difficult for the fluorescent products to be detected as the borate complex by the positive ion ESI-TOF-MS. The borate complex may be dissociated in the ion source of ESI-TOF-MS.

It was reported that 2,2-diphenyl-1-oxa-3-oxonia-2-boratanaphthalene (DOOB) could react with primary amine by dehydration condensation, and a fluorescent product was formed as shown in Figure 6A.[28] Based on this fact, we estimated that, in our fluorescent reaction, the boron atom coordinated to hydroxyl groups in two catechol molecules, the N-terminal amino and imino groups in the peptide molecule, and the resultant product (Figure 6B) emitted the fluorescence.

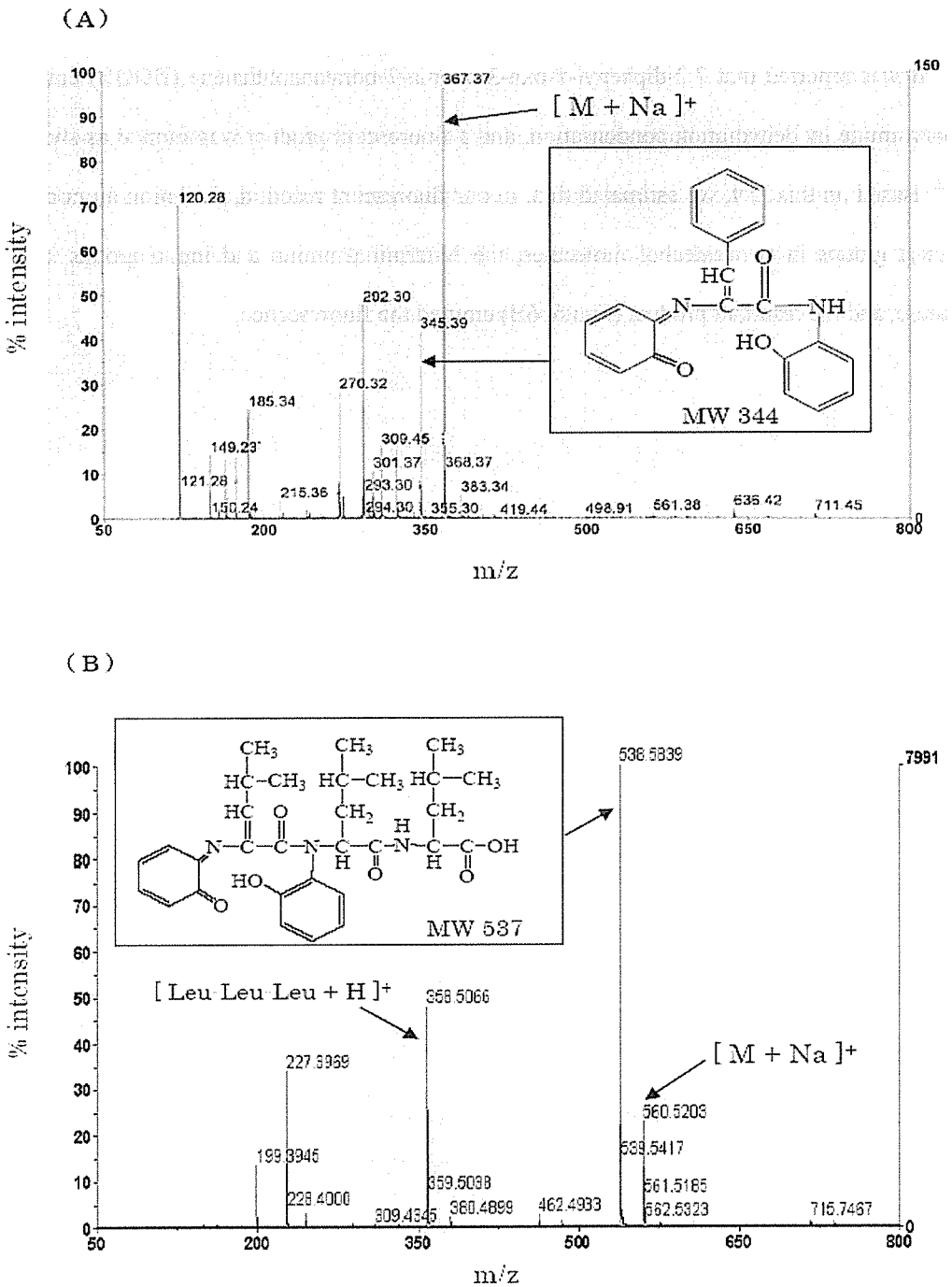

Figure 5. Positive ion ESI mass spectra of fluorescent products from Phe-NH_2 (A) and Leu-Leu-Leu (B).

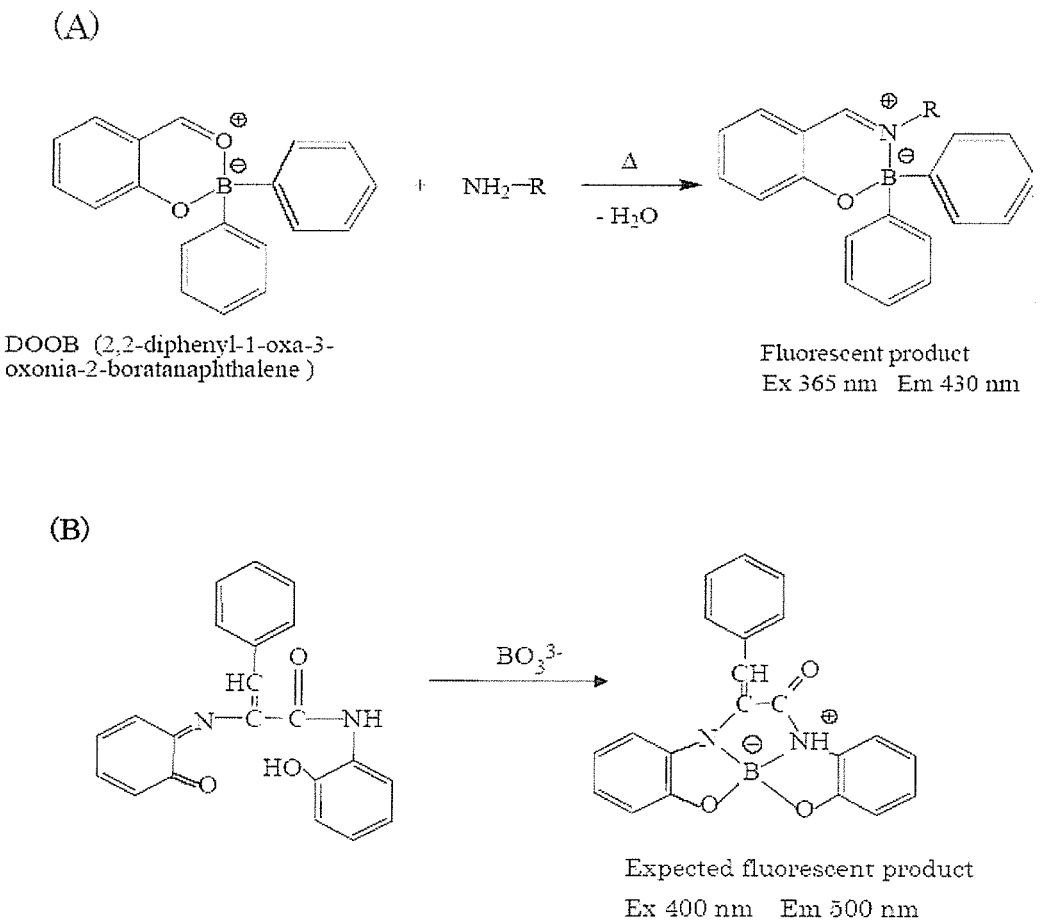

Figure 6. Structures of fluorescent products formed by the reaction of amine with DOOB reagent (A), and by the present reaction of Phe-NH2 with catechol, NaIO4 and sodium borate (B).

CONCLUSIONS

Herein a novel fluorescent reaction for peptides was developed and then applied into the RPLC analysis coupled with fluorescence detection for the quantitative determination of the peptides. Peptides first reacted with catechol and periodate under 120°C, and then the fluorescent product might be formed as a borate complex in the presence of sodium borate at pH 7.0. The peptides could be sensitively detected by RPLC, with a detection limit at a picomole level of the peptide per an injection volume. This sensitivity is 10 to100 times higher than that with UV detection for the peptides. In addition, we measured the mass spectra of the isolated fluorescent products due to Phe-NH_2 and Leu-Leu-Leu by ESI-TOF-MS. The chemical structure of the fluorescent product was estimated and two molecules of catechol were possibly involved into the peptide, and a fluorescent boron complex is formed by coordinate bonds to the amino and imino groups in the N-terminal amino acid of the peptide, and the hydroxyl group in catechol.

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Fluorescence detection of amino acids in the post-cleavage conversions for peptide Sequencing

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Abbreviations used: RPLC, reversed-phase liquid chromatography; CIA, 4-(1'-cyanoisoindolyl)aniline; PITC, phenylisothiocyanate; ATZ, anilinothiazolinone; PTC, phenylthiocarbamoyl; PTH, phenylthiohydantoin; GF, glass fiber membrane; TEA, triethylamine ; TFA, trifluoroacetic acid ; DMF, *N, N*-dimethylformamide ; SDS, sodium dodecyl sulfate; UV, ultra-violet wavelength; FL, fluorescence.

Abstract

A modified Edman-degradation method is described where fluorescent derivatives of amino acids were generated from the post-cleavage products of a peptide. In the method, the target peptide was applied onto double glass fiber membranes in a small filter disk (4-mm ID), and then treated with small amounts of reagents for the manual sequencing of the peptide. The anilinothiazolinone (ATZ) of N-terminus amino-acid residue after the isolation from the solid-phase membranes was reacted with a primary amine, 4-(1'-cyanoisoindolyl)aniline (CIA) to form a more stable and sensitive fluorescent derivative, phenylthiocarbamoyl-CIA. Average yield of 85 % was obtained in neutral pH conditions for the CIA reaction. The ATZ-CIA amino acids were separated by reversed-phase liquid chromatography, and detected by fluorometry. The lower limits of the detection for amino acids after the Edman degradation was 0.16-0.52 pmol (S/N=3) on the column. The sensitivity was approximately 10 times higher than ultra-violet absorbance detection of phenylthiohydantoin products in the conventional Edman degradation. The suitability of the method has been demonstrated by the sensitive sequencing of insulin chain-B composed of 30 amino acids.

Key words: Amino acids; Peptide sequencing; Fluorescence detection; Insulin

The amino acid composition and sequence analysis of peptides or proteins are necessary for the investigation of structure–functional relationships. The primary structure is one of the most important determinants of physiological activities of peptides and proteins. This is because the amino-acid sequences can estimate the secondary and

tertiary structure of proteins, which are necessary for analyzing biological functions. The amino acid structures, which occur in any given natural location, reflect the local pathways of biosynthesis. The knowledge of peptide structure is vital for the design of pharmaceutical peptide drugs. Recent advances in the drug designs include amino acid engineering where the primary sequence of peptide or protein is modified ranging from a single residue to a large-scale manipulation of the amino acid sequence [1-3].

Currently, peptide- or protein-sequencing determination is accomplished either directly by sequencing based on Edman-degradation principle or indirectly by sequencing the gene corresponding to the peptide or protein using an established DNA-sequencing method. In the Edman method, one cycle, which results in stepwise removal of one amino acid from the amino-terminal end of a peptide, consists of three conventional steps (Fig. 1). Firstly, phenylisothiocyanate (PITC) is coupled with N-terminus of the peptide under basic conditions. In the second step, the treatment with anhydrous acid removes the N-terminus amino-acid residue as an unstable anilinothiazolinone (ATZ), which is then isomerized to a more stable phenylthiohydantoin (PTH) by the treatment with hydrous acid for the subsequent analysis. The PTH derivatives absorb in the ultra-violet wavelength (UV) region and are separated by reversed-phase liquid chromatography (RPLC) as the product at the end of each degradation cycle.

Due to the low sensitivity for the UV detection of PTH derivatives, a large amount of sample has been required. For the sensitive analysis of isolated peptide or protein samples, an improvement method is highly required. Numerous techniques have been described for generating fluorescent amino-acid derivatives as to enhance the sensitivity of thiohydantoin-amino acids. Several Edman-type fluorescent reagents instead of the PITC reagent have been reported [4-8]. However, these reagents are not routinely used since the coupling yield of peptides with the fluorescent reagents is poorer than PITC, and/or

their by-products interfere with the sensitive detection of amino acids. The coupling and cleavage steps may kinetically be disfavored by the large size of the fluorophores due to steric and/or electrostatic hindrances.

To overcome these disadvantages, we previously synthesized a new fluorescent Edman-type reagent, 4-(1'-cyanoisoindolyl)phenylisothiocyanate (CIPIC) that was found to be highly reactive with amino acids and peptides [9]. However, we found that the CIPIC derivative of peptide after coupling is not readily cleaved at the N-terminus after the treatment with trifluoroacetic acid (TFA). In our recent work, we have shown that CIPIC and its analogue, 4-(1'-cyanoisoindolyl)aniline (CIA) can be used as a fluorescent and chemiluminescent reagent [10]. They induced strong chemiluminescence in alkaline conditions and its sensitivity was high. Although PTH-amino acid has been considered as the final product in each degradation process, ATZ-amino acid after the cleavage can be reacted with 4-aminofluorescein to generate phenylcarbamoyl (PTC)-aminofluorescein [11]. However, a low yield was generally observed with hydrophilic amino acids, and this reaction was pH dependent and thus required buffering to maintain the pH.

In this paper, we describe a manual RPLC method with fluorescence (FL) detection for the sensitive sequencing of peptide based on the Edman principle with some modifications. The target peptide was absorbed on double glass fiber (GF) membranes in a small filter disk to improve its retaining ability without polybrene or covalent attachment of peptides [12-15], and this small disk made the treatment with small amounts of reagents possible. In our current method, ATZ-amino acid derivatives were reacted with CIA to form highly sensitive fluorescent derivatives, PTC-CIA-amino acids (Fig. 1). The product was stable and identified as the final luminescent product instead of the conventional PTH-UV detectable product. With the CIA treatment at the ATZ-ring opening stage, racemization of amino acids [16] due to the conventional conditions may be avoided.

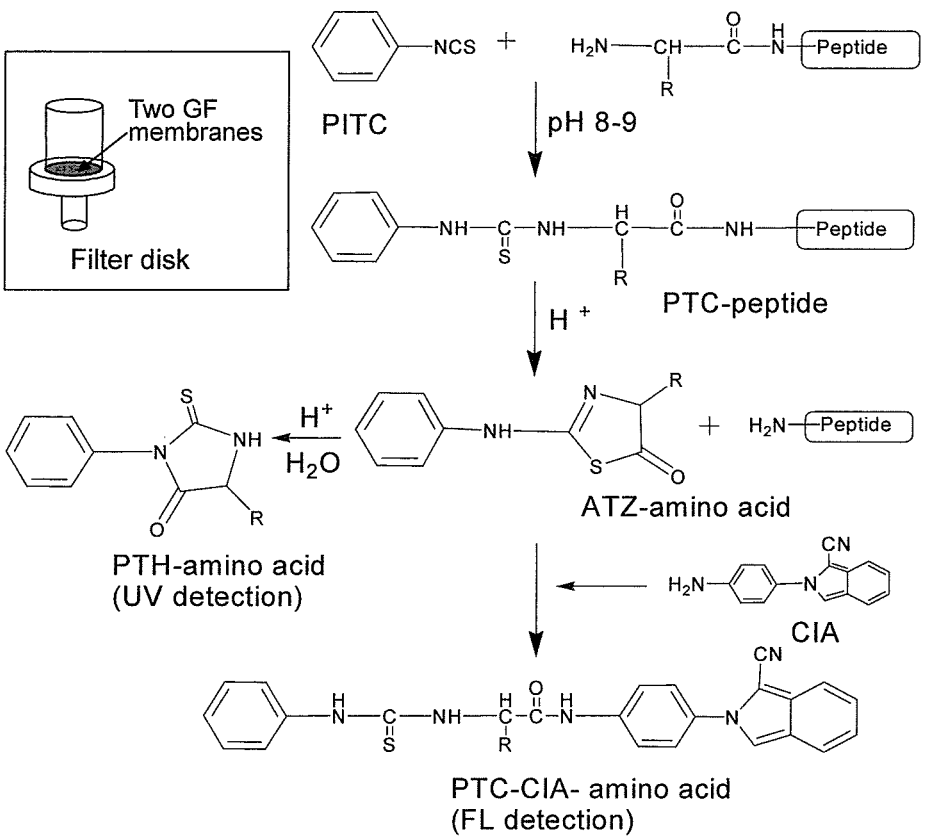


Fig. 1. Reaction scheme for the sensitization of ATZ-amino acids derivatives with CIA. The picture (inset) shows the design of a filter disk of polypropylene type supplied with a PTFE filter. Two GF membranes were set in the disk as depicted.

Materials and methods

Materials and apparatus

Trifluoroacetic acid (TFA), phenylisothiocyanate (PITC), triethylamine (TEA), TFA-treated glass fiber (GF) membranes were purchased from Wako (Osaka, Japan). Filter

disks (Dismic-3 polytetrafluoroethylene, 0.5-μm pore size, 4-mm ID) were purchased from Advantec-Toyo (Tokyo, Japan). To assemble the disk, two pieces of GF membranes were set in the filter disk (Fig. 1). All amino acids amides were purchased from Bachem (Bubendorf, Germany). Insulins chain-A and B containing oxidized Cys residues were obtained from Sigma (St Louis, MO, USA). All other chemicals were of analytical or guaranteed reagent grade and were used without further purification. Water was purified on a Milli-Q system WR 600 A from Millipore (Molsheim, France).

The RPLC system consisted of a PU-980 Intelligent pump, a DG-890-50 3-line degasser unit, an LG 980-02 gradient unit, an FP 920 FL detector, and an UV 970 detector from Jasco (Tokyo, Japan). FL detection was performed at 400 nm with excitation at 340 nm. The wavelength of UV detection was 269 nm. The reversed- phase column for separation was a Wakosil PTH-column (250 x 4.6-mm ID, 5-μm particle size) from Wako (Osaka, Japan). The flow rate was kept at 1.0 mL/min.

Preparation of standard ATZ-amino acids

Amino-acid amides were dissolved in water. Each amide (5 nmol/5 μL) was applied on GF membranes in a filter disk (4-mm ID), and then dried under reduced pressure. A portion (7.5 μL) of 5 % (w/v) PITC in CH_3CN and 5 μL of 15 % (v/v) TEA in CH_3CN were applied on the membrane and then the mixture was heated at 50 °C for 30 min. After drying in vacuum, the excess reagents and by-products were removed by washing twice with 30 μL of *n*-propyl chloride and again completely dried. A portion (10 μL) of 100 % TFA was added on the membrane and heated at 50 °C for 3 min, and after drying; the ATZ-amino acid was eluted with 100 μL of CH_3CN and kept in ice cold water. A portion (10 μL) of the reaction mixture was injected into RPLC for ATZ analysis.

The formation of PTC-CIA-amino acid

The ATZ-amino acid (approximately 100 pmol) in 20 μL of the eluate was reacted with 20 μL of 30 mM CIA in CH_3CN at 80 °C for 15 min in an open tube. After the reaction, the residue was dissolved in 40 μL of CH_3CN and an aliquot (10 μL) was injected into RPLC and monitored simultaneously by FL and UV detectors.

Sequencing of peptide

The peptide (12.5 nmol/5 μL) was dissolved in water and applied on the GF membranes and dried under reduced pressure. A portion (7.5 μL) of 5 % (v/v) PITC in CH_3CN and 5 μL of 15 % (v/v) TEA in CH_3CN were added to the disk and the mixture heated at 50 °C for 30 min. After drying for 5 min, the GF membranes in the filter disk were washed with 30 μL of toluene (2 times) and dried for 10 min. A portion (20μL) of 100 % TFA was placed in an eppendorf tube and the filter disk was inserted over it and then, the tube was heated for 5 min at 80 °C for gas-phase reaction. The filter disk was removed and then dried for 5 min. ATZ-amino acid was extracted with 30 μL of ethyl acetate and a portion (20 μL) of the eluate was mixed with 20 μL of 30 mM CIA in CH_3CN, and heated for 15 min at 80 °C in an open tube. The residue was dissolved in 40 μL of CH_3CN, and an aliquot (10 μL) was injected into RPLC equipped with a FL detector. The membranes were subsequently washed with 30 μL of toluene (2 times) and after drying for about 10 min under reduced pressure, the peptide residue on membrane was subjected to the next cycle.

Results and discussion

Optimization of reaction conditions with CIA

To improve the detectable sensitivity of the sequencing, we used a fluorescent amine, CIA [10] to sensitize ATZ intermediates in the Edman sequencing procedure. CIA gave a strong fluorescent product with ATZ-amino acid derivatives. Amino acid amides of Gly-NH_2, Val-NH_2, Ser-NH_2, and Glu-NH_2 were used for the optimization of reaction conditions, because the amide type of amino acids shows similar reactivity to that of peptide. Each amide was reacted with PITC on two GF membranes in a filter disk and then converted to the corresponding ATZ-amino acid after treatment with TFA. After complete elution of the ATZ-amino acid from the membranes with CH_3CN, the ATZ-amino acid was then reacted with CIA dissolved in different solvents in order to identify a suitable solvent for the ATZ-CIA reaction. The reaction proceeded smoothly in organic solvents such as CH_3CN and DMF, but was deteriorated in methanol, chloroform and *n*-butyl chloride, showing only 20-35% of the yield obtained with CH_3CN or DMF. CH_3CN was selected due to its high volatility.

The concentration of CIA was an important factor that affected the derivatization reactions. The influence of concentration on the product yield was investigated with various CIA concentrations of 1.0-50 mM (Fig. 2). There was a rapid increase in the production of PTC-CIA-amino acid up to 30 mM and thereafter only a gradual increase was observed. Thus, the concentration of 30 mM was conveniently selected for the derivatization.

To investigate the effect of pH on the formation of PTC-CIA derivatives, the derivatization reaction was carried out in varied concentrations (10-50 % v/v in CH_3CN)

of pyridine, TEA or TFA. It was observed that the product yield decreased significantly under basic and acidic conditions and the highest FL intensity was obtained in neutral pH conditions. The results led us to use neutral conditions which actually simplifies the reaction and also convenient to protect the ODS column which is suitable between pH 2-7.

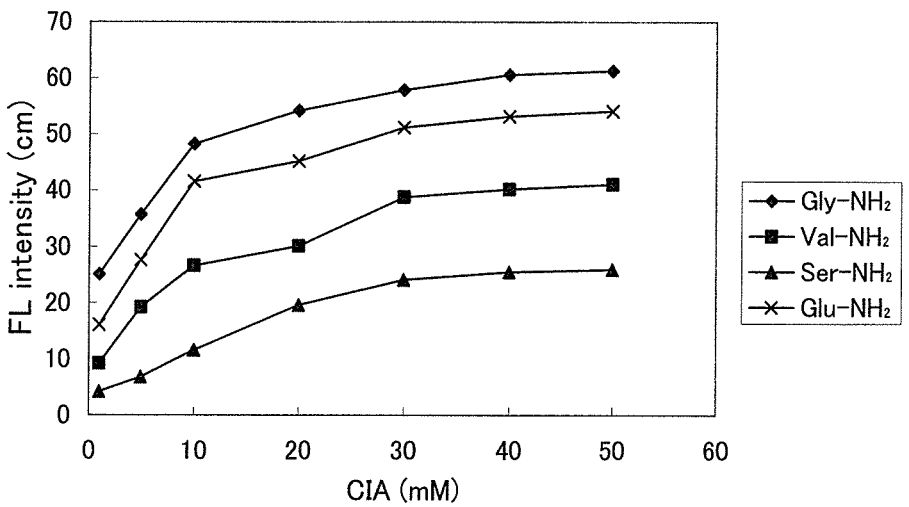


Fig. 2. Effect of CIA concentration on the formation of PTC-CIA-amino acids in the derivatization reaction at 80 °C for 15 min. A portion (10 μL) of ATZ-amino acid in CH_3CN was reacted with 10 μL of 1.0-50 mM CIA in CH_3CN. FL intensity was based on peak heights of PTC-CIA derivatives. Each point represents the mean value of three replicate experiments.

The product yields were estimated to study the reactivity of ATZ-amino acid with CIA. The estimation was based on measuring the ATZ-amino acids by RPLC with UV detection, before and after the CIA reaction. The peak height of the ATZ-amino acid before the CIA reaction was taken as 100%. The peak height of ATZ-amino acid after the CIA reaction was calculated to obtain the yield corresponding to PTC-CIA-amino acid. Under the

optimum conditions, the yield of PTC-CIA derivative was 93 % for ATZ-Gly, 90 % for ATZ-Val, 88 % for ATZ-Ser and 69 % for ATZ-Glu.

RPLC separation

Table 1. RPLC identification of PTC-CIA-amino acids derivatives

Amino acids	Retention time (min)	Peak height (cm)	Amino Acids	Retention time (min)	Peak height (cm)
Gln	10.1	7.0	Ala	23.2	12.5
Asn	10.8	6.3	Arg	24.6	5.4
Asp	13.2	6.8	Ser	28.8	3.6
Glu	13.6	8.3	Met	31.4	7.5
Thr	15.1	5.9	Val	39.6	7.3
Gly	17.2	13.4	Lys	41.6	6.9
Pro	18.6	9.8	Trp	49.2	6.1
Cys	20.8	4.4	Ile	56.4	8.0
Tyr	22.0	8.8	Leu	56.4	6.3
His	24.0	4.8	Phe	57.6	5.1

Mobile phase consisted of CH_3CN, 0.12 M CH_3COOH with 0.14 % SDS (w/v), and H_2O (55:10:35). Peak height indicates the relative FL intensity of PTC-CIA derivatives.

The retention times and relative FL intensities were measured for the PTC-CIA derivatives of 20 amino acids obtained from their amino-acid amides (5.0 nmol each on membrane) (Table 1). We used a reversed-phase column (250 x 4.6 mm ID) maintaining the column temperature at 40 °C. With this column, most of the PTC-CIA derivatives were separated by isocratic elution of 55 % CH_3CN containing 12 mM acetic acid and 0.014 % (w/v) SDS. The isomeric amino acids of Leu and Ile could not be separated. Some amino acids for example, Arg, Cys and Ser gave comparatively less intensity. The

difference of the FL intensities among the derivatives might be depended on the influence of side chains of individual amino acids for the reactivity with CIA. Their PTC-CIA derivatives were stable for the separation period. There was also no trace of degradation of labile amino acids such as Ser and Thr, which have a tendency to undergo degradation in aqueous acidic conditions required for PTH conversion.

Manual sequencing of peptide

Several organic solvents were investigated to remove excess PITC, TEA, TFA, and by-products usually urea-type compounds as well as to strongly retain the peptide on the GF membrane during washing. It was also important to remove those interfering substances on the membrane for the continuous sequencing procedures. As shown in Fig. 3, washing with toluene gave the highest yield of the CIA products from the amino acid residues at 2^{nd} and 3^{rd} cycles in the sequencing of insulin chain-A. The production of the CIA derivatives after washing the membrane with *n*-propyl chloride or *n*-butyl chloride was decreased, because the PITC-coupling peptide was eluted with these solvents. It is important to suitable solvents not only for washing, but also for extracting ATZ-amino acids from the membrane without the loss of peptide. The use of double GF membranes was found to decrease the loss of both small and large peptides from the membrane. Toluene proved to be effective solvent for washing as it had high retention of the peptides on the membranes. In addition, ethyl acetate was selected for the extraction of ATZ-amino acid. Washing the membrane with toluene before the start of next cycle resulted in an increased yield for coupling with PITC.

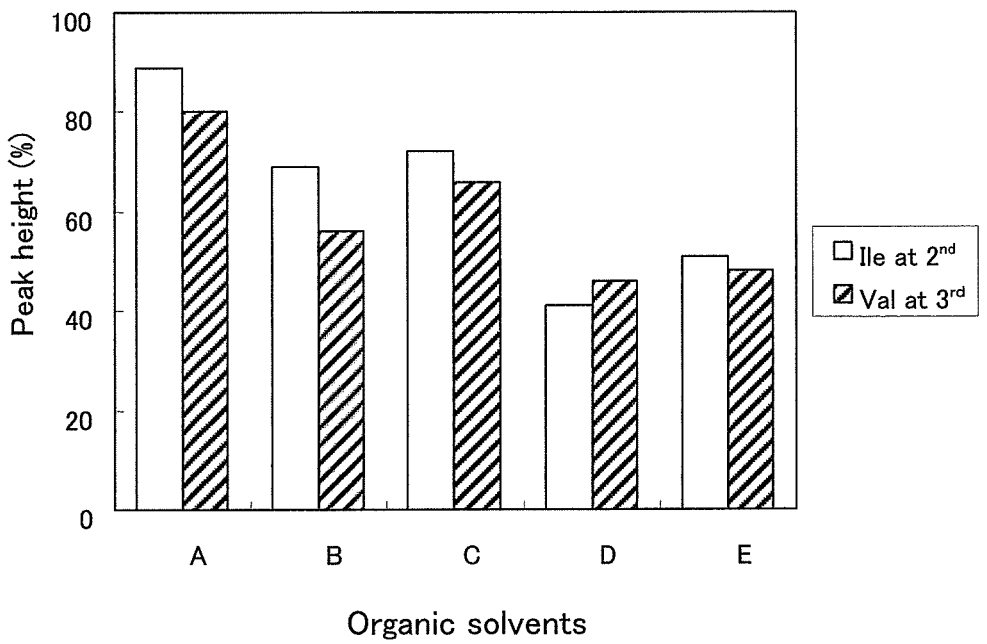
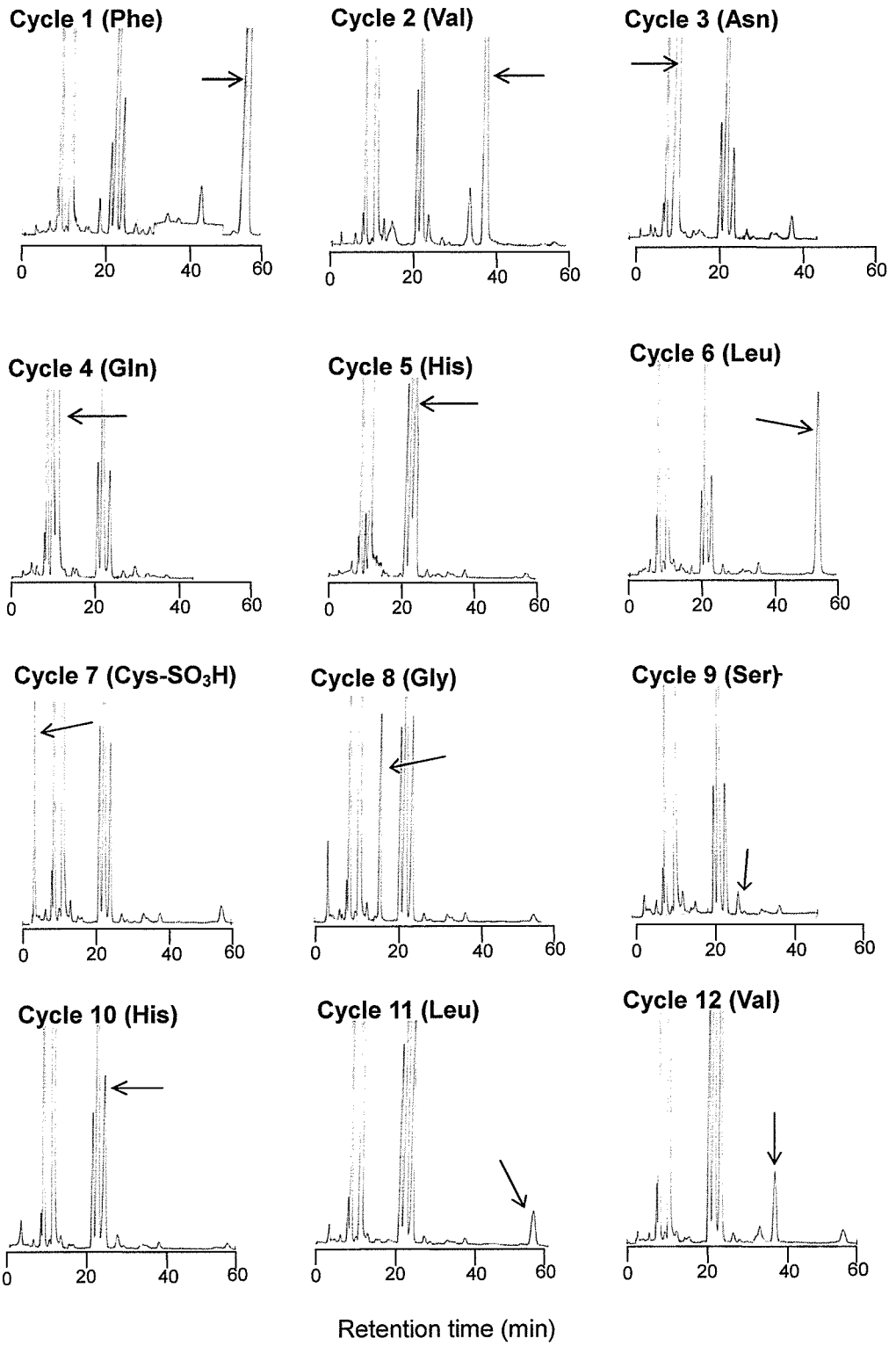


Fig. 3. Effect of solvents for washing after PITC coupling on the production of PTC-CIA-amino acid of insulin chain A at the selected cycles of 2nd cycle (Ile) and 3rd cycle (Val). Solvents: A, toluene; B, ethyl acetate; C, *n*-heptane; D, *n*-propyl chloride; E, *n*-butyl chloride. Sequencing was performed according to the procedures in the experimental section. Peak heights obtained from Ile- and Val-amides were taken as a reference, 100 %, respectively. Each point represents the mean value of three replicate experiments.

Cleavage of the PTC-peptide was performed with anhydrous TFA. It was found that the direct addition of the TFA liquid onto membrane resulted in increasing the formation of unfavorable TFA-caused artifact. This artifact was disturbed the reaction with CIA and next coupling with PITC. Therefore we used the gas-phase cleavage reaction for

sequencing. This gas-phase reaction was effective on the decrease of the artifacts, and also had the advantage of quicker drying of the GF membranes after the TFA treatment. Thus the unstable ATZ-derivatives could be immediately extracted for the CIA reaction. For the efficient cleavage with TFA vapor, temperature was increased to 80 °C for the 5-min reaction. From the chromatogram obtained, the cleavage was almost complete after 5 min. Long reaction time was accompanied by a decrease of the CIA products which may be attributed to pre-conversion of ATZ intermediates.

The suitability of the method was examined by sequencing of insulin chain-B composed of 30 amino acid residues. Fig. 4 shows the chromatograms obtained by sequencing up to 21 cycles. The peaks appearing at 21, 22 and 24 min were CIA impurities and/or by-products that were reactive to CIA. The peak appearing at 10 min was due to excess CIA. Under the above conditions, sequencing was successfully achieved up to 20 cycles. Beyond 20 cycles, identification of amino acids became difficult because of low peak height of the corresponding PTC-CIA derivatives. This might be caused by continuous loss of peptide fragments from the membrane during washing and extraction processes at each cycle. As can be seen from the chromatograms, a steady decline of peak heights was observed before the 9^{th} cycle (Ser residue). Comparing the peak heights from Leu at 6^{th}, 11^{th}, 15^{th}, and 17^{th} cycles, showed a small repetitive yield after the 9^{th} cycle. Most of the PTC-CIA-amino acids from the peptide could be identified on the chromatograms, including oxidized Cys (Cys-SO_3H), although Tyr (16^{th} cycle), Ala (14^{th} cycle) and His (5^{th} and 10^{th} cycles) derivatives co-eluted with by-product peaks.


Cycle 1 (Phe)
Cycle 2 (Val)
Cycle 3 (Asn)
Cycle 4 (Gln)
Cycle 5 (His)
Cycle 6 (Leu)
Cycle 7 (Cys-SO3H)
Cycle 8 (Gly)
Cycle 9 (Ser)
Cycle 10 (His)
Cycle 11 (Leu)
Cycle 12 (Val)
0
20
40
60
Retention time (min)