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Optical resolution of phenylthiohydantoin-amino acids by capillary electrophoresis for protein sequencing

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Abstract

An advanced method is described for the complete separation of phenylthiohydantoin (PTH)-DL-amino acids for protein sequencing. Optical resolution of all standard PTH-DL-amino acids was successfully developed using some chiral selectors, although the resolution of only PTH-DL-His and Lys could not be confirmed, due to low reproducibility and the presence of impurities.

In addition, mixed chiral selectors for making a single electrolyte with the ability to optically resolve all standard PTH-DL-amino acids were investigated. Using the only resulting electrolyte, sequence determination of [D-Ala^2]-methionine enkephalin, with DL differentiation, was performed.

Keywords: Enantiomer separation; Protein sequencing; Amino acids

1. Introduction

Biological interest in D-type amino acids has increased recently, although it is well known that bioactivity is often changed by replacing L-type amino acids with those of the D-type [1–7]. We have previously reported the optical resolution of phenylthiohydantoin (PTH)-DL-amino acids in capillary electrophoresis (CE) and the fundamental system for sequence analysis of a peptide including D-type amino acid residues [8]. The proposed system consisted of a protein sequencer with high-performance liquid chromatography (HPLC) and CE, and was demonstrated to be useful for sequence analysis with DL differentiation.

However, the optical resolution of all standard PTH-DL-amino acids was still incomplete, although seven amino acids were resolved by CE. It is necessary, therefore, to prepare electrolytes for optically resolving all standard amino acids.

This report describes an advanced method for the optical resolution of all standard PTH-DL-amino acids, i.e., nineteen amino acids (with the exception of Gly, which does not have chirality). In addition, a

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mixed electrolyte with chiral selectors for making a single electrolyte that has the ability to optically resolve all standard amino acids was developed. This work constitutes an important step in the realization of a protein sequencer analysis system with DL differentiation using CE.

2. Experimental

2.1. Reagents and materials

The PTH-D-, PTH-L- and PTH-DL-amino acids used in this study were manually derivatized from free D-, L- and DL-amino acids, respectively, except for commercially available materials, and these were then purified by HPLC [8]. Amino acids of the D- and L-types were used for identification of the D- and L peaks resolved in CE.

We used the following reagents and materials: [D-Ala^2]-methionine enkephalin (L-Tyr-D-Ala-Gly-L-Phe-L-Met) (Sigma, St. Louis, MO, USA); HPLC-grade acetonitrile, sodium dodecyl sulfate (SDS), sequencer-grade trifluoroacetic acid (TFA), sequencer-grade phenyl isothiocyanate (PITC), saponin (from tea seeds), α-cyclodextrin (α-CD) and β-cyclodextrin (β-CD) (Wako, Osaka, Japan); digitonin (Wako, and Nacalai Tesque, Kyoto, Japan); *o*-trimethyl-β-cyclodextrin (TM-β-CD), β-escin and glycyrrhizin ammoniacal hydrate (Funakoshi, Tokyo, Japan). Water was purified by deionization, followed by distillation. All other reagents were of analytical grade and were used without further purification.

2.2. Apparatus

CE was performed on a JASCO CE-800 system (Tokyo, Japan). The uncoated capillary (50 cm total length, 30 cm effective length and 50 μm I.D.) was purchased from GL Science (Tokyo, Japan). Samples were injected at the cathode end using a gravimetric method (height of 5 cm for 5–40 s). The buffer conditions are given in the table and figure captions.

Reversed-phase HPLC was performed on a JASCO 900 series HPLC system with a JASCO CrestPak C18T-5 column (250×4.6 mm I.D.; pore size, 5 μm).

Amino acid sequence determination was carried out using an Applied Biosystems Model 471A protein sequencer.

Other relevant information was described in a previous report [8].

2.3. Manual preparation of PTH-amino acids

Various amino acids or peptides (0.1–1 mg), dissolved in dimethylallylamine–propanol–water (1.7:30:20, v/v) or methanol–water–triethylamine (7:1:1, v/v) (100 μl) and PITC (5–10 μl), were vortex-mixed and heated at 55°C for 30 min. After the coupling reaction, the solvent was removed by evaporation and sublimation at 55°C. TFA (12.5–25%, v/v) in water or 1 *M* HCl (100 μl) was added to the resultant residue, which was heated at 55°C for 40 min or 80°C for 5 min. The reaction products were extracted using ethyl acetate (3×1 ml). After drying under a stream of N_2, the PTH-amino acid obtained was washed with benzene or *n*-heptane (3×1 ml). If necessary, the product was purified by HPLC.

2.4. Purification of saponin

Crude saponin, which was commercially available, was expected to contain some effective chiral selectors, as other saponins, e.g. digitonin and β-escin, are effective as chiral selectors. However, it was not effective as a chiral selector in CE and could not be dissolved sufficiently in electrolyte solution.

As digitonin or β-escin is slightly soluble in ethanol, crude saponin was purified as follows: Crude saponin (5 g), suspended in 100 ml of ethanol, was sonicated for 1 h, filtered using filter paper and the filtrate was dried by evaporation. The final yield of extract was 100–200 mg, and the extract was effective as a chiral selector. Further investigation are in progress.

3. Results and discussion

3.1. Optical resolution of PTH-DL-amino acids by CE

We investigated the optical resolution of all PTH-DL-amino acids using some conditions; seven PTH-

amino acids (Ala, Met, Ser, Thr, Trp, Tyr and Val) could be resolved using some chiral selectors [8–14]. The results are summarized in Table 1. All of the PTH-DL-amino acids were optically separated using five electrolytes with seven chiral selectors. To our knowledge, this is the first report of the optical resolution of PTH-DL-Arg, Asn, Asp, cysteic acid (CYA), Gln, Glu, Ile, Leu, Phe and Pro in CE (Fig. 1). Although PTH-DL-His and Lys were also found to be resolved (Fig. 2), problems regarding their reproducibility of migration in CE [the relative standard deviation (R.S.D.): 13% (n=4) in His and 15% (n=5) in Lys] and the purity of these PTH-DL-amino acids remain to be resolved. The R.S.Ds. of migration for the other amino acids ranged from 0.9% (n=6) in Phe to 4.0% (n=6) in Leu.

PTH-DL-Ile was optically resolved into four peaks, as it has two asymmetric carbon atoms. It was presumed that the four peaks were *allo*-D, D, *allo*-L and L, respectively, based on HPLC data [15]. The resolution of PTH-DL-Arg, Asp, Leu and Met was still not good enough (below R_s=0.5), and better resolution will be required. Although PTH-DL-CYA was optically resolved, it will also be necessary to resolve other derivatives of Cys, for example, PTH-DL-carboxymethyl-Cys, pyridylethyl-Cys and others. Conditions for their optical resolution are under investigation.

3.2. Investigation of a single electrolyte using a mixture of chiral selectors

As shown in Table 1, although all standard PTH-DL-amino acids (nineteen), with the exception of PTH-Gly, were resolved using seven chiral selectors [i.e. digitonin, β-escin, β-TM-CD, α-CD, β-CD, glycyrrhizin ammoniacal hydrate and purified saponin (which is thought to be a chiral selector)], at present, it is not possible to optically resolve all PTH-DL-amino acids using a single electrolyte. Such an electrolyte will be required for simple and rapid analysis. We have previously confirmed that a mixed electrolyte consisting of digitonin (which can resolve Val, but not Trp) and TM-β-CD (for Trp, but not for Val) was effective for optically resolving both PTH-DL-Val and PTH-DL-Trp [8]. Therefore, we investigated a single mixed electrolyte for the resolution for all PTH-amino acids.

Three chiral selectors, β-escin, digitonin and TM-β-CD, have the ability to resolve twelve, eight and seven amino acids, respectively. If three chiral selectors, β-escin, digitonin and TM-β-CD, are mixed together to give a single electrolyte, it should have be able to resolve all PTH-DL-amino acids. However, preparation of the mixture was difficult, due to precipitation. Therefore, two mixtures of chiral selectors, i.e. digitonin–TM-β-CD (type I) and digitonin–β-escin (type II) mixtures, were examined. The results are summarized in Table 2. Although based on the additivity of each resolving power, it was expected that types I and II might be able to resolve twelve and fifteen amino acids, respectively. However, type I and II mixtures had the ability to resolve eight and thirteen amino acids, respectively. The ability of type I to optically resolve CYA, Glu, His and Lys and of type II to resolve Arg and Trp was much decreased, or lost completely. In the case of Ile by type I, the ability to resolve the four peaks, *allo*-D, D, *allo*-L and L, was increased compared to that obtained from a single selector (data not shown).

It seems that studies using combinations of chiral selectors (and their concentrations) are important for obtaining a complete single electrolyte with high resolving ability.

3.3. Application to the sequence analysis of a peptide including a D-amino acid

As a model peptide, [D-Ala^2]-methionine enkephalin (200 pmol, 118 ng), was used for identification of D-amino acid residues in sequencing. The ratio (D/D+L) of D-Ala in [D-Ala^2]-methionine enkephalin was 98–99%, which was checked by Marfey's method [16–18].

PTH-Amino acids were manually fractionated from the protein sequencer. After drying, the obtained residue was dissolved in 20 μl of 50% (v/v) acetonitrile and analyzed by CE for DL differentiation. If the derivatization reaction proceeds to completion, the sample concentration is theoretically 10 μ*M*.

Determination of the amino acid sequence with DL differentiation in [D-Ala^2]-methionine enkephalin could be performed using only electrolyte type II. The chirality of Tyr in step 1 and Ala in step 2 were

Table 1
Summary of the optical resolution of PTH-DL-amino acids

Amino acids	Chiral selectors	R_s [a]	t_m (min)[b]	Buffer type[c]
Ala	Digitonin	1.07	37.79, 39.67	1
		0.68	51.25, 52.24	2
	β-Escin	0.59	23.68, 24.07	1
	Purified saponin	0.63	14.17, 14.46	1
Arg	β-Escin	0.36	9.80, 9.92	1
	α-CD	+		1
	β-CD	+		1
Asn	β-Escin	0.69	36.24, 36.92	1
Asp	β-Escin	0.43	27.71, 28.05	1
Cys(CYA)	TM-β-CD	0.53	12.77, 12.98	5
Gln	β-Escin	0.79	24.38, 24.75	1
Glu	β-Escin	0.52	26.25, 26.58	1
	Digitonin	+		1
	GAH[d]	4.02	11.15, 12.28	3
His*	TM-β-CD	1.01	24.26, 24.72	5
	β-CD	+		1
Ile	Digitonin	1.59	24.34, 26.13	2
		0.62	13.95, 14.33	1
	TM-β-CD	4.30	28.79, 32.43	5
	β-Escin	0.74	13.23, 13.70	1
	GAH	2.37	14.17, 15.33	3
	α-CD	+		1
	β-CD	+		1
Leu	β-Escin	0.44	12.25, 12.33	1
Lys*	β-CD	1.22	8.40, 9.00	1
	TM-β-CD	0.48	39.93, 40.57	5
Met	β-Escin	0.47	15.77, 15.93	1
	Digitonin	0.60	14.63, 14.81	1
		0.37	26.68, 27.00	2
Phe	TM-β-CD	0.68	40.30, 40.90	5
	Digitonin	0.42	16.50, 16.60	1
Pro	TM-β-CD	1.74	32.90, 33.73	5
	β-CD	1.27	10.08, 10.86	1
	α-CD	0.97	8.60, 9.10	1
Ser	β-Escin	0.34	41.82, 42.60	1
Thr	β-Escin	0.94	47.83, 49.38	1
Trp	TM-β-CD	5.94	29.47, 33.48	5
		1.14	11.23, 11.90	1
	Digitonin	0.27	18.17, 18.33	2
		0.34	15.07, 15.21	4
Tyr	Digitonin	0.78	11.35, 11.49	1
	TM-β-CD	+		5
Val	Digitonin	0.71	18.53, 18.94	1
		0.59	28.92, 29.33	2
		0.36	13.98, 14.13	4
	β-Escin	0.73	16.03, 16.26	1
	Purified saponin	0.55	9.30, 9.60	1
	GAH	0.92	22.70, 23.31	3

[a] R_s=Resolution. [b] t_m=Migration time. [c] 1=50 m*M* sodium phosphate (pH 3.0)–25–50 m*M* chiral selector–50 m*M* SDS. 2=[50 m*M* sodium phosphate (pH 3.0)–25 m*M* chiral selector–50 m*M* SDS]–methanol (80:20, v/v). 3=[50 m*M* sodium phosphate (pH 3.0)–1.0–3.0% (w/v) chiral selector–50 m*M* SDS]–methanol (95:5, v/v). 4=50 m*M* sodium phosphate (pH 3.0)–50 m*M* chiral selector–50 m*M* SDS–1–7 *M* urea. 5=(10 m*M* formic acid–25–40 m*M* chiral selector–50 m*M* SDS)–methanol (95:5, v/v). [d] GAH=Glycyrrhizin ammoniacal hydrate. + Indicates that R_s values could not be obtained due to the minimal amount of resolution that occurred. * Identification for DL peaks is still incomplete.

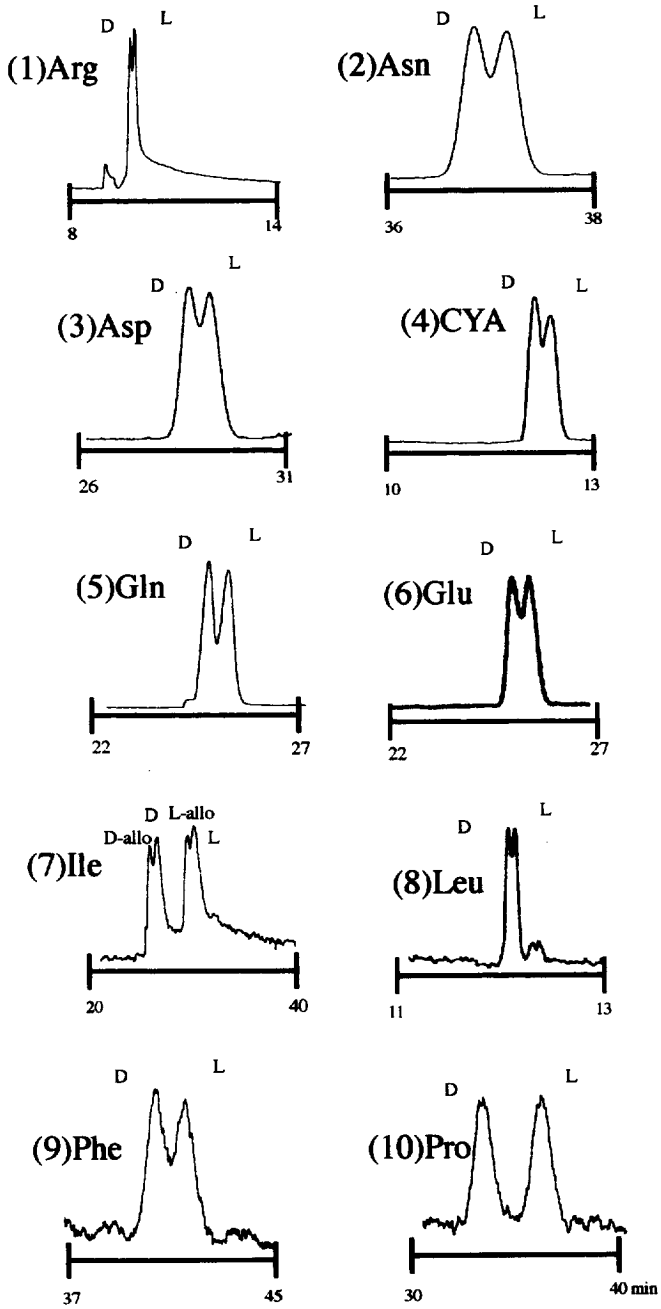

Fig. 1. Optical resolution of (1) PTH-DL-Arg, (2) Asn, (3) Asp, (4) CYA, (5) Gln, (6) Glu, (7) Ile, (8) Leu, (9) Phe and (10) Pro in CE. Conditions: electrolyte, (1)(2)(3)(5)(6) and (8), 50 m*M* sodium phosphate, pH 3.0, containing 50 m*M* SDS and 25 m*M* β-escin; (4)(9) and (10), 10 m*M* formic acid containing 50 m*M* SDS, 30–40 m*M* TM-β-CD and 5% (v/v) methanol; (7), 50 m*M* sodium phosphate, pH 3.0, containing 50 m*M* SDS, 25 m*M* digitonin and 20% (v/v) methanol. Other conditions were as described in Section 2.

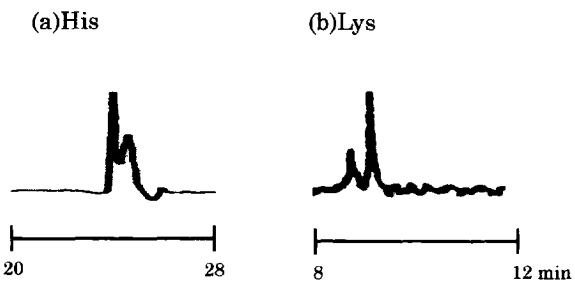

Fig. 2. Optical resolution of (a) PTH-DL-His and (b) Lys in CE. Conditions: electrolyte, (a) 10 m*M* formic acid containing 50 m*M* SDS, 30–40 m*M* TM-β-CD and 5% (v/v) methanol. The sample was injected at the anode end using a gravimetric method (5 cm height for 30 s); (b) 50 m*M* sodium phosphate, pH 3.0, containing 50 m*M* SDS and 25 m*M* β-CD. Other conditions were as described in Section 2.

Table 2
Summary of the optical resolution of PTH-DL-amino acids using a mixed electrolyte

Amino acids	Type I[a]		Type II[b]	
	R_s[c]	t_m (min)[d]	R_s	t_m (min)
Ala	1.38	97.80, 99.90	1.20	31.14, 31.90
Arg	–		x	
Asn	–		1.03	47.96, 49.14
Asp	–		0.89	33.70, 34.29
Cys(CYA)	x		–	
Gln	–		0.93	35.00, 35.62
Glu	x		1.04	31.03, 31.63
His	x		–	
Ile	3.48	33.38, 35.34	0.90	19.27, 19.71
Leu	–		0.54	19.14, 19.29
Lys	x		–	
Met	0.98	52.79, 54.07	0.50	22.03, 22.31
Phe	1.27	29.90, 30.50	0.78	18.17, 18.35
Pro	0.73	48.90, 49.80	–	
Ser	–		0.64	50.68, 51.78
Thr	–		0.46	42.87, 43.98
Trp	1.14	23.25, 23.60	x	
Tyr	1.41	40.46, 41.57	1.06	22.53, 22.85
Val	1.39	43.40, 44.76	1.33	22.74, 23.12

[a] 10 m*M* formic acid–50 m*M* sodium phosphate (pH 3.0)–35 m*M* TM-β-CD–25 m*M* digitonin–50 m*M* SDS.
[b] 50 m*M* sodium phosphate (pH 3.0)–25 m*M* β-escin–25 m*M* digitonin–50 m*M* SDS.
[c] R_s=Resolution.
[d] t_m=Migration time.
– Indicates that the mixed electrolyte did not have the resolving ability.
x Indicates that the mixed electrolyte did not have the resolving ability, although either selector had the ability.

identified as L-type and D-type, respectively, as shown in Fig. 3. Gly in step 3 gave a single peak because it does not have chirality. Each peak identification was confirmed by the addition of D-type or L-type amino acid, as described previously [8]. Other amino acids residues (Phe and Met) were also identified and confirmed as being of the L-type, in the same manner. The D/D+L ratio for Ala and the L/D+L ratios for Tyr, Phe and Met were 60–70% (n=20).

Despite the racemization caused, identification of the enantiomers could be accomplished [8]. It is important to suppress the racemization ratio because of the precision of the chirality identification. Further investigation are in progress.

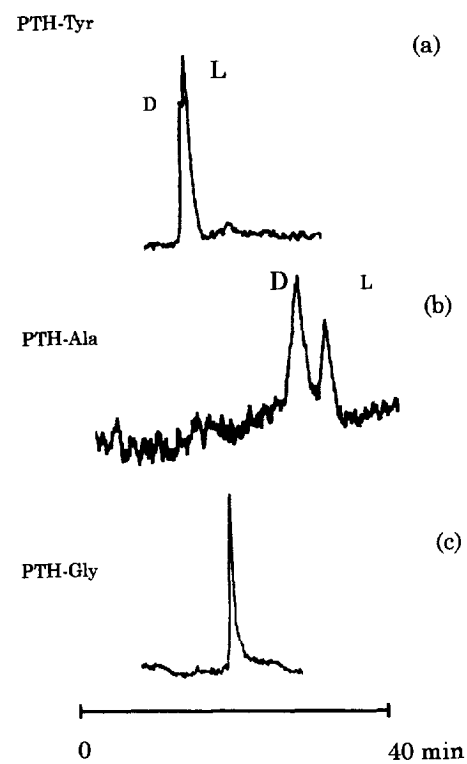

Fig. 3. Sequence analysis of [D-Ala^2]-methionine enkephalin. Conditions: electrolyte, Type II (50 m*M* sodium phosphate, pH 3.0, containing 50 m*M* SDS, 25 m*M* β-escin and 25 m*M* digitonin). Other conditions were as described in Section 2. (a) Step 1 (PTH-L-Tyr); (b) step 2 (PTH-D-Ala) and (c) step 3 (PTH-Gly).

4. Conclusion

We accomplished the optical resolution of all standard PTH-DL-amino acids (nineteen) by CE (Table 1), and showed that a single electrolyte was a powerful and effective technique for the resolution of PTH-DL-amino acids (Table 2). The resulting electrolyte has the ability to optically resolve thirteen PTH-DL-amino acids. The amino acid sequence with DL differentiation for [D-Ala^2]-methionine enkephalin was determined by our system using only a single electrolyte (Fig. 3). In the future, the development of an application for DL differentiation using a mixed electrolyte will be very useful for improving the sequence analysis system.

However, there are still several problems to be solved: (1) Better optical resolution will be required, especially for PTH-DL-His and Lys; (2) racemization for all standard PTH-amino acids must be investigated during derivatization; (3) the difference in sensitivity levels between HPLC and CE must be improved. The electrophoretic concentration in CE may be useful; and (4) automation between the protein sequencer and the CE system will be necessary.

At present, we are investigating solutions to the problems described above. In the future, this system will be useful in the life sciences because of the increasing importance of D-type amino acids.

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