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Optical resolution of phenylthiohydantoin-amino acids and identification of phenylthiohydantoin-D-amino acid residue of [D-Ala^2]-methionine enkephalin by capillary electrophoresis

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Abstract

We propose a system of protein sequence analysis with DL differentiation using capillary electrophoresis (CE). This system consists of a protein sequencer and a CE. After fractionation of phenylthiohydantoin (PTH)-amino acids from the protein sequencer, optical resolution for each PTH-amino acid is performed by CE using some chiral selectors such as digitonin, *o*-trimethyl-β-cyclodextrin (TM-β-CD) and others. In addition, optical resolution of all standard PTH-DL-amino acids including PTH-DL-carboxymethyl-Cys (CM-Cys) and cysteic acid (CYA) except for PTH-DL-Lys was successfully developed. The resolution of PTH-DL-Lys could not be reconfirmed due to low reproducibility and the impurities. As a model peptide, [D-Ala^2]-methionine enkephalin (L-Tyr–D-Ala–Gly–L-Phe–L-Met), was used and the sequence with DL differentiation was completely determined. © 1998 Elsevier Science B.V.

Keywords: Enantiomer separation; Protein sequencing; Amino acids; Enkephalin; PTH derivatives

1. Introduction

Life on earth metabolize only L-stereoisomer amino acids except for some bacteria [1–5]. The origin and significance of the in vivo existence of the D-stereoisomer are unclear, although it is well-known that the bioactivity is often changed by replacing the L-stereoisomer with the D-stereoisomer amino acids in the field of synthesis chemistry and pharmacy [6,7]. Recently, however, D-stereoisomer amino acids have been discovered in lower species of animals [8–11], crystalline lens [12,13], and mammalian organs and blood [5,14–19], as well as free amino acids in various tissues. The biological interest in D-stereoisomer amino acids has recently increased.

In this paper, we describe the optical resolution of all standard PTH-amino acids using capillary electrophoresis (CE) which has some advantages, e.g. rapid analysis, high resolution and miniaturized total chemical analysis. Furthermore, we propose a fundamental system for sequence analysis of a peptide including D-amino acid residues. The system consists of a protein sequencer with high-performance liquid chromatography (HPLC) and CE. As a model pep-

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tide, an opioid peptide, [D-Ala^2]-methionine enkephalin, was used. In this case, since complete PTH-DL-amino acid separation at the same run was not required, optical resolution was expected to be easily accomplished.

2. Experimental

2.1. Reagents and materials

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 the commercially available materials, and then purified by HPLC [20]. PTH-D-, PTH-L- and PTH-DL-carboxymethyl-Cys (CM-Cys) were derivatized from D-, L- and DL-CM-Cys which were synthesized from free D-, L- and DL-Cys, respectively, in the usual manner. D- and L-Stereoisomers were used for identification of 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 ammonical hydrate (GAH) (Funakoshi, Tokyo, Japan). Saponin was purified as described previously [21]. Water was purified by deionization and then distillation. All other reagents were analytical grade and used without further purification.

2.2. Apparatus

CE was performed on a Jasco CE-800 and CE-900 series (Tokyo, Japan). The uncoated capillary (50 cm (30 cm effective length)×50 μm internal diameter) was purchased from GL Science (Tokyo, Japan). Samples were injected at the cathode using a gravity method at 5 cm height for 5–40 s. The buffer conditions are given in the table and figure captions. The separation voltage was −15 kV with detection by absorbance at 269 nm, at room temperature (ca. 26°C).

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 with an Applied Biosystems Model 471A protein sequencer.

The other information was described in previous report [20].

2.3. Manual standard preparation of PTH-amino acids

The preparation is described elsewhere [20,21] and was followed with a slight modification. A peptide or an amino acid (0.01–0.1 mg) dissolved in dimethylallylamine–propanol–water (1.7:30:20, v/v) (100 μl) and PITC (5 μl) were vortex-mixed and heated at 55°C for 30 min. After the coupling reaction, the mixture was washed three times with 300 μl of *n*-heptane. The aqueous phase was lyophilized for 20 min and sublimated at 55°C for 25 min, and 50 μl of TFA was added to the resultant residue, which was heated at 55°C for 10 min. After drying under a stream of N_2, the resultant residue was mixed with distilled water (100 μl) and extracted using ethyl acetate (3×250 μl). The combined organic solvent containing an amino acid residue was dried under a stream of N_2. One hundred μl of 1 *M* HCl was added to the resultant residue, which was heated at 80°C for 5 min. The reaction product was extracted by ethyl acetate (3×250 μl). After drying under a stream of N_2, the PTH-amino acids obtained were dissolved in 50% (v/v) acetonitrile and subjected to HPLC or CE.

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 almost the same CE conditions; seven PTH-amino acids (Ala, Met, Ser, Thr, Trp, Tyr, Val) [20,22–27] and another 10 PTH-amino

acids [Arg, Asn, Asp, cysteic acid (CYA), Gln, Glu, Ile, Leu, Phe, Pro] [21] could be resolved. Although the remaining two PTH-amino acids (His, Lys) [21] were also found to be resolved, problems regarding their reproducibility of migration in CE and the purity of these amino acids remains to be solved. We reconfirmed that all of the PTH-DL-amino acids were optically separated using seven chiral selectors: β-escin, digitonin, TM-β-CD, β-CD, α-CD, GAH and purified saponin, have the abilities to resolve twelve (Ala, Arg, Asn, Asp, Gln, Glu, Ile, Leu, Met, Ser, Thr, Val), eight (Ala, Glu, Ile, Met, Phe, Trp, Tyr, Val), eight (CYA, His, Ile, Lys, Phe, Pro, Trp, Tyr), four (Arg, His, Ile, Pro), three (Arg, Ile, Pro), three (Glu, Ile, Val) and two (Ala, Val) amino acids, respectively. The best results for each amino acid are summarized in Table 1.

From our experiments, we found that His was resolved successfully under borate buffer (Fig. 1a). The peak identification was confirmed by the addition of D- and/or L-stereoisomer (Fig. 1b) [20]. This injection was performed at the opposite electrode (anode) against other PTH-amino acids. Identification for DL peaks of Lys was still incomplete. The R.S.D. values of migration for 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. Although GAH has high resolution ability for Glu ($R_s=4.02$), Ile ($R_s=2.37$) and Val ($R_s=0.92$), the baseline noise was much increased.

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

Amino acids	Chiral selectors	R_s[a]	Buffer type[b]
Ala	Digitonin	1.58	1
Arg	β-Escin	0.36	1
Asn	β-Escin	0.69	1
Asp	β-Escin	0.43	1
CYA	TM-β-CD	0.53	2
Gln	β-Escin	0.79	1
Glu	β-Escin	0.52	1
His	β-CD	0.71	3
Ile	TM-β-CD	4.30	2
Leu	β-Escin	0.44	1
Lys[c]	TM-β-CD	0.48	1
Met	Digitonin	0.60	1
Phe	TM-β-CD	0.68	2
Pro	TM-β-CD	1.74	2
Ser	β-Escin	0.34	1
Thr	β-Escin	0.94	1
Trp	TM-β-CD	5.94	2
Tyr	Digitonin	0.78	1
Val	Digitonin	0.71	1

[a] R_s=resolution.
[b] (1) 50 m*M* sodium phosphate (pH 3.0)–25–50 m*M* chiral selector–50 m*M* SDS; (2) (10 m*M* formic acid–25–40 m*M* chiral selector–50 m*M* SDS)–methanol (95:5, v/v); (3) 50 m*M* sodium borate (pH 9.3)–25 m*M* chiral selector–50 m*M* SDS.
[c] Identification for DL peaks is still incomplete.

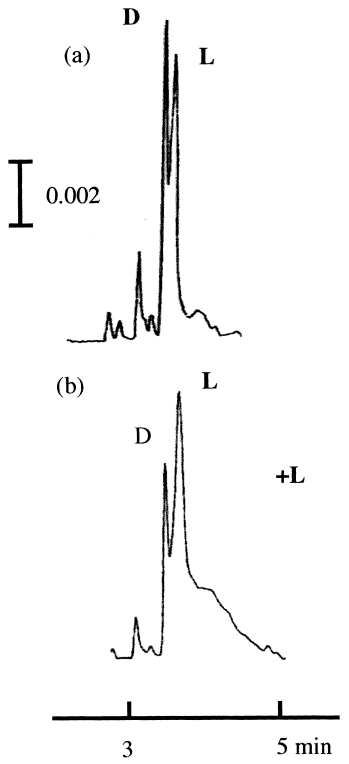

Fig. 1. Optical resolution of PTH-DL-His (a). L-Stereoisomer was added to DL-isomers (b). Electrolyte: 50 m*M* sodium borate (pH 9.3)–25 m*M* β-CD–50 m*M* SDS. The injection was performed at opposite electrode (anode) against other PTH-amino acids. Other conditions were as in Section 2.

As shown in Table 1, all PTH-DL-amino acids except PTH-Gly are resolved using four chiral selectors. However, a single electrolyte for optical resolution of all PTH-amino acids will be required in the future. For example, we have confirmed that a mixed electrolyte of digitonin, TM-β-CD and β-escin is effective to achieve optical resolution of 15 PTH-DL-amino acids (Table 2). In this condition, we accomplished the optical resolution of PTH-DL-CM-

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

Amino acids	R_s[a]	t_m (min)[b]
Ala	1.26	30.74, 31.67
Arg	0.75	11.66, 11.85
Asn	1.61	61.33, 64.35
Asp	1.33	33.16, 34.29
CM-Cys[d]	0.53	20.42, 20.73
CYA	[c]	
Gln	0.65	34.93, 35.61
Glu	0.83	28.28, 28.85
His	[c]	
Ile	3.14	12.57, 13.12
Leu	[c]	
Lys	[c]	
Met	0.85	16.27, 16.51
Phe	0.79	10.83, 10.94
Pro	[c]	
Ser	0.94	54.20, 55.80
Thr[e]	0.33	40.18, 40.85
Trp	0.22	10.24, 10.30
Tyr	1.12	16.48, 16.76
Val	1.16	16.84, 17.16

Conditions: 10 m*M* formic acid–17.5 m*M* TM-β-CD–12.5 m*M* digitonin–12.5 m*M* β-escin–50 m*M* SDS.
[a] R_s=resolution.
[b] t_m=migration time.
[c] Indicates that the mixed electrolyte did not have the resolving ability, although either selector was capable of doing so.
[d] The mixed electrolyte had the resolving ability, although both selectors were incapable of doing so.
[e] The measurement was performed as follows. The liquid level of the electrolyte solution in the cathode reservoir was 4 cm higher than that in the anode to shorten analysis time.

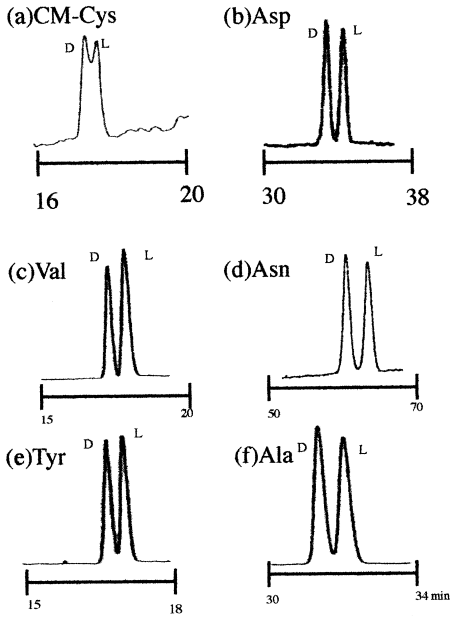

Fig. 2. Optical resolution of typical PTH-DL-amino acids using a mixed electrolyte. Conditions were as in Table 2 and Section 2.

Cys which was one of derivatives for Cys (Fig. 2a), although both selectors were incapable of resolution. On the other hand, PTH-DL-Cya could not be resolved even though TM-β-CD had the ability. Compared with our data, as described before [21], better results were totally obtained. Typical electropherograms are shown in Fig. 2.

3.2. Investigation of racemization in PTH-derivatization

Racemization of PTH-amino acids obtained from a protein sequencer can be expected [20,28–31]. Partial racemization was not a serious problem in our case, but a promising result, because a minor L or D peak produced by racemization was useful as an internal standard. Only complete racemization (50%) during derivatization must be avoided. From our experiments, we found that the racemization (D or L/D+L) for any PTH-D- or L-amino acid was 0–40% in any condition, changing reaction temperature and reaction time at cyclization/cleavage and conversion steps.

3.3. Identification of the PTH-D-Ala residue obtained from the protein sequencer for [D-Ala^2]-methionine enkephalin

As a model peptide, [D-Ala^2]-methionine enkephalin (L-Tyr–D-Ala–Gly–L-Phe–L-Met, 200 pmol, 118 ng), was used for identification of D-amino acid residue 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 [3,32,33].

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 com-

pletion, the sample concentration is theoretically 10 μ*M*.

Determination of the chirality in [D-Ala^2]-methionine enkephalin was as follows (Fig. 3):

In step 1; Tyr was identified as an L-stereoisomer. However, high racemization was observed. The L/D+L ratio was 60%.

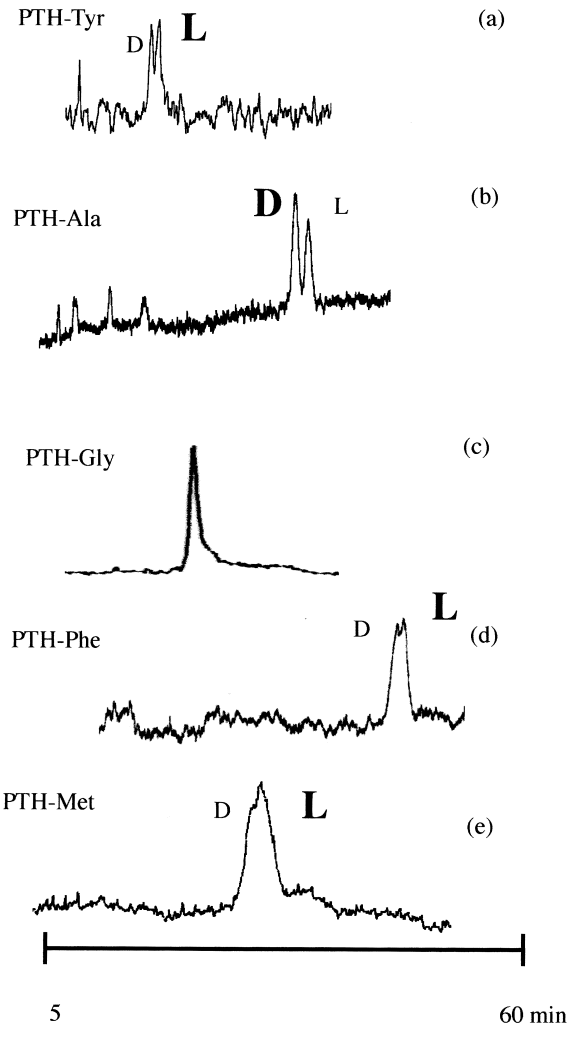

Fig. 3. Sequence analysis of [D-Ala^2]-methionine enkephalin. (a) Step 1 (PTH-L-Tyr), (b) step 2 (PTH-D-Ala), (c) step 3 (PTH-Gly), (d) step 4 (PTH-L-Phe) and (c) step 5 (PTH-L-Met). Electrolyte: (a) and (b) 50 m*M* sodium phosphate (pH 3.0)–25 m*M* digitonin–50 m*M* SDS; (c) and (d) (10 m*M* formic acid–40 m*M* TM-β-CD–50 m*M* SDS)–methanol (95:5, v/v); (e) 50 m*M* sodium phosphate (pH 3.0)–25 m*M* β-escin–50 m*M* SDS. Other conditions were as in Section 2.

In step 2; Ala was identified as a D-stereoisomer. The D/D+L ratio was 60%.

In step 3; Gly gave a single peak because it does not have chirality.

In step 4; Phe was identified as an L-stereoisomer. The L/D+L ratio was 65%.

In step 5; Met was identified as an L-stereoisomer. The L/D+L ratio was 63%.

Each peak identification was confirmed by the addition of D- or L-stereoisomer amino acid in the same manner, as described in Section 3.1. The D/D+L ratio for Ala and the L/D+L ratios for Tyr, Phe and Met were 60–75% (n=30).

Identification of enantiomers could be accomplished, despite the racemization [20]. However, it is important to suppress the racemization ratio for the precision of the chirality identification. Further investigations are in progress with the Edman sequencing method using boron trifluoride (BF_3) complexes [31].

4. Conclusion

We have proposed a system of protein sequence analysis with DL differentiation using capillary electrophoresis and demonstrated its potential. This method is available not only for protein sequencing but also identification of configuration of free amino acids in various biological fluid.

However, there are still several problems to be solved: (1) better optical resolution will be required, especially for PTH-DL-Lys and Cys derivatives; (2) racemization for all standard PTH-amino acids must be investigated during derivatization. The use of BF_3 complexes are useful at the cyclization/cleavage step [31]; (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.

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