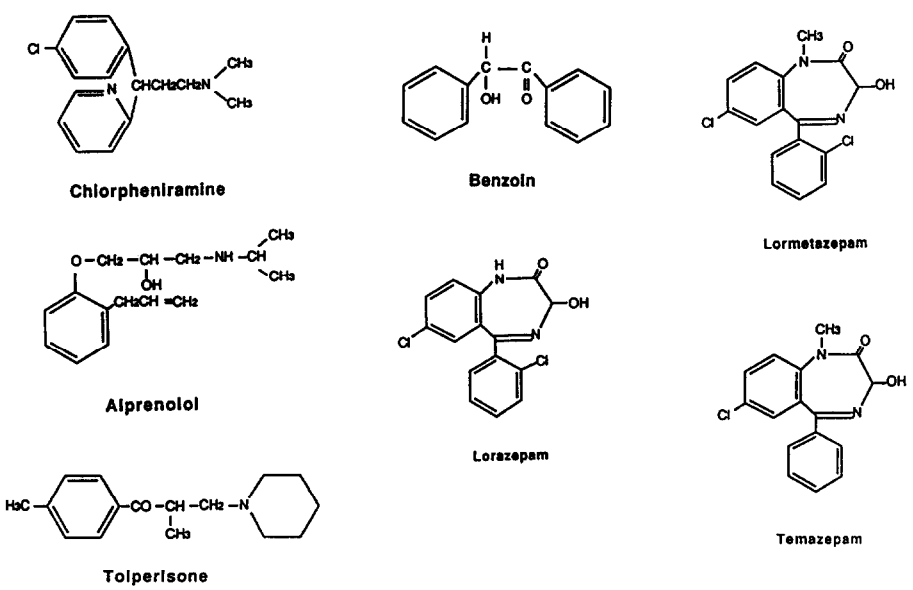

Fig. 1. The structures of enantiomers used in this study.

SIL-6B autoinjector, a C-R4A integrator and an SCL-6B system controller (all from Shimadzu, Kyoto, Japan). The flow-rate was maintained at 0.8 ml/min. All separations were carried out at 25°C using a CO-1093C column oven (Uniflows, Tokyo, Japan). Chromatographic parameters such as capacity factor (k'), enantioseparation factor (α) and resolution (R_s) were calculated from data obtained.

Sample preparation

A known amount of racemic solute was dissolved in methanol or water and the solution was diluted with the eluent to the desired concentration. A 20-μl aliquot of the sample solution was loaded onto a column. The loaded amount was 0.2–0.5 μg.

RESULTS

Comparison of base silica materials

We prepared two lysozyme-bonded materials, which are based on porous silica materials having average pore sizes of 120 and 300 Å. Table I illustrates the carbon and sulphur contents of aminopropyl and lysozyme phases, respectively, and the surface coverages of aminopropyl and lysozyme phases. Although the surface coverages of aminopropyl phases were similar with both the materials, that of lysozyme phases of the 300-Å materials was higher than that of the 120-Å materials. However, note that the amounts of bonded lysozyme proteins should be 11.7 and 5.6 μmol/g for the 120- and 300-Å material, respectively. Table II illustrates the capacity factors and enantioseparation factors of chlorpheniramine and temazepam on the two lysozyme-bonded columns based on the 120- and 300-Å materials. The lysozyme-bonded column prepared from the 120-Å base silica materials contained more bonded proteins and gave longer retentions for chlorpheniramine and temazepam enantiomers than that prepared from the 300-Å base silica materials, while the former column had lower enantioselectivity. Thus, the lysozyme-bonded materials prepared from a 300-Å base silica material were used in the experiments described below.

TABLE II

COMPARISON OF BASE SILICA MATERIALS FOR CAPACITY FACTOR OF FIRST-ELUTED (k'_1) CHLORPHENIRAMINE OR TEMAZEPAM AND ENANTIOSELECTIVITY (α) ON A LYSOZYME-BONDED MATERIAL

Pore size of base silica (Å)[a]	Chlorpheniramine[b]		Temazepam[b]	
	k'_1	α	k'_1	α
120	12.9	1.16	5.94	1.27
300	6.50	1.37	3.78	1.93

[a] The particle diameter of the base silica materials used was 5 μm.
[b] HPLC conditions are as follows: eluent, 20 m*M* phosphate buffer (pH 6.5) including 1.5% 2-propanol; flow-rate, 0.8 ml/min.

TABLE I

COMPARISON OF BASE SILICA MATERIALS AND SURFACE COVERAGES

Pore size of base silica (Å)	Specific surface area (m^2/g)	Aminopropyl phase		Lysozyme phase	
		Carbon content	Surface coverage (μmol/m^2)[a]	Sulphur content	Surface coverage (μmol/m^2)[b]
120	300	3.23	3.3	0.37	0.039
300	100	1.25	3.6	0.18	0.056

[a] Estimated from the elemental analysis data of nitrogen.
[b] Estimated from the elemental analysis data of sulphur.

TABLE III

COMPARISON OF REACTION pH FOR CAPACITY FACTOR OF FIRST-ELUTED (k'_1) CHLORPHENIRAMINE OR TEMAZEPAM AND ENANTIOSELECTIVITY (α) ON A LYSOZYME-BONDED MATERIAL

Reaction pH[a]	Chlorpheniramine[b]		Temazepam[b]	
	k'_1	α	k'_1	α
5.5	6.18	1.34	2.88	1.86
6.5	6.50	1.37	3.78	1.93
8.0	3.56	1.27	2.68	1.76

[a] Reaction pH for bonding of lysozyme.
[b] HPLC conditions are as follows: eluent, 20 m*M* phosphate buffer (pH 6.5) including 1.5% 2-propanol; flow-rate, 0.8 ml/min.

Reaction pH for bonding of lysozyme

The reaction pH for binding of a lysozyme protein to DSC-activated aminopropyl silica gels was examined at pH 5.5, 6.5 and 8.0. Table III shows the capacity factors and enantioseparation factors of chlorpheniramine and temazepam on the lysozyme-bonded columns based on the different reaction pH values. Maximum retention and enantioselectivity of chlorpheniramine and temazepam were obtained at a reaction pH of 6.5. Thus, pH 6.5 was chosen as the reaction pH.

Effect of eluent pH on retention, enantioselectivity and resolution of basic and uncharged solutes

Chiral resolution of acidic, basic and uncharged enantiomers was examined on a lysozyme-bonded material. Basic and uncharged enantiomers were resolved. Although we attempted to optimize mobile phase conditions, acidic enantiomers such as 2-arylpropionic acid derivatives were not resolved. Next, we examined various factors affecting retention, enantioselectivity and resolution of basic and uncharged solutes on the lysozyme-bonded column. Table IV shows the effect of eluent pH on the retention, enantioselectivity and resolution of basic and uncharged solutes. The capacity factors of basic solutes increased with an increase in eluent pH, while the enantioseparation factors and resolution were highest at eluent pH values of 7.0 and 7.5. The capacity factors of uncharged solutes remained almost unchanged despite changes in eluent pH. The enantioseparation factors and resolution of benzoin remained unchanged, while those of benzodiazepines, especially lormetazepam and temazepan, increased with an increase in eluent pH. Fig. 2 shows chromatograms of temazepam enantiomers obtained when the eluent pH was varied from 6.0 to 7.5. It is interesting that although the retention time of the first-eluted enantiomer of

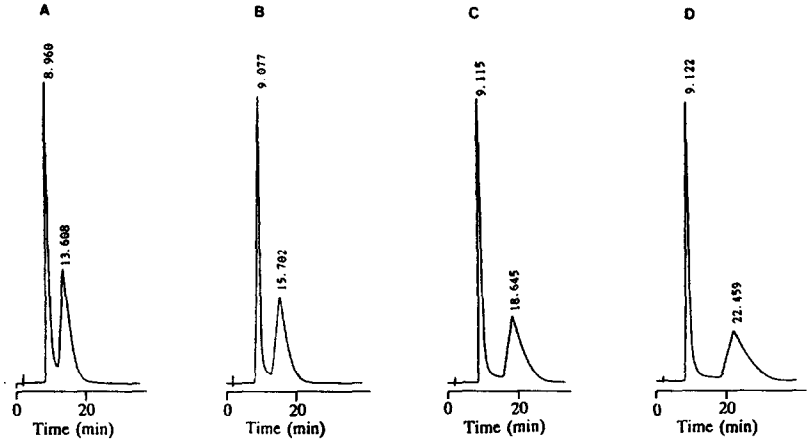

Fig. 2. Chromatograms of temazepam on the lysozyme-bonded silica column. Eluents used are a mixture of 1.5% 2-propanol and 20 m*M* phosphate buffer, whose pH is as follows: (A) 6.0; (B) 6.5; (C) 7.0; (D) 7.5. Concentration of racemic temazepam is 200 μg/ml. Other conditions: column temperature, 25°C; flow-rate, 0.8 ml/min; detection, 254 nm; injection volume, 2 μl.

TABLE IV

EFFECT OF pH ON RETENTION AND ENANTIOSELECTIVITY OF BASIC AND UNCHARGED SOLUTES ON A LYSOZYME-BONDED MATERIAL

Phosphate buffer (20 m*M*)–2-propanol (98.5:1.5, v/v) was used as an eluent.

	pH 6.0[a]			pH 6.5[a]			pH 7.0[a]			pH 7.5[a]		
	k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s
Chlorpheniramine	3.66	1.24	0.80	6.50	1.37	1.05	12.2	1.48	1.50	23.0	1.42	1.20
Alprenolol	1.76	1.12	0.23	4.08	1.23	0.41	8.76	1.30	0.60	15.6	1.32	0.56
Tolperisone	1.54	1.00		2.29	1.14	0.35	4.14	1.19	0.51	7.27	1.16	0.53
Benzoin	1.89	1.30	1.39	1.94	1.30	1.38	2.00	1.30	1.44	2.00	1.28	1.33
Lorazepam	6.65	1.00		5.93	1.08	0.21	6.00	1.09	0.28	6.02	1.10	0.34
Lormetazepam	5.80	1.07	<0.2	6.75	1.19	0.26	6.90	1.35	0.69	6.67	1.49	0.86
Temazepam	3.70	1.66	1.32	3.78	1.93	1.36	3.84	2.34	1.58	3.81	2.34	2.04

[a] Buffer pH.

TABLE V

EFFECT OF 2-PROPANOL CONTENT ON RETENTION AND ENANTIOSELECTIVITY OF BASIC AND UNCHARGED SOLUTES ON A LYSOZYME-BONDED MATERIAL

A mixture of 20 m*M* phosphate buffer (pH 7.0) and 2-propanol was used as an eluent.

	1.5%[a]			3.0%[a]			4.5%[a]			6.0%[a]		
	k_1'	α	R_s	k_1'	α	R_s	k_1'	α	R_s	k_1'	α	R_s
Chlorpheniramine	12.2	1.48	1.51	8.54	1.56	1.11	6.54	1.23	0.86	6.49	1.19	0.69
Alprenolol	8.76	1.30	0.60	5.37	1.24	0.56	4.09	1.19	0.52	3.14	1.16	0.44
Tolperisone	4.14	1.19	0.51	2.83	1.12	0.34	2.17	1.09	0.26	2.21	1.00	
Benzoin	2.00	1.30	1.44	1.64	1.23	1.23	1.43	1.20	1.07	1.30	1.17	1.12
Lorazepam	6.00	1.09	0.28	5.00	1.09	0.32	4.41	1.09	0.26	3.99	1.08	<0.2
Lormetazepam	6.90	1.35	0.69	5.42	1.20	0.35	4.61	1.09	<0.2	4.04	1.05	<0.2
Temazepam	3.84	2.34	1.58	3.14	1.89	1.72	2.73	1.63	1.65	2.44	1.55	1.45

[a] Percentage of 2-propanol.

temazepam is constant, that of the second-eluted enantiomer increases with an increase in eluent pH. As a result, the enantioseparation factor of temazepam increased with an increase in eluent pH.

Effect of organic modifier content on retention, enantioselectivity and resolution of basic and uncharged solutes

Table V shows the effect of 2-propanol content on retention, enantioselectivity and resolution of basic and uncharged solutes on a lysozyme-bonded column. The capacity factors, enantioselectivity and resolution generally decreased with an increase in 2-propanol content.

Effect of buffer concentration on retention, enantioselectivity and resolution of basic and uncharged solutes

Table VI shows the effect of phosphate buffer concentration on retention, enantioselectivity and resolution of basic and uncharged solutes on

TABLE VI

EFFECT OF PHOSPHATE BUFFER CONCENTRATION ON RETENTION AND ENANTIOSELECTIVITY OF BASIC AND UNCHARGED SOLUTES ON A LYSOZYME-BONDED MATERIAL

A mixture of phosphate buffer (pH 7.0) and 1.5% 2-propanol was used as an eluent.

	5 m*M*[a]			20 m*M*[a]			50 m*M*[a]			100 m*M*[a]		
	k_1'	α	R_s	k_1'	α	R_s	k_1'	α	R_s	k_1'	α	R_s
Chlorpheniramine	15.6	1.47	1.71	12.2	1.48	1.51	10.2	1.47	1.54	10.1	1.31	0.92
Benzoin	2.17	1.37	1.76	2.00	1.30	1.44	2.09	1.28	1.24	2.33	1.26	0.89
Temazepam	3.90	2.33	1.47	3.84	2.34	1.58	4.15	2.43	1.44	4.20	2.08	1.36

[a] Concentration of phosphate buffer.

a lysozyme-bonded column. The capacity factor of a basic solute, chlorpheniramine, decreased with an increase in the buffer concentration from 5 to 50 m*M*, while those of uncharged solutes, benzoin and temazepam, remained almost unchanged. The enantioselectivity and resolution of chlopheniramine remained unchanged over buffer concentrations 5–50 m*M*, while at the buffer concentrations of 100 m*M* they were decreased. Those of benzoin were decreased with an increase in buffer concentration, while enantioselectivity and resolution of temazepam were maximum at a buffer concentration of 50 and 20 m*M*, respectively.

Effect of type of organic modifier on enantioselectivity of basic and uncharged solutes

Fig. 3 illustrates the effect of type of organic modifier on enantioselectivity of chlorpheniramine and lorazepam when the organic modifier content was varied so as to give almost the same retention times. The highest enantioselectivity was obtained with methanol, ethanol and 2-propanol for chlorpheniramine. *tert.*-Butanol gave the highest enantioselectivity for lorazepam. Fig. 4A and B shows the separation of benzoin and chlorpheniramine enantiomers when *tert.*-butanol and 2-propanol were used as organic modifiers.

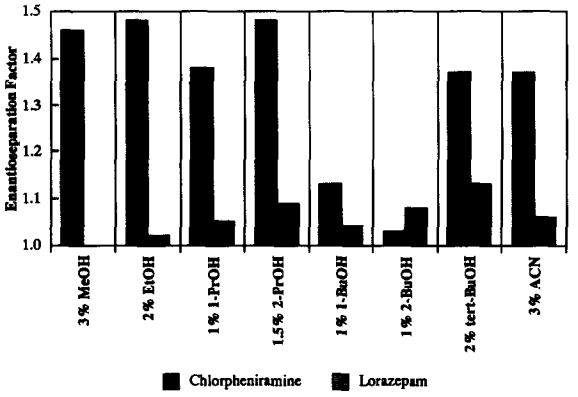

Fig. 3. Effect of type of organic modifier on enantioselectivity of chlorpheniramine and lorazepam. Eluents used were a mixture of 20 m*M* phosphate buffer (pH 7.0) and organic modifier. Organic modifiers used were methanol (MeOH), ethanol (EtOH), 1-propanol (1-PrOH), 2-propanol (2-PrOH), 1-butanol (1-BuOH), 2-butanol (2-BuOH), *tert.*-butanol (*tert.*-BuOH) and acetonitrile (ACN). Other conditions: column temperature, 25°C; flow-rate, 0.8 ml/min; detection, 254 nm.

DISCUSSION

Miwa *et al.* [6] reported that a basic protein, avidin, retained and resolved an acidic solute such as 2-arylpropionic acid derivatives and that an acidic protein, ovomucoid, retained and resolved basic solutes such as β-blockers. However, a lysozyme-bonded column resolved basic solutes with no resolution of acidic solutes. This suggests that the charged states on chiral recognition site(s) should be important. The chiral binding site(s), including carboxylate ion(s), should be present in a lysozyme molecule.

The lysozyme-bonded column prepared from a 300-Å base silica material gave higher enantioselectivity than that prepared from a 120-Å base silica material, despite the higher amounts of bonded proteins and longer retentions for solutes on the latter materials. This means that

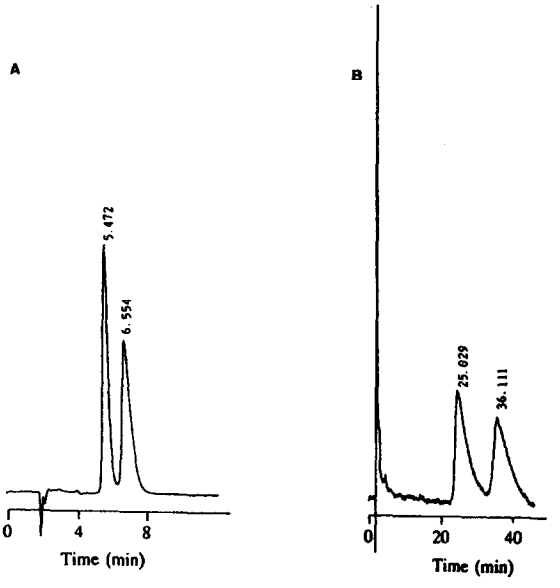

Fig. 4. Chromatograms of (A) benzoin and (B) chlorpheniramine on the lysozyme-bonded silica column. Concentrations of racemic benzoin and chlorpheniramine are 10 and 20 μg/ml. Chromatographic conditions were as follows: eluents, 20 m*M* phosphate buffer (pH 7.5)–*tert.*-butanol (98:2, v/v) for A and 20 m*M* phosphate buffer (pH 7.0)–2-propanol (98.5:1.5, v/v); column temperature, 25°C; flow-rate, 0.8 ml/min; detection, 254 nm; injection volume, 10 μl.

the bonded-lysozyme proteins do not function effectively for chiral recognition of solutes on the latter materials. In other words, one of the bonded proteins works for chiral interaction(s) with a solute, but the other does not.

The effects of eluent pH, organic modifier content and buffer concentrations on retention and enantioselectivity of basic and uncharged solutes reveal that hydrophobic and electrostatic interactions should play an important role in the retention and enantioselective properties of a lysozyme-bonded column.

In conclusion, a lysozyme-bonded column could be used for chiral resolution of basic and uncharged solutes. We are now trying to apply the lysozyme-bonded column to the chiral separation of propranolol and its ester derivatives, and to locate binding site(s) of a chiral compound on a lysozyme protein.

REFERENCES

1 S.R. Narayanan, *J. Pharm. Biomed. Anal.*, 10 (1992) 251; and references cited therein.
2 S. Allenmark, *J. Liq. Chromatogr.*, 9 (1986) 425.
3 E. Domenici, C. Bertucci, P. Salvadori, G. Felix, I. Cahagne, S. Montellier and I.W. Wainer, *Chromatographia*, 29 (1990) 170.
4 J. Hermansson, *J. Chromatogr.*, 269 (1983) 71.
5 T. Miwa, M. Ichikawa, M. Tsuno, T. Hattori, T. Miyakawa, M. Kayano and Y. Miyake, *Chem. Pharm. Bull.*, 35 (1987) 682.
6 T. Miwa, T. Miyakawa and T. Miyake, *J. Chromatogr.*, 457 (1988) 227.
7 P. Erlandsson, I. Marle, L. Hansson, R. Isaksson, C. Pettersson and G. Pettersson, *J. Am. Chem. Soc.*, 112 (1990) 4573.
8 S. Thelohan, P. Jadaud and I.W. Wainer, *Chromatographia*, 28 (1989) 551.
9 I.W. Wainer, P. Jadaud, G.R. Schombaum, S.V. Kadodkar and M.P. Henry, *Chromatographia*, 25 (1988) 903.
10 J. Haginaka, Ch. Seyama, H. Yasuda, H. Fujima and H. Wada, *J. Chromatogr.*, 592 (1992) 301.
11 J. Haginaka, T. Murashima, Ch. Seyama, H. Fujima and H. Wada, *J. Chromatogr.*, 631 (1992) 183.
12 P. Erlandsson and S. Nilsson, *J. Chromatogr.*, 482 (1989) 35.
13 T.C. Pinkerton and J. Haginaka, presented at the *16th International Symposium on Column Liquid Chromatography, Baltimore, MD, June 1992*, abstract 287.
14 L. Stevens, *Comp. Biochem. Physiol.*, 100B (1991) 1.
15 K. Kurachi, L.C. Sieker and L.H. Jensen, *J. Mol. Biol.*, 101 (1976) 11.
16 C. Redfield and C.M. Dobson, *Biochemistry*, 27 (1988) 122.