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

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Abstract

This is an initial report to propose a protein sequence analysis system with DL differentiation using capillary electrophoresis (CE). This system consists of a protein sequencer and a CE system. After fractionation of phenylthiohydantoin (PTH)-amino acids using a protein sequencer, optical resolution for each PTH-amino acid is performed by CE using some chiral selectors such as digitonin, β-escin and others. 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 determined, with the exception of the fourth amino acid, L-Phe, using our proposed system.

Keywords: Chiral selectors; Amino acids, PTH derivatives; [D-Ala^2]-Methionine enkephalin; Enkephalin

1. Introduction

Most species, with the exception of some bacteria and aged organs, metabolize only L-type amino acids [1–4]. The origin and significance of the existence of the D-type are unclear. Recently, however, D-type amino acids have been discovered in the neuropeptides of lower species of animals, e.g. spiders and snails [5–7]. Furthermore, the existence in mammalian mitochondria is also suggested [8,9]. Biological interest in D-type amino acids has recently increased, although it is well known that the bioactivity is often changed by replacing L-type amino acids with those of the D-type in the field of synthesis chemistry and pharmacy [10,11].

In this paper, we describe the optical resolution of PTH-amino acids using capillary electrophoresis (CE) which has some advantages, e.g. rapid analysis time, high resolution and miniaturized total chemical analysis, and, furthermore, we propose a fundamental system for the sequence analysis of a peptide including D-amino acid residues. As a model peptide, an opioid peptide, [D-Ala^2]-methionine enkephalin was used and the system consisted of a protein

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sequencer with high-performance liquid chromatography (HPLC) and CE. In this case, since all PTH-DL-amino acid separation at the same run was not required, optical resolution was expected to accomplish with ease. In addition, the advantage of this system is that the position of D-type amino acids in a peptide or protein sequence can be specified, even if an amino acid appears more than once in the peptide or protein.

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 they were then purified by HPLC as described in Section 2. D- and L-Types were used for peak identification.

We used the following reagents and materials: PTH-DL-Ala, PTH-DL-Ile, PTH-DL-Met, PTH-DL-Ser, PTH-DL-Thr, PTH-DL-Trp, PTH-DL-Val, PTH-L-Arg, PTH-L-Asp, PTH-L-Asn, PTH-L-Cys, PTH-L-Gln, PTH-L-Glu, PTH-L-His, PTH-L-Leu, PTH-L-Lys, PTH-L-Phe, PTH-L-Pro, PTH-L-Tyr and PTH-Gly (Wako, Osaka, Japan); [L-Ala^2]-Methionine enkephalin (L-Tyr-L-Ala-Gly-L-Phe-L-Met) and [D-Ala^2]-methionine enkephalin (L-Tyr-D-Ala-Gly-L-Phe-L-Met) (Sigma, St. Louis, MO, USA); L-Ala-L-Ser (Kokusan, Tokyo, Japan); HPLC-grade acetonitrile, sodium dodecyl sulfate (SDS), sequencer-grade trifluoroacetic acid (TFA), sequencer-grade phenyl isothiocyanate (PITC), α-cyclodextrin (α-CD) and β-cyclodextrin (β-CD) (Wako); digitonin (Wako and Nacalai Tesque, Kyoto, Japan); *o*-trimethyl-β-cyclodextrin (TM-β-CD) and β-escin (Funakoshi, Tokyo, Japan). Water was purified by deionization and then distillation. All other reagents were of analytical grade and used without further purification.

2.2. Capillary electrophoresis

Capillary electrophoresis was performed on a JASCO CE-800 system (Tokyo, Japan). The uncoated capillary (50 cm total length, 30 cm effective length and 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 system consisted of 50 m*M* sodium phosphate, pH 3.0, 50 m*M* SDS and 15–25 m*M* chiral selectors, such as digitonin, TM-β-CD and β-escin. The separation voltage was -15 kV with detection by absorbance at 269 nm, at room temperature (ca. 26°C). Between injections, the capillary was cleaned for 2 min (using an aspirator) with the buffer and, before the first injection on the day, the capillary was cleaned for 10 min (using an aspirator) with the following: 0.1 *M* NaOH, water, 1 *M* phosphoric acid, water and then with the buffer, in order to retain the reproducibility of the separations. When the efficiency was decreased drastically, the capillary was cleaned (using an aspirator) consecutively with acetonitrile, ethyl acetate, acetonitrile, 0.1 *M* NaOH–acetone (1:1, v/v), water, 1 *M* phosphoric acid, water, and finally, the buffer, for 15 min.

2.3. HPLC

Reversed-phase HPLC (RP-HPLC) was performed on a JASCO 900 series HPLC system with a JASCO CrestPak C_{18} T-5 column (250×4.6 mm I.D.; pore size, 5 μm). The eluate was monitored by absorbance at 269 nm. Samples (PTH-amino acids) were separated using an isocratic elution with acetonitrile–35 m*M* sodium acetate, pH 4.5–5.1 (39:61, v/v).

2.4. 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. 12.5–25% (v/v) TFA in water or 1 *M* HCl (100 μl) was added to the resultant residue, which was heated at 55°C for 40 min or at 80°C for 5 min. The reaction products were extracted using ethyl acetate (1 ml; three times). After drying the sample under a stream of N_2, the obtained PTH-amino acid was washed with benzene or *n*-heptane (1 ml; three times). If necessary, the product was purified by HPLC.

2.5. Protein sequencing

Amino acid sequence determination was carried out with an Applied Biosystems Model 471A protein sequencer. The reaction conditions for PTH-derivatization were optimized using an ABI operation program.

3. Results and discussion

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

The optical resolution of some PTH-amino acids has been accomplished by Terabe et al. [12], Nishi and Terabe [13], Otsuka and Terabe [14], Ishihama and Terabe [15] and by Otsuka et al. [16–18] using some chiral selectors (Table 1). In addition, the

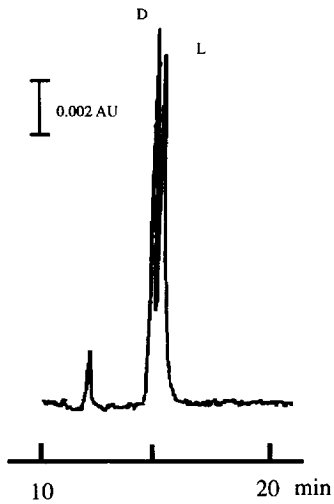

Fig. 1. Optical resolution of a mixture containing PTH-D-Tyr and PTH-L-Tyr. Conditions: electrolyte, 50 m*M* sodium phosphate, pH 3.0 containing 50 m*M* SDS and 25 m*M* digitonin. Other conditions were as described in Section 2.

Table 1
Optical resolution of PTH-DL-amino acids by CE

Amino acid	Chiral selector	Additive	Reference
Ala	Digitonin	SDS	[14]
	β-Escin	SDS	[15]
Met	SDGlu[a]	SDS–urea	[16]
	β-Escin	SDS	[15]
	CHAPS[b]	SDS–urea–methanol	[18]
Ser	β-Escin	SDS	[15]
Thr	β-Escin	SDS	[15]
Trp	Digitonin	SDS	[14]
	SDVal[c]		[14]
	SDVal	SDS–urea	[17]
	SDGlu	SDS–urea	[16]
	β-Escin	SDS	[15]
	STGlu[d]–CHAPS	SDS–urea–methanol	[18]
	TM-β-CD	SDS	*
Tyr	Digitonin	SDS	*
Val	Digitonin	SDS	[14]
	SDVal		[14]
	SDVal	SDS–urea	[17]
	SDGlu	SDS–urea	[16]
	Digitonin	STDC[e]–urea	[16]
	β-Escin	SDS	[15]
	STGlu–CHAPS	SDS–urea–methanol	[18]

* This optical resolution was performed in this work (Fig. 1, Fig. 2).
[a] Sodium N-dodecanoyl-L-glutamate.
[b] 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate.
[c] Sodium N-dodecanoyl-L-valinate.
[d] Sodium N-tetradecanoyl-L-glutamate.
[e] Sodium taurodeoxycholate.

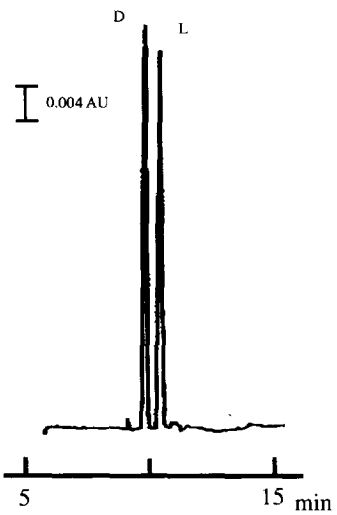

Fig. 2. Optical resolution of PTH-DL-Trp. Conditions: electrolyte, 50 m*M* sodium phosphate, pH 3.0 containing 50 m*M* SDS and 25 m*M* TM-β-CD. Other conditions were as described in Section 2.

optical resolution of PTH-DL-Tyr (Fig. 1) and PTH-DL-Trp (Fig. 2) was obtained using a digitonin–SDS and a TM-β-CD–SDS system, respectively. Although PTH-DL-Ile and PTH-DL-Leu were also resolved, problems regarding their reproducibility of migration in CE and the purity of these PTH-DL-amino acids remain to be solved. Other PTH-DL-amino acids could not be optically resolved using chiral selectors, such as digitonin, β-escin, some CDs, *n*-octyl-β-D-glucoside, *n*-octyl-β-D-thioglucoside and some cholic acid groups. It is important to note the the resolving power of digitonin varies depending on the manufacturer and the reagent lot.

As shown in Table 1, although only seven PTH-DL-amino acids are resolved at present using five chiral selectors, a single electrolyte for the optical resolution of all PTH-amino acids, with the exception of PTH-Gly, will be required in the future. For example, we have confirmed that a mixed electrolyte of digitonin (having resolving ability for Val, but not Trp) and TM-β-CD (for Trp, but not for Val) is effective to optically resolve both PTH-DL-Val and PTH-DL-Trp (data not shown).

3.2. Investigation of racemization in PTH-derivatization

Racemization of PTH-amino acids obtained from a protein sequencer can be expected. Therefore, the study of racemization was performed using free D- and L-Val, free D- and L-Ala and L-Ala-L-Ser. PTH-derivatization was manually performed using the reaction conditions described in Section 2. As a result, partial racemization of only free D- and L-Ala sometimes occurred, as shown in Fig. 3 (8% partial racemization case). Racemization of free D- and L-Val (Fig. 4) and of L-Ala of L-Ala-L-Ser (data not shown) was not observed. In the case of free L-Ala, the racemization ratio (D/D+L) was 0–40% (calculated using the migration time with a correction value) [19]. There was no observation up to 40% racemization. The conversion step is most doubtful reaction for racemization because of the use of strong acid with high temperature. Therefore, racemization can be controlled by altering the reaction conditions,

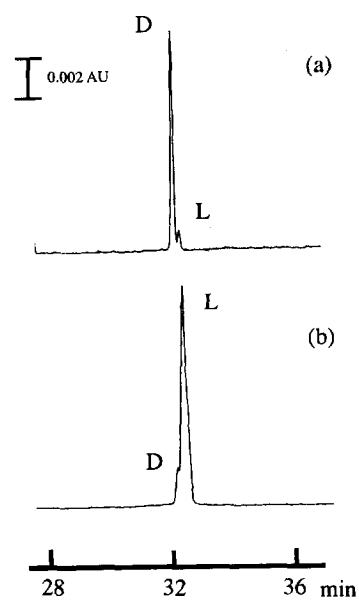

Fig. 3. Racemization of manually synthesized PTH-D-Ala and PTH-L-Ala. Conditions: electrolyte, 50 m*M* sodium phosphate. pH 3.0 containing 50 m*M* SDS and 25 m*M* digitonin. Other conditions were as described in Section 2. The ratios of (a) L/D+L in PTH-D-Ala and (b) D/D+L in PTH-L-Ala, were 8.1 and 8.4%, respectively.

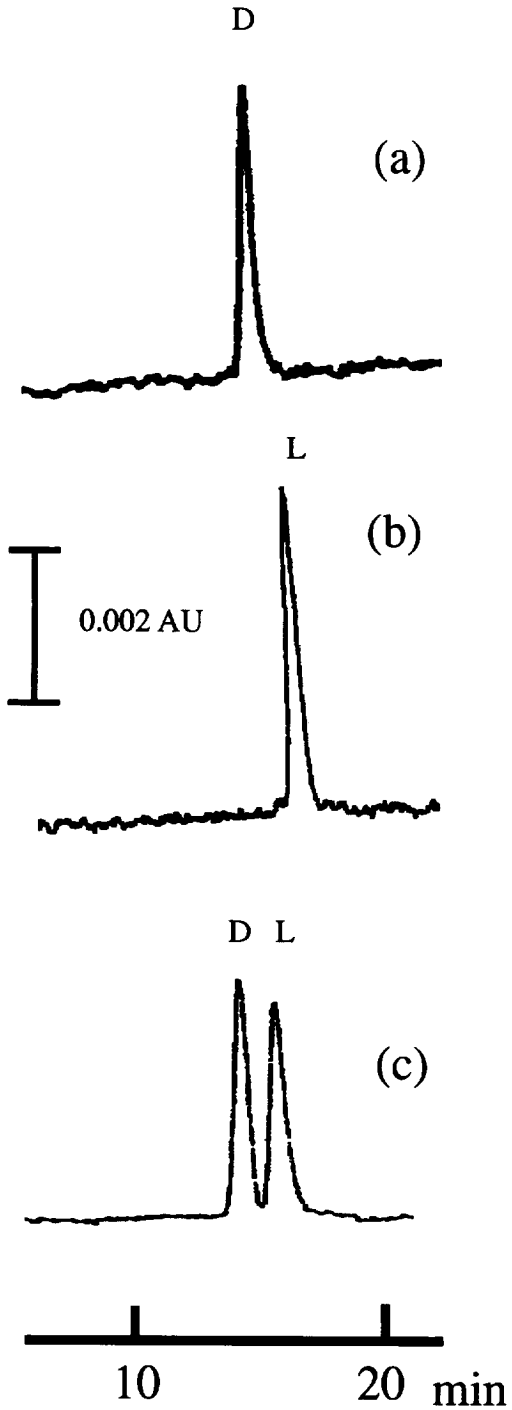

Fig. 4. Racemization of manually synthesized PTH-D-Val and PTH-L-Val. Conditions: electrolyte, 50 m*M* sodium phosphate, pH 3.0 containing 50 m*M* SDS and 25 m*M* digitonin. Other conditions were as described in Section 2. The ratios of (a) L/D+L in PTH-D-Val and (b) D/D+L in PTH-L-Val were both 0%. (c) A mixture of PTH-D-Val and PTH-L-Val.

although the cause of racemization is still unclear. When PTH-D- or L-Ala, Met, Trp, Tyr and Val were manually synthesized using some reaction conditions, complete racemization did not occur. Further investigation of the reaction conditions is still being undertaken.

Partial racemization was not a serious problem in our case, but rather, was 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.

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

As model peptides, [D-Ala^2]-methionine enkephalin (L-Tyr-D-Ala-Gly-L-Phe-L-Met, 200 pmole, 118 ng), was used for identification of the D-amino acid residue and [L-Ala^2]-methionine enkephalin (L-Tyr-L-Ala-Gly-L-Phe-L-Met) was used as a reference. They both have same sequence except that the chirality of the second amino acid (Ala residue) is different. The ratio (D/D+L) of D-Ala in [D-Ala^2]-methionine enkephalin and the ratio (L/D+L) of L-Ala in [L-Ala^2]-methionine enkephalin were checked using Marfey's method [3,20,21]. The ratio of each of them was 98–99%.

PTH-amino acids were manually fractionated using the HPLC system of 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 completely, the sample concentration is 10 μM.

Determination of the chirality in [D-Ala^2]-methionine enkephalin was as follows (Fig. 5):

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

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

In step 3; Gly is not necessary for identification because it does not have chirality.

In step 4; Phe could not be identified due to the lack of a method for optically resolving PTH-DL-Phe.

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

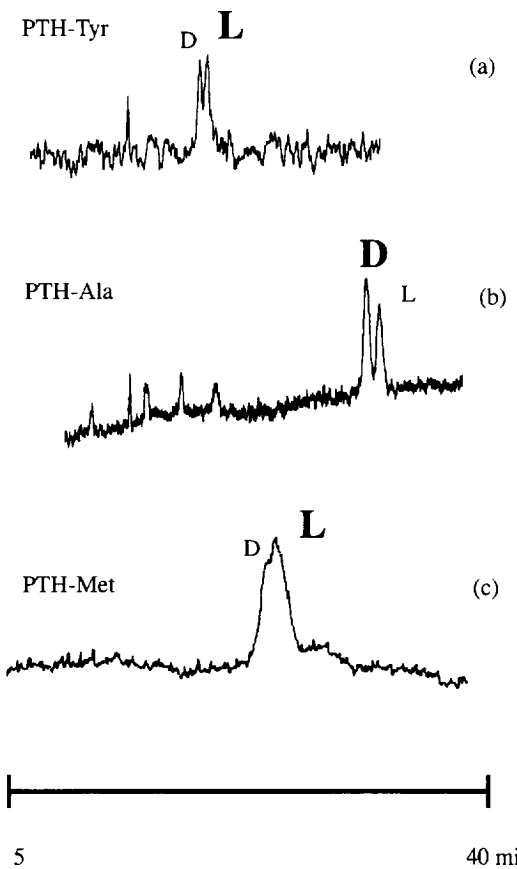

Fig. 5. Sequence analysis of [D-Ala^2]-methionine enkephalin. Conditions: electrolyte, 50 m*M* sodium phosphate, pH 3.0 containing 50 m*M* SDS and 25 m*M* chiral selector (steps 1 and 2, digitonin; step 5, β-escin). Other conditions were as described in Section 2. (a) Step 1 (PTH-L-Tyr); (b) step 2 (PTH-D-Ala) and (c) step 5 (PTH-L-Met).

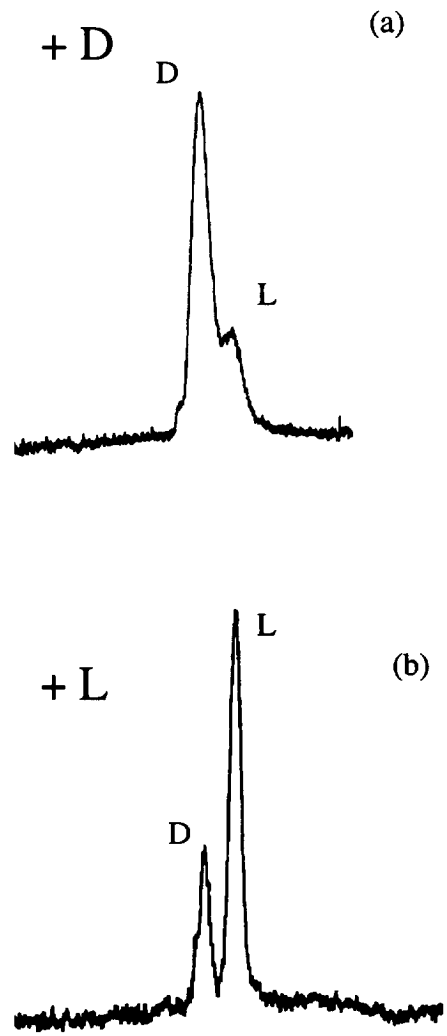

Fig. 6. Reconfirmation of D-Ala in [D-Ala^2]-methionine enkephalin. (a) D-Type and (b) L-type were added to each fraction of step 2, respectively. Conditions: electrolyte, 50 m*M* sodium phosphate, pH 3.0 containing 50 m*M* SDS and 25 m*M* digitonin. Other conditions were as described in Section 2.

The peak identification of D-Ala (step 2) was reconfirmed by addition of D-type or L-type as shown in Fig. 6. Other PTH-amino acids were also reconfirmed in the same manner. In the case of [L-Ala^2]-methionine enkephalin, the same results were obtained as for [D-Ala^2]methionine enkephalin, with the exception of the result obtained for step 2. In step 2, Ala was obviously identified as L-type (Fig. 7). The L/D+L ratio was 62%. The racemization values represent the average of two-to-four analyses.

Although the racemization ratio was higher than expected and the resolution efficiency of Met was unsatisfactory, the identification of chiral amino acids was clearly demonstrated, as can be seen from a comparison of Fig. 5b and Fig. 7 on our proposed protein sequencing–CE system. At present, other conditions for the PTH-derivatization of all amino acids on a protein sequencer are under investigation.

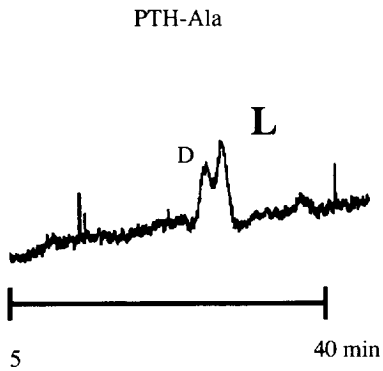

Fig. 7. Electropherogram of the second amino acid residue in [L-Ala^2]-methionine enkephalin. Conditions: electrolyte, 50 m*M* sodium phosphate, pH 3.0 containing 50 m*M* SDS and 25 m*M* digitonin. Other conditions were as described in Section 2.

Imal et al. [22,23] also have been investigating the amino acid sequence/configuration of peptides containing D–L-amino acids using some Edman reagents, on chiral HPLC columns [22,23]. The merit of our proposed system is that partial racemization in PTH-derivatization is useful for DL differentiation and the reproducibility of the migration time in CE is not a serious problem because PTH-amino acids have been identified previously using the protein sequencer. Therefore, accomplishment of simple and rapid analyses is expected.

4. Conclusion

This is the first report of DL differentiation in peptide sequence analysis using our proposed system. In the future, this system will be useful in the life sciences because of the increasing importance of studying D-type amino acids.

However, there are still several problems to be solved:

(1) Automation between the protein sequencer and the CE system will be necessary.

(2) Optical resolution for all standard PTH-amino acids (except Gly) should be complete in CE.

(3) The difference in the level of sensitivity between HPLC and CE must be improved. The electrophoretic concentration in CE may be useful.

(4) Racemization of all standard PTH-amino acids (except Gly) must be investigated during derivatization.

At present, we are trying to find solutions to the problems outlined above, including the optical resolution of PTH-DL-amino acids using HPLC which could provide better routine analysis.

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