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Amino acid sequence and D/L-configuration determination of peptides utilizing liberated N-terminus phenylthiohydantoin amino acids

Takayuki Iida, Hirokazu Matsunaga, Tomofumi Santa, Takeshi Fukushima, Hiroshi Homma, Kazuhiro Imai*

Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

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Abstract

In this paper, we examined the possibility of using conventional Edman degradation with phenyl isothiocyanate for the simultaneous determination of both the sequence and the D/L-configuration of amino acids in peptides. Boron trifluoride and HCl–methanol (1:10, v/v) were adopted as the cyclization/cleavage and conversion reagents instead of the respective use of anhydrous trifluoroacetic acid (TFA) and 20% aqueous TFA to suppress the amino acid residue racemization. The enantiomeric separation of 18 phenylthiohydantoin amino acids was achieved on two types of chiral stationary phases bonded with β-cyclodextrin. The proposed Edman procedure was applied to a synthetic β-amyloid 1–16 with all L-forms as a model peptide, affording the amino acid sequence and configuration determination up to 12 residues.

Keywords: Sequencing methods; Enantiomer separation; Amino acids

1. Introduction

Recent studies have revealed the occurrence of several peptides (or proteins) containing D-amino acid in living animals [1–3] and mammals [4–7]. The D-amino acid component of these peptides affected their biological activity [2], resistance to proteolytic digestion [3], and their three-dimensional conformation [8]. In view of these results, it now seems necessary to devise methods that allow the simultaneous determination of both the sequence and D/L configurations of the amino acids of a given peptide. However, there exist very few reports describing such procedures as racemization of amino acids has been reported to occur during the Edman sequencing procedure [9–11]. One of the methods, which relies on the diastereomer formation of an N-terminus amino acid residue with a chiral Edman reagent followed by separation by high-performance liquid chromatography (HPLC), does not theoretically exclude the possibility of racemization during the conventional Edman procedure [12–14]. The absolute chiral purity of the chiral reagent is also a prerequisite for the precise determination of the configuration.

We synthesized new non-chiral fluorescent Edman reagents, 7-[(N,N-dimethylamino)sulfonyl]-4-(2,1,3-

*Corresponding author.

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benzoxadiazolyl) isothiocyanate (DBD-NCS) as well as 7-(aminosulfonyl)-4-(2,1,3-benzoxadiazolyl) isothiocyanate (ABD-NCS) [15], and applied the DBD-NCS procedure to a sequence/configuration determination of a peptide containing D-amino acids [16,17]. The peptide was reacted with DBD-NCS and cleaved at the N-terminus amino acid residue, and then the generated DBD-thiazolinone (DBD-TZ) amino acids were enantiomerically separated on a β-cyclodextrin (β-CD) bonded chiral stationary phase. Since we found that a proton supplied by trifluoroacetic acid (TFA) caused the racemization of the DBD-TZ amino acids generated in the cyclization/cleavage reaction, an aprotic Lewis acid, boron trifluoride (BF_3), was used as the cyclization/cleavage reagent in the revised Edman procedure in order to avoid further racemization [17].

On the other hand, we employed the widely used Edman reagent, phenyl isothiocyanate (PITC), for the sequence/configuration determination of the peptide, [D-Ala^2, D-Leu^5]-enkephalin (Tyr–D-Ala–Gly–Phe–D-Leu), under the following conditions; the cyclization/cleavage reaction with TFA was performed at 50°C for 5 min, the conversion reaction with 20% aqueous TFA was done at 50°C for 10 min, and the enantiomeric separation of the liberated phenylthiohydantoin (PTH) amino acid was achieved by HPLC with β-CD bonded stationary phases [18]. The racemization ratio [=D/(D+L)·100] was at most 11% (Tyr, Ala and Leu), and the data indicated that the procedure was suitable for the amino acid sequence/configuration of peptides containing D-amino acids. However, it turned out that other PTH amino acids, such as aspartic acid and proline, were more racemized under these conditions. In addition, it was suggested that the racemization of PTH amino acids occurs in the conversion reaction [19].

In this study, we have focused our attention on the contribution to racemization in Edman degradation of the conversion reaction from the anilinothiazolinone (ATZ) amino acid to the PTH amino acid as well as the contribution of the cyclization/cleavage reaction. Under reaction conditions that were optimal for the suppression of racemization, we determined the sequence and amino acid configurations of a model peptide sample, a synthetic β-amyloid 1-16 [βA(1–16)] with all L-forms.

2. Experimental

2.1. Materials and apparatus

PITC, TFA and PTH amino acids were purchased from Wako Pure Chemicals (Osaka, Japan). BF_3 etherate and pyridine were purchased from Tokyo Chemical Industry (Tokyo, Japan). Trimethylsilyldiazomethane was purchased from Nacalai Tesque (Tokyo, Japan). Dipeptides (Asp–Ala, Arg–Phe, His–Phe, Pro–Leu, Tyr–Val) and amino acids were obtained from Sigma (St. Louis, MO, USA). Leu–Gly and Gly–Leu were obtained from Peptide Institute (Osaka, Japan). βA(1–16) peptide (Asp–Ala–Glu–Phe–Arg–His–Asp–Ser–Gly–Tyr–Glu–Val–His–His–Gln–Lys) was obtained from Backem (Bubenford, Switzerland). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). Acetonitrile and methanol used for the mobile phase were of HPLC grade. All chemicals were of analytical grade. BF_3 etherate was distilled under reduced pressure and kept in an ampoule at 4°C until use.

HPLC systems and columns were as follows. Intelligent pumps were L-6200 (Hitachi, Tokyo, Japan) and 880-PU (Jasco, Tokyo, Japan). UV detectors were L-4000H (Hitachi) and UV-8000 (Tosoh, Tokyo, Japan). The reversed-phase column was TSK-GEL ODS-80Ts (250×4.6 mm I.D., 5 μm, Tosoh). Enantiomeric separation columns were Sumichiral OA-2500(S) (250×4.6 mm I.D., 5 μm, Sumika Chemical Analysis Service, Kyoto, Japan) and Ultron ES-CD or ES-1/2PhCD (150×6.0 mm I.D., 5 μm, Shinwa Chemical Industries, Kyoto, Japan). The stationary phase of the ES-1/2PhCD column was bonded with phenylcarbamoyl β-CD with 7.5 out of 20 hydroxyl groups modified in each β-CD molecule [20].

2.2. Preparation of the phenylthiocarbamoyl (PTC) dipeptide

Dipeptides were dissolved in 50% pyridine. Ten μl of the solution (1–10 m*M*) was mixed with 10 μl of 50% pyridine, 5 μl of PITC and 20 μl of ethanol. The mixture was heated at 50°C for 10 min. After drying in a centrifugal evaporator (SPE-200,

Shimadzu) at 50°C for 5 min, 10 μl of water was added to the residue. The excess reagent and by-products were removed by washing three times with 100 μl of *n*-heptane–ethyl acetate (7:1, v/v). The aqueous phase was dried in a centrifugal evaporator at 50°C for 15 min.

2.3. Studies of racemization in the cyclization/cleavage reaction

Cyclization/cleavage reagents were TFA or 8 m*M* BF_3 etherate in acetonitrile. To several sample tubes containing PTC dipeptide (20 nmol), 10 μl of the cyclization/cleavage reagent was added and heated at 50°C for appropriate time intervals. After the cyclization/cleavage reaction, each sample was hydrolyzed by adding 40 μl of water to generate PTC amino acids followed by the addition of 100 μl of a saturated sodium bicarbonate solution for neutralization. PTC amino acids were isolated by HPLC using a reversed-phase column, which was developed with a 850:150:1 to 150:850:1 (v/v/v) linear gradient of water–acetonitrile–TFA over 15 min at a flow-rate of 1.0 ml/min. The racemization ratio of the PTC amino acid isolated was determined by HPLC as described in Table 1.

2.4. Studies of racemization in the conversion reaction

Conversion reagents were 20% TFA or HCl–methanol (1:10, v/v). The PTC amino acid was prepared from the L-amino acid by the PITC coupling reaction as described in Section 2.2. The ATZ amino acid was prepared from the PTC dipeptide with 8 m*M* BF_3 in acetonitrile as described in Section 2.3.

To a sample tube containing PTC or ATZ amino acid (10 nmol), 150 μl of the conversion reagent was added and heated at 50°C. Fifteen-μl aliquots were withdrawn after appropriate time intervals and dried under a stream of nitrogen gas. The PTH amino acid residue was reconstituted with 40 μl of the mobile phase for HPLC and the racemization ratio of the PTH amino acid was determined as described in Table 1.

2.5. Enantiomeric separation of PTH amino acids

The methyl ester derivatives of PTH–Asp or –Glu were prepared by the following method. Five μl of PTH–Asp or –Glu solution (10 m*M*) was mixed with 30 μl of benzene, 10 μl of methanol and 5 μl of trimethylsilyldiazomethane in *n*-hexane solution. The mixture was heated at 50°C for 5 min and dried under a stream of nitrogen gas.

The enantiomeric separation of PTH amino acids was carried out on ES-CD or ES-1/2PhCD columns with UV detection at 269 nm. The mobile phase was water–methanol (8:2, v/v) containing 10 m*M* formic acid at a flow-rate of 1.0 ml/min.

2.6. Sequencing of βA(1–16)

βA(1–16) was dissolved in 50% pyridine. Ten μl of the solution (100 μ*M*) was mixed with 10 μl of

Table 1
HPLC conditions for studies of racemization in the cyclization/cleavage and the conversion reaction

Amino acids	Columns	Mobile phases
PTC–Asp	OA-2500(S)	Methanol containing 5 m*M* citric acid
PTC–Arg, –His	ES-1/2PhCD	Water–methanol (9:1, v/v) containing 10 m*M* formic acid
PTC–Leu	ES-PhCD	Water–methanol (5:5, v/v) containing 10 m*M* formic acid
PTC–Pro	ES-CD	Water–methanol (8:2, v/v) containing 10 m*M* formic acid
PTC–Tyr	OA-2500(S)	Methanol containing 1 m*M* citric acid
PTH–Arg, –His	ES-1/2PhCD	Water–methanol (9:1, v/v) containing 10 m*M* formic acid
PTH–Asp, –Leu	ES-CD	Water–methanol (8:2, v/v) containing 10 m*M* formic acid
PTH–Pro	ES-CD	Water–methanol (7:3, v/v) containing 10 m*M* formic acid

The flow-rate was at 1.0 ml/min. The wavelength of UV detection for PTC and PTH was 254 nm and 269 nm, respectively.

50% pyridine, 5 μl of PITC and 20 μl of ethanol. The mixture was heated at 50°C for 30 min. The coupling and washing procedures were the same as described in Section 2.2. To the residue of the PTC peptide, 30 μl of 8 m*M* BF_3 in acetonitrile was added and the mixture was heated at 50°C for 5 min. After drying under a stream of nitrogen gas, 20 μl of water was added to the residue. The solution was extracted twice with 100 μl of *n*-heptane–ethyl acetate (1:5, v/v). The aqueous phase was dried in a centrifugal evaporator at 50°C for 10 min and subjected to the next cycle. The combined organic phase was dried under a stream of nitrogen gas and mixed with 30 μl of HCl–methanol (1:10, v/v). The mixture was heated at 50°C for 30 min. After the conversion reaction, the solution was dried under a stream of nitrogen gas. The residue was dissolved in 100 μl of the mobile phase for HPLC. Forty μl of this solution was injected into the ES-CD column and ES-1/2CD column. The HPLC conditions were as described in Section 2.5.

3. Results and discussion

3.1. Studies of racemization in the cyclization/cleavage reaction

The racemization ratio of DBD-TZ amino acids during the cyclization/cleavage reaction was easily determined by HPLC as described in a pervious study [17]. However, ATZ amino acids were unstable in the mobile phase of the HPLC column used to obtain the racemization ratio. Therefore, in this experiment, ATZ amino acids were hydrolyzed to their more stable PTC forms [10] to determine the racemization ratio. Racemization during the hydrolysis reaction of the ATZ amino acids was scarcely observed; for example, the racemization ratios of aspartic acid and proline after hydrolysis were only 3% and 4%, respectively.

Based on previous results showing that aspartic acid and proline residues in the DBD-labeled peptide are subject to a high degree of racemization in the cyclization/cleavage reaction containing the TFA reagent [17], we adopted them as model amino acids in our racemization study. A gradual increase in the racemization ratio of these amino acids was observed

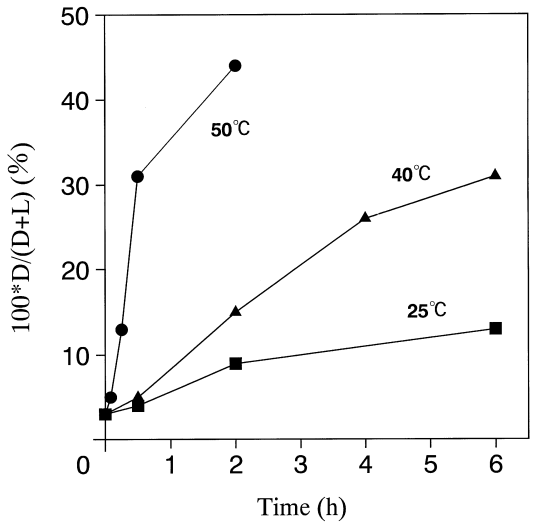

Fig. 1. Racemization of ATZ–Asp in the cyclization/cleavage reaction containing TFA.

during the time course of the cyclization/cleavage reaction containing the TFA reagent (Fig. 1). Other amino acids (arginine, leucine and tyrosine) were also racemized during the 30 min reaction (17%, 16% and 7%, respectively). These results indicate that ATZ amino acids are also racemized by the TFA reagent, because of the exchange between the hydrogen atom at the α-position of the amino acid and an acidic hydrogen of TFA as described for DBD-TZ amino acids [17]. The racemization ratio of proline remained constant at 4% in the cyclization/cleavage reaction containing the BF_3 reagent (Fig. 2a). Then the time course of the yield of Pro–Leu dipeptide in the cyclization/cleavage reaction containing either BF_3 or TFA was examined, since the N-terminus proline residue of a peptide is known to be particularly resistant to cleavage [21]. As shown in Fig. 2b, BF_3 gave a higher yield than TFA, and the cyclization/cleavage reaction was complete after 5 min at 50°C with 8 m*M* BF_3, suggesting that BF_3 was appropriate for both PITC and DBD-NCS [17] in the cyclization/cleavage reaction.

3.2. Studies of racemization in the conversion reaction

ATZ amino acids are converted to PTH amino acids under strong acidic conditions, such as 20%

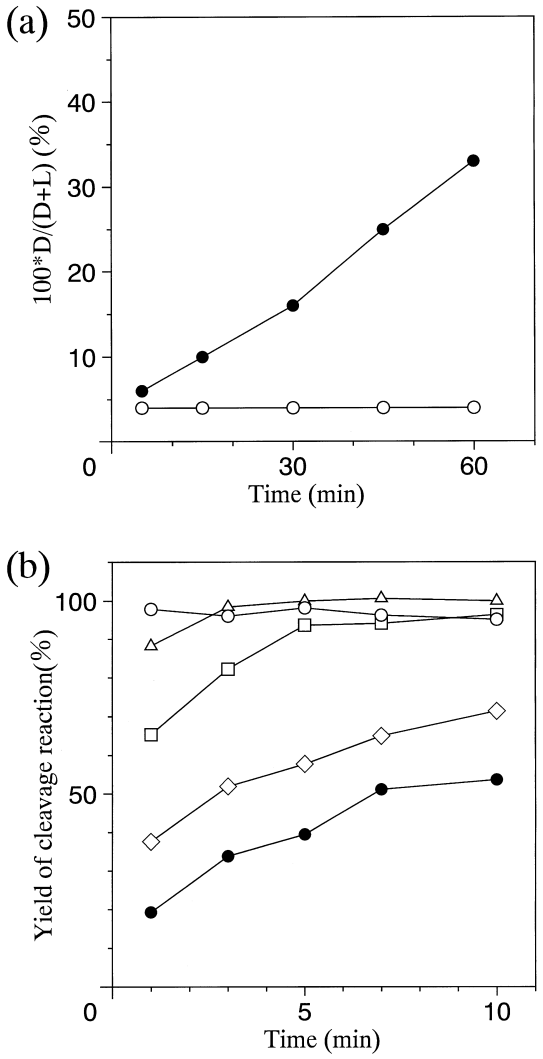

Fig. 2. (a) Racemization and (b) yield of ATZ–Pro in the cyclization/cleavage reaction at 50°C. The closed symbol (●) and the open symbols (◇, □, △, ○) show the results obtained with TFA and BF_3 (concentration; 1 m*M*, 2 m*M*, 4 m*M*, 8 m*M*), respectively.

TFA or HCl–methanol (1:10, v/v), through the ring opening and recyclization steps [21,22]. First, we investigated the degree of racemization in the re-cyclization step of PTH amino acids using PTC–Asp, –Arg, –Pro and –Leu. The racemization ratios in this step were found to be low (≤10%) with either 20% TFA [10] or HCl–methanol solutions. Thus, the recyclization step would appear to contribute little to racemization.

Next, the degree of racemization of ATZ amino acids during their conversion to PTH amino acids was examined using ATZ–Asp, –Arg, –His, –Pro and –Leu. The racemization ratios of amino acids in HCl–methanol solutions were lower than in solutions containing 20% TFA, especially in the case of PTH–Leu (Fig. 3). One possibility is that racemization is accelerated during the longer period of ATZ–Leu in 20% TFA than in HCl–methanol since ATZ amino acids are solvolyzed more slowly with 20% TFA

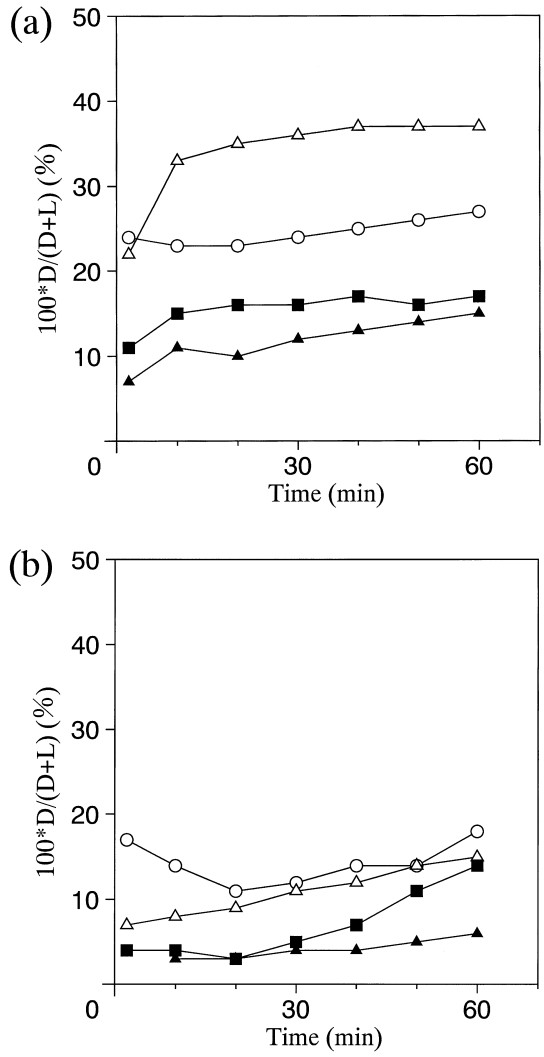

Fig. 3. Racemization of PTH amino acids in the conversion reaction at 50°C with (a) 20% TFA and (b) HCl–methanol (1:10, v/v). Symbols show the following amino acids; Asp (○), Arg (■), Pro (▲) and Leu (△).

than with HCl–methanol [21]. Therefore, the racemization in the conversion reaction with 20% TFA would appear to occur during the ring opening step rather than during the recyclization step. Considering these results, we adopted HCl–methanol as the conversion reagent.

PTH amino acids were produced with good yield after 30 min in the reaction containing HCl–methanol, except for PTH–Pro and –Gly (Fig. 4). Previous studies reported that the conversion reaction of proline and glycine was slower than that of other amino acids in the recyclization step [21]. On the other hand, the generated PTH amino acids were racemized to some extent after long reaction times; for example, the racemization ratio of PTH–Arg increased from 5 to 13% after 30 to 60 min of incubation, respectively (Fig. 3b), with good yield (Fig. 4). We made a compromise between the yield and the racemization ratio of the PTH amino acids, and employed conversion reaction conditions of HCl–methanol at 50°C for 30 min.

3.3. Enantiomeric separation of PTH amino acids

In previous papers [17,20], the enantiomeric separation of DBD-TZ or PTC amino acids was achieved on a β-CD bonded chiral stationary phase where the hydroxyl group of β-CD is modified by a phenylcarbamoyl moiety. In this study, we examined the enantiomeric separation of PTH amino acids on a column packed with the native β-CD stationary phase (ES-CD column) or with the phenylcarbamoylated β-CD stationary phase (ES-1/2PhCD column), which had 7.5 out of the 20 hydroxyl groups of each β-CD molecule modified by the phenylcarbamoyl moiety [20].

The capacity factors and the separation factors for PTH amino acids are shown in Table 2. The enantiomers of PTH amino acids were separated on the ES-CD column (PTH–Asn, –Ile, –Leu, –Phe, –Pro and –Val), on the ES-1/2PhCD column (PTH–Arg, –Gln, –His, –Lys, –Met and –Tyr), and on both columns (PTH–Ala, –Asp, –Glu, –Ser, –Thr and –Trp). In this way, the enantiomeric separation of 18 PTH amino acids was achieved. The concomitant use of these two different columns could allow the

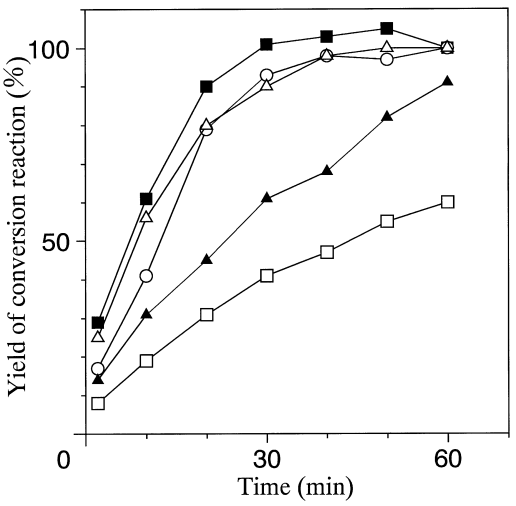

Fig. 4. Yield of PTH amino acids in the conversion reaction at 50°C with HCl–methanol (1:10, v/v). Symbols show the following amino acids; Asp (○), Arg (■), Gly (□), Pro (▲) and Leu (△).

Table 2
Capacity factors (k_1') and separation factors (α) for PTH amino acids on β-CD columns

Amino acids	ES-CD		ES-1/2PhCD	
	k_1'	α	k_1'	α
Ala	4.14 D	1.04	2.77 L	1.12
Arg	1.03	1	1.69 L	1.20
Asn	2.90 D	1.07	2.05	1
Asp[a]	3.91 D	1.21	3.99 L	1.05
Gln	4.45	1	2.95 L	1.19
Glu[a]	4.81 D	1.05	5.00 L	1.12
Gly[b]	3.48	–	2.52	–
His	0.50	1	0.90 L	1.30
Ile	10.12 D[c]	1.21	8.14	1
Leu	9.33 D	1.08	9.52	1
Lys	15.93	1	43.19 L	1.10
Met	5.89	1	6.43 L	1.08
Phe	5.50 D	1.05	10.02	1
Pro	12.22 D	1.15	12.61	1
Ser	2.94 D	1.15	1.78 L	1.07
Thr	3.20 D	1.10	2.06 L	1.16
Trp	6.60 L	1.04	10.34 L	1.07
Tyr	3.38	1	4.05 L	1.11
Val	7.78 D	1.08	5.81	1

The flow-rate was 1.0 ml/min. The mobile phase was water–methanol (8:2, v/v) containing 10 m*M* formic acid with UV detection at 269 nm.

[a] PTH–Asp and –Glu indicate those of methyl ester.

[b] Gly has no chirality.

[c] This is D-*allo*-Ile, which is a stereoisomer of D-Ile at the β-carbon position.

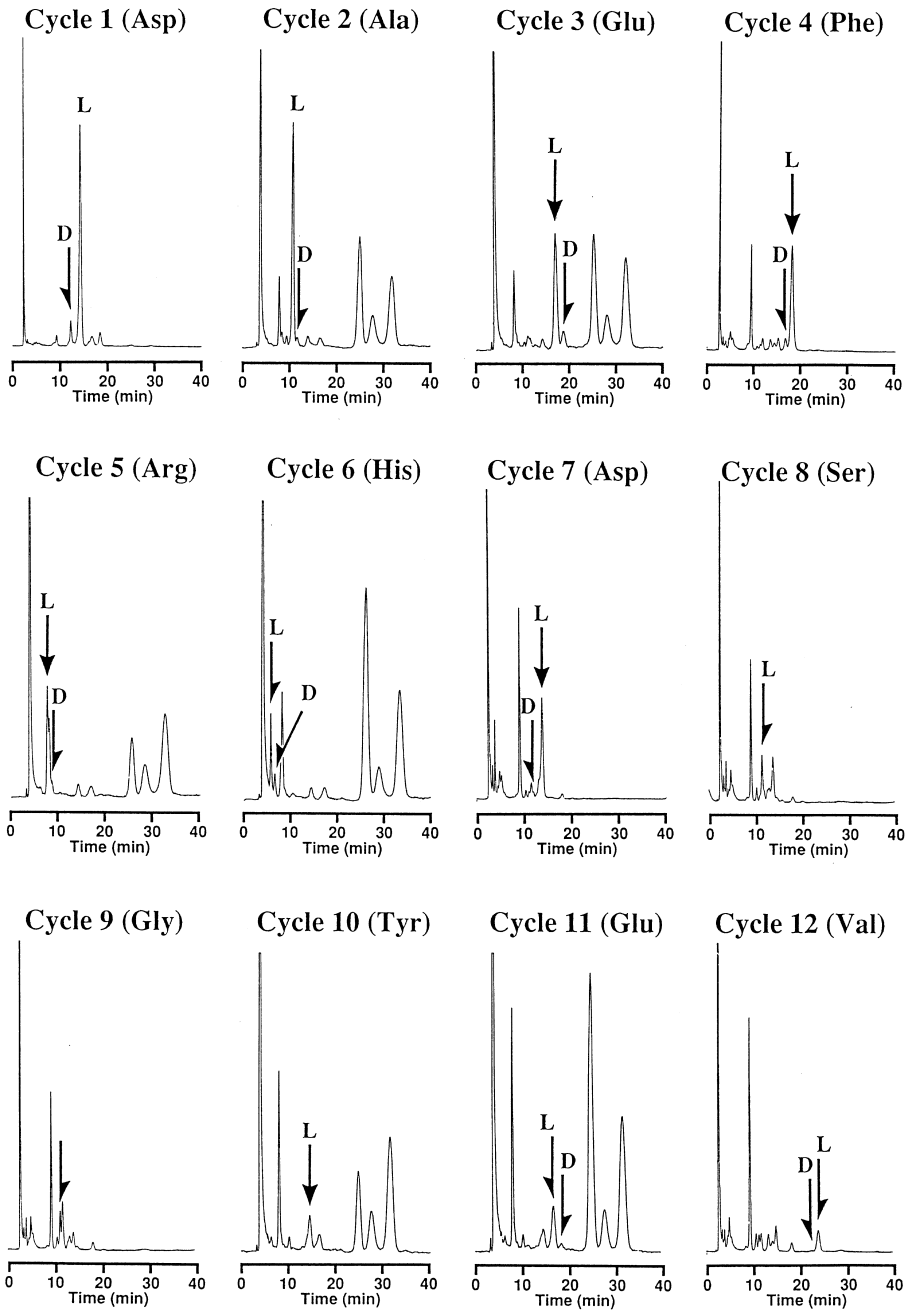

Fig. 5. Separation and D/L-configuration determination of the amino acids of the β-amyloid peptide (1–16). HPLC conditions as in Table 2. The PTH amino acid in each cycle was determined by the ES-CD column (cycles 1, 4, 7, 8, 9 and 12) and ES-1/2PhCD column (cycles 2, 3, 5, 6, 10 and 11). After cycle 13, PTH amino acids were not detected.

determination of both the D/L-configuration and the identification of each PTH amino acid. For example, PTH–L-Asp and –L-Tyr were eluted at the same time on the ES-1/2PhCD column ($k'=3.99$ and 4.05, respectively), but were separated on the ES-CD column ($k'=4.73$ and 3.38, respectively). The detection limit of PTH–Gly on either column was 1–2 pmol, indicating that the detection limit of PTH amino acids was superior to that of PTC amino acids (5–20 pmol) [20].

3.4. Sequencing of βA(1–16)

To demonstrate the applicability of this sequencing procedure to a real sample, the sequencing analysis of the βA(1–16) peptide was carried out. Since a natural βA peptide occurring in the brain of patients with Alzheimer's disease includes isoaspartic acid as well as D-Asp [6], the sequence determination of the natural peptide would be suspended at the isoaspartic acid residue [5]. Therefore, in this experiment, the commercially available peptide composed of L-amino acids was adopted. The amino acid sequence was identified from cycles 1 to 12 (Fig. 5). Sequencing beyond cycle 13 was difficult due to the low yield of PTH amino acids and the disturbance from many interfering peaks. The D/L-configuration of all the amino acids was determined as the L-form, although a certain amount of the D-form was also detected due to racemization: cycles 1 (Asp; racemization ratio 11%), 4 (Phe; 9%), 7 (Asp; 1%), 8 (Ser; D-form not identified), 9 (Gly; no chirality), and 12(Val; 3%) using the ES-CD column, and cycles 2 (Ala; 4%), 3 (Glu; 14%), 5 (Arg; 7%), 6 (His; 25%), 10 (Tyr; D-form not identified) and 11 (Glu; 9%) using the ES-1/2PhCD column. This was due essentially to the racemization of the histidine residue (cycle 6). It was difficult to suppress the racemization of the histidine residue in the conversion reaction; the racemization ratio was 9% at the recyclization step as described in Section 3.2, and was 22% at the ring opening and recyclization steps. This result suggests that most of the racemization of histidine occurred at the ring opening step. However, the reason why histidine residue specifically tended to racemize is not yet clear. It should be noted that the racemization of serine residue was not observed (cycle 8), since the β-elimination reaction of the hydroxyl moiety of serine would predominate over the racemization [17]. The repetitive yield of this sequencing procedure was 86.7% as calculated by the Asp^{1} and Asp^{7}, and appeared to be lower than when an automatic sequencer as the regular Edman procedure is used. As the results, we consider that the new procedure is good enough to determine the L- and D-configuration of amino acids in peptides.

In conclusion, we developed a new sequence/configuration determination procedure for peptides by the conventional Edman degradation procedure utilizing PITC. To suppress the racemization of amino acids, BF_3 and HCl–methanol were used in the cyclization/cleavage and the conversion reaction, respectively. The enantiomeric separation of PTH amino acids was achieved on two columns packed with a β-CD bonded chiral stationary phase. Since all the individual PTH amino acids can not be separated at present, the identification of individual PTH amino acids at the N-terminus of peptides should be carried out first by reversed-phase HPLC, and then the D/L-configuration should be determined by HPLC with a chiral stationary phase. Further study should make possible the adaptation of the present sequencing procedure by commercially available automatic sequencers using the PITC reagent.

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