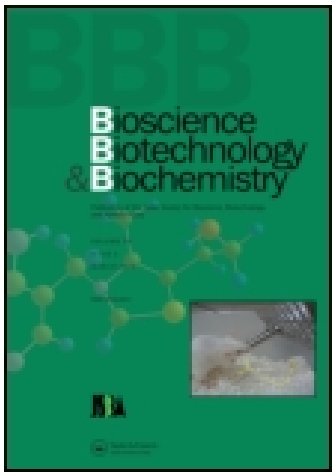

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Rapid Paper

Potential D,L-Amino Acid Sequence Analysis of Peptides from the C-Terminus

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A model tripeptide, Gly-L-Leu-L-Phe, was immobilized with activated aminomethyl polystyrene, and its C-terminal was reduced to an alcohol. This peptidyl alcohol was selectively hydrolyzed at the C-terminal amide bond to afford a polymer-supported dipeptide (Gly-L-Leu) and amino alcohol (Phe-OH). The amino alcohol, including its absolute configuration, was determined by labelling with (+)-MNB–COOH, and the dipeptide was reused for a determination of its C-terminal amino acids. The D,L-amino acids of the tripeptide were sequentially determined from the C-terminus.

Key words: C-terminal; sequence analysis; D,L-amino acid; chiral amino alcohol

Some methods for sequencing peptides from the C-terminus have recently been developed[1–4] to give additional information to the results of the Edman method.[5] Among them, the most promising would be the thiohydantoin method proposed by Bailey *et al.*,[3] while the other methods need more elaboration. Hamada *et al.* have reported the determination of C-terminal amino acids by reducing the C-terminal carboxylic ester to a hydroxy methyl group (peptidyl alcohol) and then completely hydrolyzing into a mixture of amino acids and an amino alcohol.[6] We found that the D,L of the amino acids could be determined by an HPLC analysis of the amino alcohols labelled with the chiral fluorescent converting reagent, (+)-MNB carboxylic acid,[7,8] and furthermore that the peptidyl alcohol was selectively hydrolyzed under specific acidic conditions at the C-terminal amide linkage to release an amino alcohol and a one-component-shortened peptide. In this paper, we describe the sequential determination of the amino acids and their D,L-configurations from the C-terminus by using a model tripeptide, Gly-L-Leu-L-Phe.

Materials and Methods

Chemicals. Amino alcohols were prepared from the corresponding amino acids according to the procedure reported by Yonemitsu *et al.*[6,9] Aminomethyl polystyrene beads were purchased from Sigma as was Disuccinimidy suberate (DSS). The tripeptides and other reagents were purchased from Wako Pure Chemical Industries.

Immobilization of Gly-L-Leu-L-Phe (GLF). Attachment of the tripeptide, GLF, to 50% cross-linked aminomethyl polystyrene beads (26 μmol amine/g) was carried out according to the procedure reported by Boyd *et al.*[2] DSS (15 mg) was dissolved in *N*-methylpyrrolidone (760 μl, NMP) containing 10% (v/v) pyridine and diisopropylethylamine (DIEA). The polystyrene beads (100 mg) were shaken with the DSS solution for 1 h at room temperature and then washed with NMP. The GLF ethyl ester (10 mg) was dissolved in 360 μl NMP containing 10% pyridine, and the polystyrene beads were added to it. The mixture was shaken overnight at room temperature, before the beads were sequentially washed with NMP, CH_3CN, and EtOH, and then dried under a vacuum.

Sequential analysis of polymer supported GLF. Polymer-supported GLF was treated with EtOH containing 1% $NaBH_4$ at 50°C for 1 h and then washed with 50% EtOH. The resin was suspended in a mixture of EtOH and 33% aq. HCl (1 : 1, 300 μl) and stirred at 70°C for 2 h, before being extracted with 50% EtOH. The resulting extract was evaporated to dryness and dissolved in a carbonate buffer (pH 9.0, 500 μl) for subsequent conversion with (+)-MNB–COCl. To the separated resin was added 10% $SOCl_2$ in EtOH, the mixture being kept at 50°C for 1 h, and then the resin was washed with EtOH. The foregoing procedure was repeated twice more for the sequential determination of the tripeptide.

Conversion of the amino alcohols with (+)-MNB–COCl. A mixture of an amino alcohol (up to 100 μM) and (+)-MNB–COCl (an acetonitrile solution, 3300 μM, 100 μl) in a carbonate buffer (pH 9.0, 10 mM, 100 μl) was stirred for 10 min at room temperature. To the mixture was added L-cysteic acid (4000 μM, 100 μl) in the same buffer, and the resulting solution was stirred for 30 min to decompose the excess reagent.

HPLC conditions. HPLC separation was conducted in a reversed-phase column (Capcell pak UG-120, 4.6 mm × 150 mm, Shiseido) held at 40°C and connected with a pump (CCMP, Toshou), a fluorescence detector (FPS-920, Nippon Bunko) and an integrator (HP-3396A, Hewlett Packard). The flow rate was 1.0 ml/min and the eluent was monitored at λ_{ex} = 310 nm and λ_{em} = 380 nm. A citrate buffer (pH 6.5, 10 mM), tetrahydrofuran and acetonitrile were mixed in the ratio of 85 : 12 : 3 (v/v/v) for mobile phase A and 40 : 48 : 12 (v/v/v) for B. Linear gradient elution was done for 40 min from 30% to 80% B.

Results and Discussion

HPLC determination of the amino acids and their D,L-configuration as corresponding amino alcohols

Since amino acids are quantitatively converted without racemization to their corresponding amino alcohols by esterification and subsequent $NaBH_4$ reduction, the HPLC fluorometric determination of the amino acids as their corresponding amino alcohols was studied. Fifteen kinds of amino alcohols were labelled with (+)-MNB carbonyl chloride[7] in a carbonate buffer (pH 9.0) to afford mono-*N*-acylated derivatives. These were separated by reversed-phase HPLC with linear gradient elution was shown in Fig. 1A. The (±)-MNB carbonylated L-amino alcohols, which correspond to (+)-MNB carbonylated D,L-amino alcohols, were also clearly separated under these conditions (Fig. 1B). Pro-OH and D,L of Thr-OH and Ser-OH could not be determined under these conditions because Pro-OH could

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Abbreviations: MNB–COOH. 2-methyl-2-β-naphthyl-1,3-benzodioxole-4-carboxylic acid; Phe-OH, phenylalaninol; Leu-OH, leucinol; Gly-OH, glycinol; Pro-OH, prolinol; Thr-OH, threoninol; Ser-OH, serinol; Trp-OH, tryptophanol; Cys-OH, cysteinol.

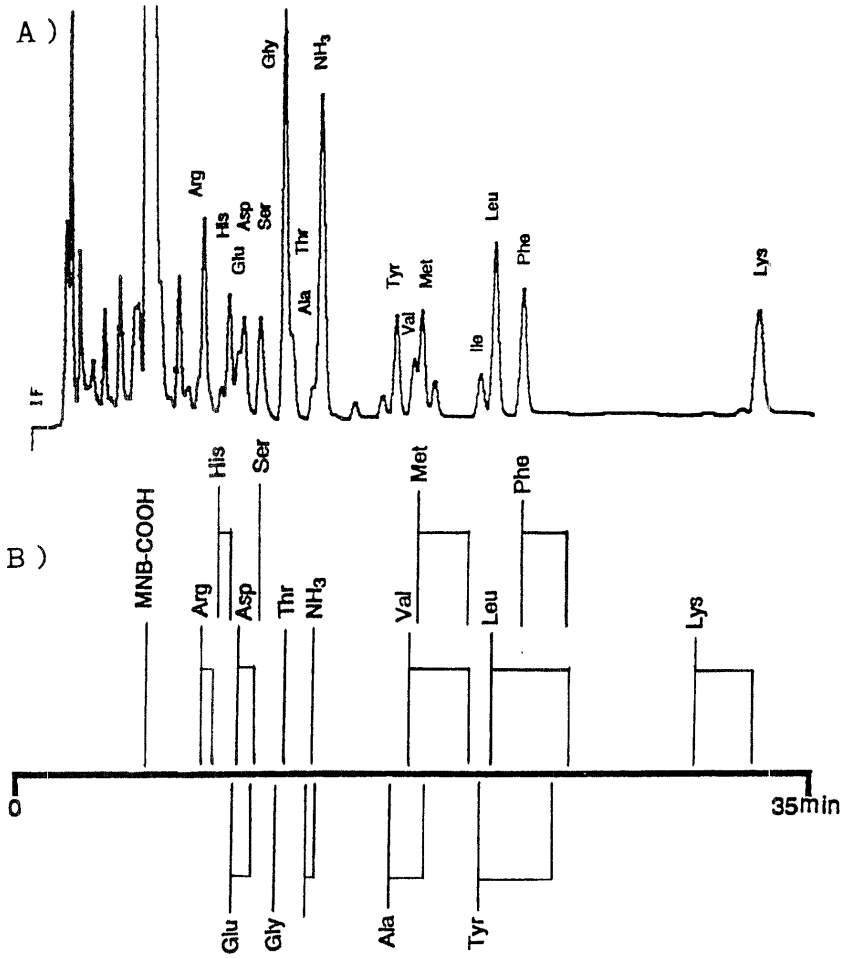

Fig. 1. HPLC Determination of (+)-MNB-carbonylated L-Amino Alcohols (1A) and Their D,L (1B).

HPLC conditions: mobile phase A, pH 6.5 buffer : THF : CH_3CN (85 : 12 : 3, v/v/v); mobile phase B, pH 6.5 buffer : THF : CH_3CN (40 : 48 : 12, v/v/v) linear gradient from 30% to 80% B in 40 min. 1.0 ml/min flow rate, Capcell pak UG-120 column (150 mm × 4.6 mm). Fluorescence detection ($Ex = 310$ nm, $Em = 380$ nm) at 40°C.

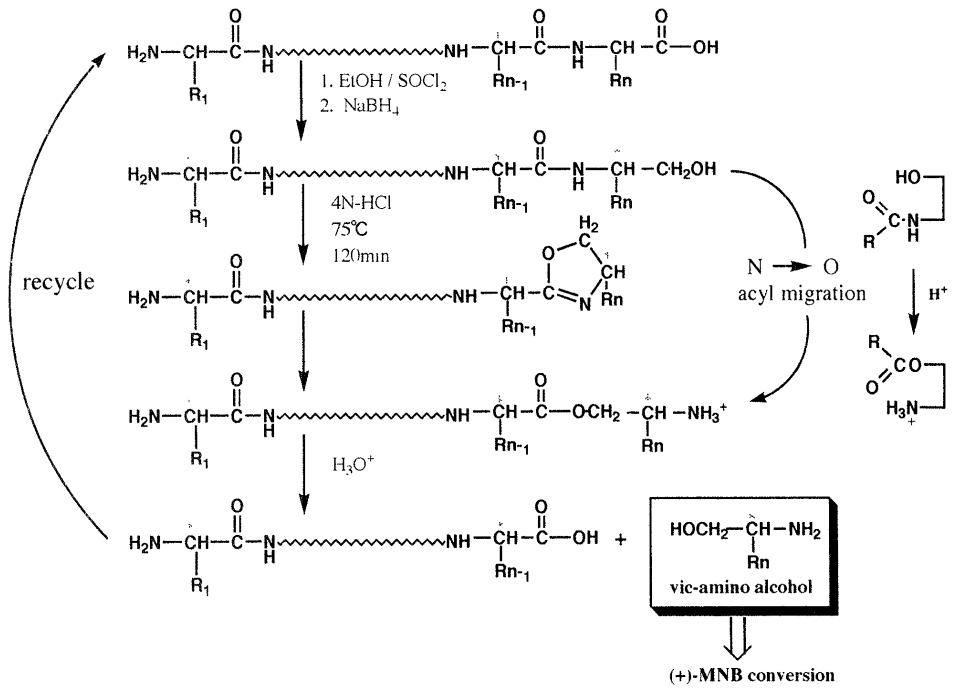

Fig. 2. Strategy for the Sequential Analysis of Peptides from the C-Terminal.

not be labelled, D,L of Thr-OH could not be separated, and Ser gave achiral Ser-OH by reduction. Trp-OH and Cys-OH could not be detected by fluorometry because they gave very weak fluorescence due to quenching, but they could be determined by UV detection (nmole level). The amide groups of Gln and Asn were transformed to hydroxyl groups

Table Hydrolysis of Leu-Gly-Gly-OH under Various Acidic Conditions

$$\underset{\text{(L-G-G-OH)}}{\text{Leu-Gly-Gly-OH}} \xrightarrow{\text{HCl aq}} \underset{\text{(LG)}}{\text{Leu-Gly-COOH}} + \underset{\text{(G-OH)}}{\text{Ethanolamine}}$$

Entry	HCl conc. (N)	Time (h)	Temp. (°C)	Recovery (%)			
				L-G-G-OH	LG	G-OH	Leu
1	1 N	14	r.t.	100	0	0	0
2	1 N	2.5	40	100	0	0	0
3	4 N	3.4	50	55	49	35	0
4	4 N	1	75	32	43	49	2.7
5	5 N	2	75	5	78	62	4
6	6 N	1	75	0	0	80	90

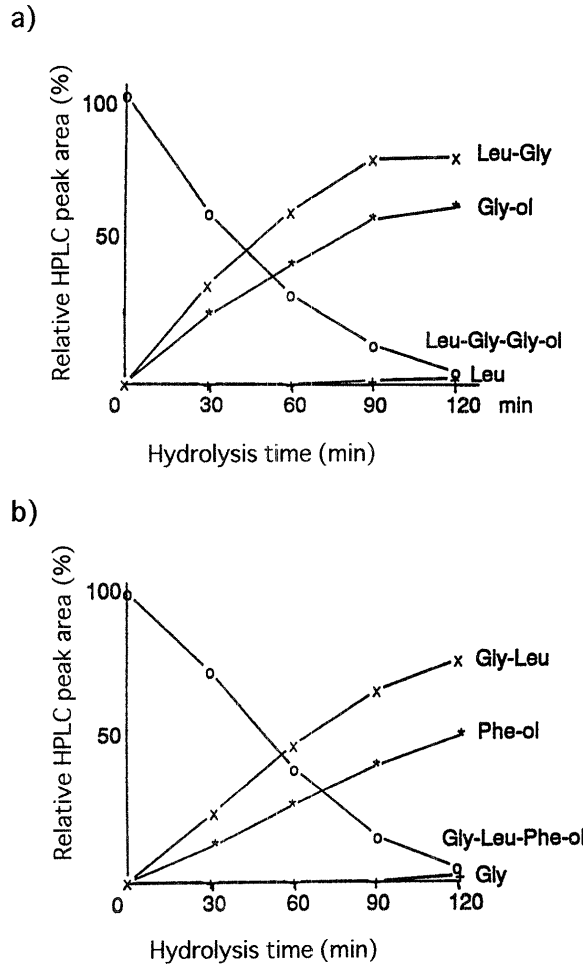

Fig. 3. Time–Course fot the Selective Hydrolysis of a) Leu-Gly-Gly-OH and b) Gly-Leu-Gly-OH and b) Gly-Leu-Phe-OH with 4 N HCl at 75°C.

by the esterification and reduction process, and Gln and Asn would further be hydrolyzed to Glu and Asp, respectively, during acid hydrolysis of the amide bond of the C-terminal amino alcohol. Therefore, the differentiation of Gln from Gl and of Asn from As in a peptide by this method would be very difficult. However, these results show that most of the amino acids and their D,L could be determined as their corresponding amino alcohols. The detection limit for MNB carbonylated amino alcohols was 0.1 pmol in the column.[7]

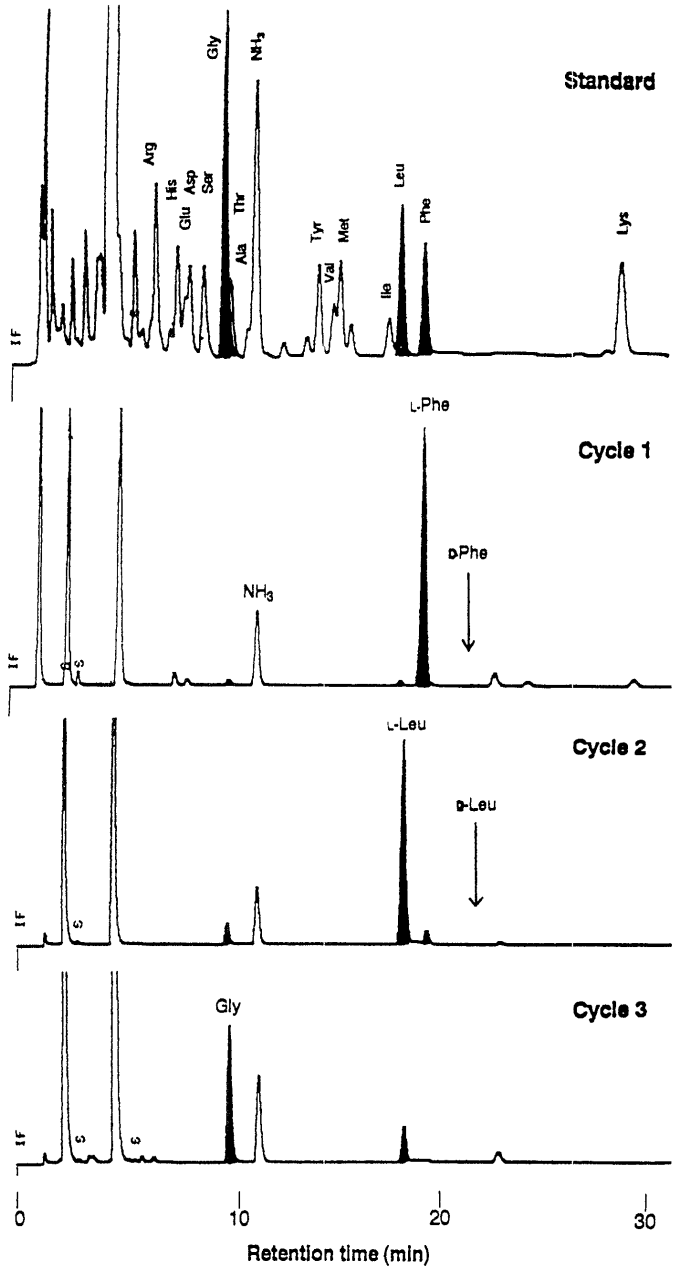

Fig. 4. Solid-phase Sequential Determination of Gly-L-Leu-L-Phe.

Selective hydrolysis of the C-terminal amide bond of the peptidyl alcohol

Since the C-terminal amide bond of a peptidyl alcohol is

the amide bond of the vicinal amino alcohol, the $N \rightarrow O$ acyl migration[10] under acidic conditions can be expected. Once this $N \rightarrow O$ acyl migration takes place, the ester could be hydrolyzed under much milder acidic conditions than those for peptide cleavage (Fig. 2). Therefore, we studied the conditions for selective hydrolysis of the C-terminal amide bond by using two tripeptidyl alcohols, Leu-Gly-Gly-OH and Gly-Leu-Phe-OH, under several acidic conditions. We found that 4 N HCl at 75°C for 2 h could selectively hydrolyze the C-terminal amide bond as shown in the Table and Fig. 3. The recoveries of dipeptides Leu-Gly and Gly-Leu, and of amino alcohols Gly-OH and Phe-OH, were 83%, 80%, 62%, and 44%, respectively. The released amino alcohol can be used for determining of the C-terminal amino acid, and the peptide can be recycled for determining the next C-terminal amino acid. In order to easily separate the resulting amino alcohol from the peptide, the peptide was immobilized on aminomethyl polystyrene resin[2] as detailed in Materials and Methods section.

Sequential analysis of a model tripeptide from the C-terminus

As a model study, Gly-L-Leu-L-Phe-OH immobilized on aminomethyl polystyrene resin was subjected to a sequential analysis from the C-terminus. The amino alcohol released during every cycle was labeled with (+)-MNB–COCl and determined by HPLC, with the results shown in Fig. 4. L-Leu-OH but no D-Leu-OH in the first cycle and L-Phe-OH but no D-Phe-OH in the second cycle were respectively determined. These results indicate that no racemization occurred during the analysis. In the third cycle, Gly-OH was determined. Thus, the tripeptide was determined to be Gly-L-Leu-L-Phe. Although the present study was on a very simple example, the results indicate a promising method for the sequential analysis of peptides from the C-terminus. Further elaboration of this method is being investigated in our laboratory.

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