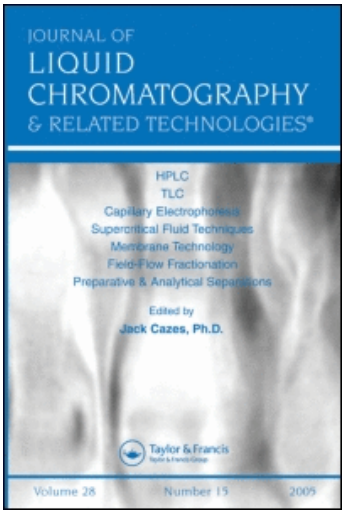

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Identification of Chirality of Phenylthiohydantoin-D-Amino Acid Residue of [D-ala²]-Metthionine Enkephalin by Capillary Electrophoresis: Suppression and Control of Racemization Ratio in the Edman Sequencing Method

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IDENTIFICATION OF CHIRALITY OF PHENYLTHIOHYDANTOIN-D-AMINO ACID RESIDUE OF [D-ALA^2]-METHIONINE ENKEPHALIN BY CAPILLARY ELECTROPHORESIS: SUPPRESSION AND CONTROL OF RACEMIZATION RATIO IN THE EDMAN SEQUENCING METHOD

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ABSTRACT

This paper describes the suppression and control of the racemization ratio (D or L/D+L) of phenylthiohydantoin (PTH) amino acids in the Edman sequencing method. Most of the racemization occurs in the cyclization/cleavage step. Although optimization of partial racemization using a mixture of TFA and boron trifluoride (BF_3)-ethyl ether complex, which is effective in suppressing racemization in the cyclization/cleavage reaction. The partial racemization in PTH derivatization is often useful for DL differentiation, because a minor L- or D-peak produced by racemization can be used as an internal standard in CE. Using the partial racemization method with mixed acids as a cyclization/cleavage reagent, the sequence determination of [D-Ala^2]-methionine enkephalin, with DL differentiation, was achieved on a sequencer.

INTRODUCTION

Recently, D-type amino acids in peptides and proteins have been discovered in neuropeptides of lower species of animals,[1-4] human lens,[5,6] mammalian mitochondria,[7,8] and human erytbrocytes,[9] as well as in free amino acids in various tissues.[10-19] Therefore, the biological interest for peptides or proteins containing D-type amino acids has been extremely great.

We have previously reported the optical resolution of phenylthiohydantoin (PTH)-DL-amino acids in capillary electrophoresis (CE) and the fundamental systems for sequence analysis of a peptide containing D-type amino acid residue.[20,21]

Our proposed system, comprising a protein sequencer with high performance liquid chromatography (HPLC) and capillary electrophoresis (CE), has been proven to be useful in sequence analysis with DL differentiation.

However, several problems remain. The most important is that racemization occurs during the Edman reaction.[20,21] The racemization ratios (D or L/D+L) during the reaction are often over 40 %. The large partial racemization disrupts the determination of D- or L-type amino acid. Therefore, it is necessary to establish a method of suppressing and controlling racemization. Imai et al. have demonstrated that the replacement reaction with trifluoroacetic acid (TFA) during the cyclization/cleavage reaction in the sequencing procedures correlated sufficiently with the racemization of the

liberated/derivatized amino acids, and that aprotic acid, i.e., the Lewis acid boron trifluoride (BF_3)-ethyl ether complex. was effective in suppressing racemization.[22-24]

This report describes the suppression and control of the racemization ratio (D or L/D+L) of phenylthiohydantoin (PTH) amino acids in the Edman sequencing method. Using a partial racemization method with a mixed acid (TFA and BF_3-ethyl ether complex), sequence determination of [D-Ala^2]-methionine enkephalin, with DL differentiation, was achieved on a sequencer.

EXPERIMENTAL

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.[20] Amino acids of the D-, L-, and DL-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, MW 587.7) (Sigma, St. Louis, MO, USA); HPLC-grade acetonitrile, sodium dodecyl sulfate (SDS), sequencer-grade trifluoroacetic acid (TFA), mercaptoethanol and sequencer-grade phenyl isothiocyanate (PITC) (Wako, Osaka, Japan); digitonin (Wako, Osaka, Japan and Nacalai Tesque, Kyoto, Japan); o-trimethyl-β -cyclodextrin (TM-β-CD) and β-escin (Funakoshi, Tokyo, Japan); boron trifluoride (BF_3)-ethyl ether complex (Tokyo Kasei, Tokyo, Japan); BF_3-acetic acid complex and BF_3-methanol complex (Aldrich, Milwaukee, WI, USA). Water was purified by deionization, followed by distillation. All other reagents were of analytical grade and were used without further purification.

Apparatus

CE was performed on a JASCO CE-800. 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 10-40 s). The separation voltage was -15 kV with detection by absorbance at 269 nm, at room temp. (ca 26°C). The buffer conditions are given in the figure captions. Reversed-phase HPLC was

performed on a JASCO 900 series HPLC system with a JASCO CrestPak C18T-5 column (250 x 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.[20]

Manual Standard Preparation of PTH-Amino Acids from a Peptide

The preparation is described elsewhere[20,21] and was followed with slight modification. A peptide (0.01 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, BF_3 complex, or a mixture of TFA/BF_3-ethyl ether complex was added to the resultant residue, which was heated at 55°C for 10 min. After drying under a stream of N_2 gas, the resultant residue was mixed with distilled water (100 μL) and extracted using ethyl acetate (3 x 250 μL). The combined organic solvent containing anilinothiazolinone (ATZ)-amino acids was dried under a stream of N_2. 100 μL of 1M HCl was added to the resultant residue, which was heated at 80°C for 5 min. The reaction product was extracted with ethyl acetate (3 x 250 μL). After drying under a stream of N_2 gas, PTH-amino acid obtained was dissolved in 50% (v/v) acetonitrile and subjected to HPLC.

Preparation of a Mixture of TFA and BF_3-Ethyl Ether Complex

In a draft chamber, under N_2 gas, TFA and BF_3-ethyl ether complex were mixed with dried pipettes and stored below 10°C. A slight coloration seen after long storage may be disregarded.

RESULTS AND DISCUSSION

Investigation of Racemization in PTH-Derivatization Process–Cyclization/Cleavage Reaction and Conversion Reaction

Racemization during the cyclization/cleavage step has been carefully investigated by Imai et al. They demonstrated that it was caused by the

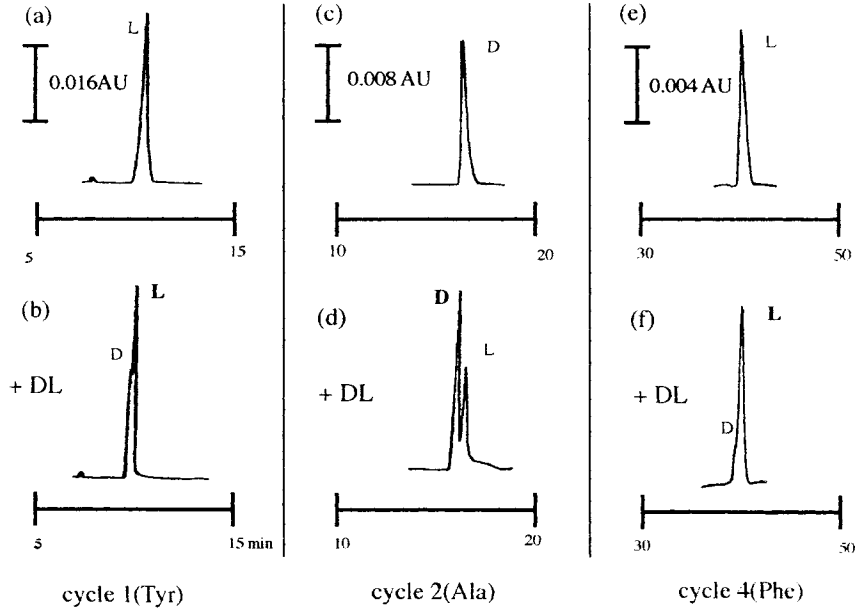


Figure 1. Manual sequence analysis of [D-Ala^2]-methionine enkephalin using BF_3-ethyl ether complex at cyclization/cleavage step. Conditions: electrolyte, (cycle 1) (cycle 2) 50 mM sodium phosphate, pH 3.0 including 50 mM SDS and 25 mM digitonin; (cycle 4) 50 mM sodium phosphate, pH 3.0 including 50 mM SDS and 40 mM TM-β-CD/methanol (90:10, v/v). Details of injection are given in Results and Discussion. Other conditions were as in the Experimental section. The PTH derivatives were injected without (a, c, and e) and with (b, d, and f) an internal standard (DL-type). Gly in cycle 3 does not have chirality. Met in cycle 5 could not be determined because of a very low yield.

replacement of a hydrogen atom by TFA, and that BF_3-ethyl ether complex was effective in retaining the D/L-amino acid configuration of the amino acid residues.[24] Although we attempted to suppress racemization at various reaction temperatures (25-55°C) and times (1-10 min) usingTFA, we could not suppress it completely and we were unable to control the racemization ratio accurately (data not shown).

When we used BF_3-ethyl ether complex in the cyclization/cleavage step, derivatized PTH-amino acids were scarcely racemized in the sequence analysis for enkephalin. (Fig. 1a, c, and e). The effect of the conversion step was expected to be slight. Since no racemization was produced in this case, a corresponding standard DL-type was added to each cycle fraction as an internal

standard. Although the optical resolution was still unsatisfactory, the chirality could be identified, but with difficulty. As a result, double peaks were obtained (Fig. 1b, d, and f). The larger peak is the one to be identified. Therefore, determination of the chirality in cycle 1[1] (L-Tyr), cycle 2 (D-Ala) and cycle 4 (L-Phe) of [D-Ala[2]]-methionine enkephalin (L-Tyr-D-Ala-Gly-L-Phe-L-Met) could be performed. Gly in cycle 3 does not have chirality. Met in cycle 5 could not be determined because of its very low yield. A sample (PTH derivative) was injected at the cathode using a gravity method (height of 5 cm for 20 sec, ca. 4 nL). The concentration of the injected sample was 0.5 mg/mL if the derivatization reaction proceeded completely. An internal standard (DL-type) was subsequently introduced at the cathode using a gravity method (height of 5 cm for 5-20 sec, ca. 1-4 nL). The concentration of the DL-type was 1 mg/mL of each. The detection limit in the CE-800 system was 131 μM (0.78 pmol). Here, we define the detection limit as the sample concentration that gives a SNR (signal-to-noise ratio) of 3.

Other BF_3 complexes, such as BF_3-acetic acid complex and BF_3-methanol complex, also have a similar ability to that of the BF_3-ethyl ether complex. However, the elimination of the reagents was much more difficult, and some side reaction products occurred. In addition, the yields were much lower. These were serious disadvantages for automation.

The racemization during the conversion step was investigated under various conditions of reaction time (1-10 min), temperature (25-80°C) and acid concentration (12.5-25% TFA, 0.1- 1M HCl). We found that racemization could not be controlled under such conditions (data not shown), although we had expected that racemization was likely to occur in the conversion step because of the strong acid under high temperature.

Stability of PTH-Amino Acids in Solution in CE

The racemization ratio in 50% acetonitrile, which is usually used for dissolution after PTH-derivatization, was checked because samples were often stored in solution prior to determination of the chirality. Fluctuation of racemization ratio of PTH-amino acids (L-Tyr and L-Gln) was measured as changes in peak areas in CE (Fig. 2).

High racemization did not cause any change at room temperature, even after 300 h. However, the ratio for L-Tyr at 60°C increased after several hours. The ratio for complete racemization (L or D/L+D) = 50%) was attained within ca. 60 h.

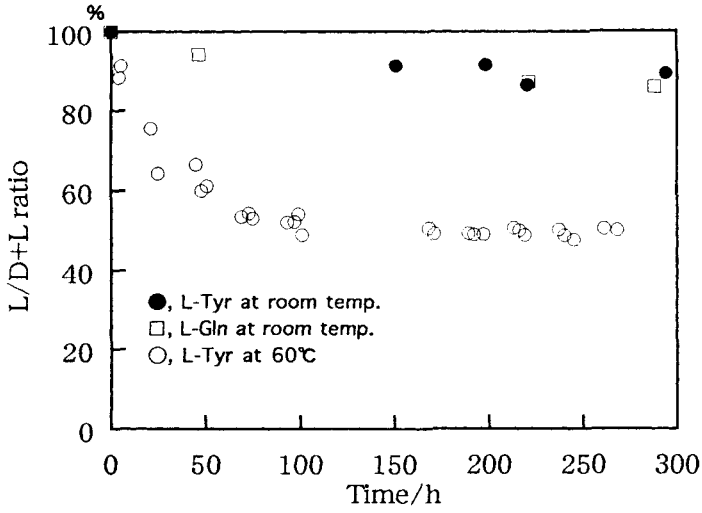


Figure 2. The racemization stability of PTH-amino acids in the solution. Conditions: electrolyte, 50 mM sodium phosphate, pH 3.0, including 50 mM SDS and 25 mM digitonin or β-escin. Other conditions were as in the Experimental section.

We reconfirmed that the main cause of the racemization was in the cyclization/cleavage reaction.[24] However, to obtain an accurate racemization ratio, the preservation technique for PTH-amino acids and all processes in the Edman degradation must be studied. We are currently investigating racemization in more detail.

Control of Racemization Ratio using a Mixture of TFA and BF_3-Ethyl Ether Complex

Low partial racemization makes it easier to identify the D/L- amino acid configuration. TFA offers high partial racemization and BF_3-ethyl ether complex offers no racemization. Therefore, we used a mixture of TFA and BF_3-ethyl ether complex to achieve controlled racemization. The relationship between the racemization ratio and BF_3-ethyl ether complex concentration in TFA is shown in Figure 3. We found that a 25% BF_3-ethyl ether complex offered adequate partial racemization (20% ratio) by the manual method, with good reproducibility. However, this mixed acid could not work on a sequencer because of its low yield and high viscosity. In addition, the white precipitation often clogs the lines. Therefore, the mixed acid was diluted with

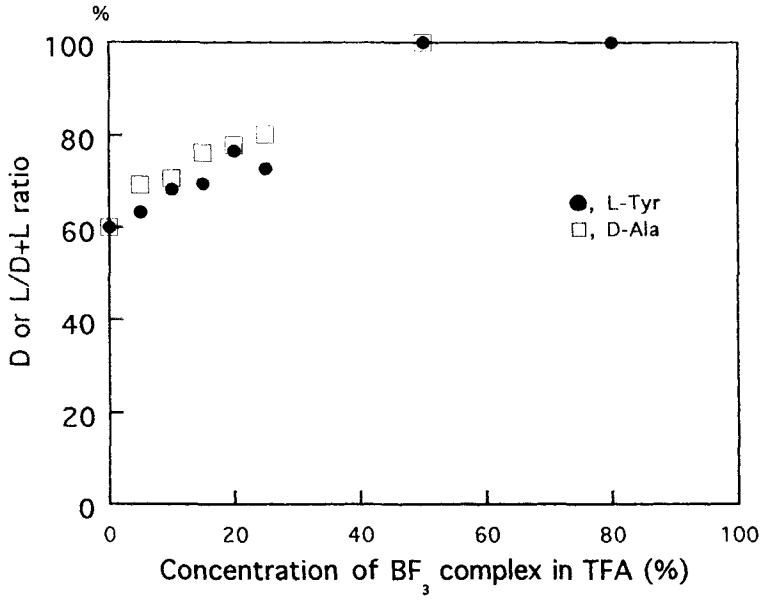
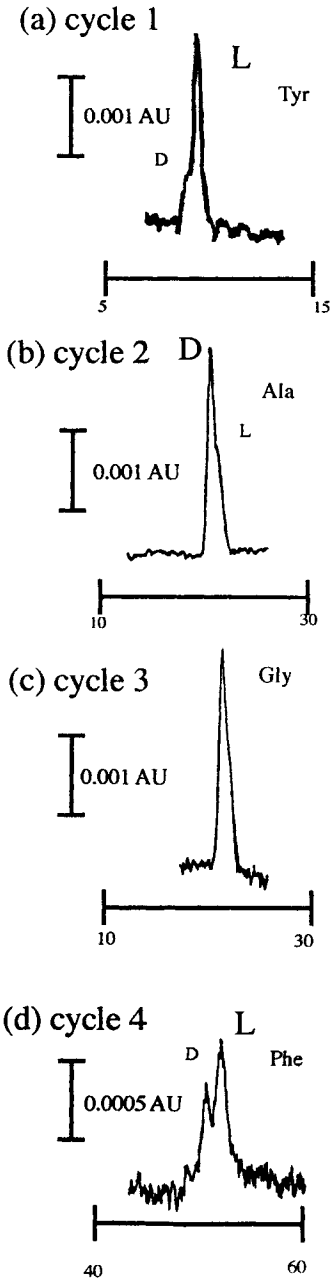


Figure 3. The relationship between racemization ratio and concentration of BF_3-ethyl ether complex in TFA. Conditions: electrolyte, 50 mM sodium phosphate, pH 3.0, including 50 mM SDS and 25 mM digitonin. Other conditions were as in the Experimental section.

1,2-dichloroethane. The resulting mixture for the sequencer was BF_3-ethyl ether complex/TFA/1,2-dichloroethane/2-mercaptoethanol (1:75:23:1, v/v). 2-Mercaptoethanol was added to scavenge peroxides and any other oxidants.[25] The partial racemization ratio was suppressed below 30% on a sequencer.

Figure 4 (right). Sequence analysis of [D-Ala^2]-methionine enkephalin on a protein sequencer using a mixture of TFA/BF_3-ethyl ether complex at cyclization/cleavage step. Conditions: electrolyte, the same as in Fig. 1; sample concentration, theoretically 10 μM (if the reaction of derivatization proceeds completely); injection, a gravity method (height of 5 cm for 40 sec, ca. 8 nL). Other conditions were as in the Experimental section. (a) cycle 1, PTH-L-Tyr; (b) cycle 2, PTH-D-Ala; (c) cycle 3, PTH-Gly; (d) cycle 4, PTH-L-Phe.



Determination of Chirality of PTH-Amino Acid Residues Obtained from Protein Sequencer for [D-Ala^2]-Methionine Enkephalin

As a model peptide, [D-Ala^2]-methionine enkephalin (L-Tyr-D-Ala-Gly-LPhe-L-Met, 200 pmol, 118 ng), was used for identification in sequencing. The ratio (D/D+L) of D-Ala in [D-Ala^2]-methionine enkephalin was 98-99%, as verified by the Marfey's method.[26,27] 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 derivatizationreaction is completed, the sample concentration is theoretically 10 μM. The PTH derivatives were injected at the cathode using a gravity method (height of 5 cm for 40 sec, ca. 8 nL). The mixture (BF_3-ethyl ether complex/TFA/1,2-dichloroethane/2-mercaptoethanol (1:75:23:1, v/v) was used instead of TFA at the cyclization/cleavage step. The nature of the acid was maintained on a sequencer without corrosion for one week.

The determination of the chirality in [D-Ala^2]-methionine enkephalin was as follows:

In cycle 1: Tyr was identified as L-type. L/D+L ratio was 80%.
In cycle 2: Ala was identified as D-type. D/D+L ratio was 80%.
In cycle 3: Gly is not necessary for identification because of no chirality.
In cycle 4: Phe was identified as L-type. L/D+L ratio was 70%.

The ratios were calculated from the peak areas obtained from an integrator. Met in cycle 5 could not be determined because of the very low yield. The sequence analysis with DL differentiation was easily accomplished, as shown in Fig. 4, although the resolution (R_s) values were insufficient. The R_s values of cycles 1 and 2 were not obtained due to the minimal amount of resolution that occurred. The R_s value of cycle 4 was 0.86.

CONCLUSION

We overcame a major racemization problem in sequencing. We achieved the control of low partial racemization on a sequencer for easy DL identification in CE, using the mixed acid (BF_3-ethyl ether complex/TFA/1,2-dichloroethane/2-mercaptoethanol (1:75:23:1, v/v) in the cyclization/cleavage step. If an accurate D/L ratio is required, racemization can be suppressed completely using only BF_3 complex. Therefore, this method is available not only for protein sequencing but also for identifying the

configuration of free amino acids in various biological fluids. In the future, this system will be useful in the life sciences because of the increase in the importance of D-type amino acids.

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