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Short Communication

Liquid chromatography–mass spectrometry for simultaneous analyses of iminodipeptides containing an N-terminal or a C-terminal proline

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ABSTRACT

Simultaneous analyses of synthetic iminodipeptides containing an N-terminal proline or a C-terminal proline have been demonstrated using liquid chromatography–mass spectrometry with an atmospheric pressure ionization interface system. The separation of iminodipeptides was carried out on a reversed-phase high-performance liquid chromatographic column using 0.1% aqueous trifluoroacetic acid–methanol (75:25, v/v, pH 2.0) as mobile phase. Very intense protonated molecular ions $[M+H]^+$ of various synthetic iminodipeptides, Pro-Gly, Gly-Pro, Pro-Ala, Ala-Pro, Pro-Val, Val-Pro, Pro-Leu and Leu-Pro, were observed. Pro-Gly (Pro-X) and Gly-Pro (X-Pro) have the same protonated molecular ion (m/z 173), but the peaks of these compounds on the mass chromatograms were clearly distinguished by the differences of the retention times and mass spectra. The synthetic iminodipeptides containing an N-terminal proline added to urine samples from a patient with prolidase deficiency were also distinguished from iminodipeptides containing a C-terminal proline in urine samples from a patient with prolidase deficiency by scanning the $[M+H]^+$ ion of each iminodipeptide. We established the method to measure simultaneously the various iminodipeptides containing an N-terminal or a C-terminal proline in biological samples.

INTRODUCTION

It is known that patients with iminodipeptiduria excrete in the urine large amounts of iminodipeptides containing a C-terminal proline, owing to a hereditary prolidase deficiency [1–3].

High-performance liquid chromatography (HPLC) is one of the most widely used methods for the analysis of peptide mixtures [4]. Recently, analyses of mixtures of non-volatile compounds have become increasingly important, and liquid chromatography combined with atmospheric pressure ionization mass spectrometry (LC–API-MS) shows promise as a new analytical method in various fields [5]. We have previously reported the protonated molecular ions $[M + H]^+$ of a series of synthetic iminodipeptides containing a C-terminal proline, and various

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endogenous iminodipeptides in the urine of patients with iminodipeptiduria have been identified by this method [6].

The present paper describes the protonated molecular ions $[M + H]^+$ of synthetic iminodipeptides containing an N-terminal proline, and the identification of various synthetic N-terminal proline iminodipeptides added to control human urine.

EXPERIMENTAL

Reagents

Pro-Gly, Pro-Ala, Pro-Val, Pro-Leu, Pro-Ile, Pro-Met, Pro-Pro, Pro-Hyp, Gly-Pro, Ala-Pro and Leu-Pro were purchased from Sigma. All other chemicals were analytical grade.

Instrumentation

A Hitachi L-6200 HPLC instrument equipped with a 5-μm Inertsil ODS-packed column (150 mm × 4.6 mm I.D.) from Gasukuro Kogyo (Tokyo, Japan) was connected to a Hitachi M80B mass spectrometer–computer system through an API interface [7]. The nebulizer and vaporizer temperatures were 255 and 390°C, respectively. HPLC analyses of synthetic and urinary iminodipeptides were carried out with a mobile phase of 0.1% trifluoroacetic acid–methanol (75:25, v/v) at a flow-rate of 0.9 ml/min.

Isolation of iminodipeptides from urine

Control human urine was obtained from laboratory personnel, and various synthetic iminodipeptides containing an N-terminal proline were added to the normal human urine.

A column containing 5 ml of Chelex 100 (Na^+, 100–200 mesh, Bio-Rad, Richmond, CA, U.S.A.) was prepared for the collection of urinary peptides according to the following method. The column was washed with 50 ml of water, then 20 ml of saturated copper sulphate were added to the column, which was allowed to stand overnight. The column was washed with water, and then buffered with 0.01 *M* borate buffer (pH 11.0). Each 3-ml sample of a control human urine and of a control urine sample that contained synthetic N-terminal proline iminodipeptides was adjusted to pH 11.0 with 1 *M* sodium hydroxide, transferred to the buffered Chelex column prepared as above, and washed with 30 ml of 0.01 *M* borate buffer (pH 11.0). The effluent and washings were combined, and evaporated under reduced pressure at 40°C. The residue containing peptides was dissolved in water, made weakly acidic (pH 3.0) with 2 *M* hydrochloric acid, and filtered. The filtrate was transferred to a column containing 10 ml of Diaion SK-1 (H^+, 100 mesh, Mitsubishi Kasei, Tokyo, Japan), and washed with 50 ml of water. The peptides were eluted with 2 *M* ammonia.

The eluate was evaporated to dryness under reduced pressure. The residue was dissolved in a small amount (1 ml) of water, and subjected to LC–API-MS.

RESULTS AND DISCUSSION

LC–API-MS analyses of various C-terminal proline iminodipeptides have been reported in a previous paper [6]. This paper describes the simultaneous analyses of various iminodipeptides containing an N-terminal or a C-terminal proline in biological samples using LC–API-MS. The mass chromatograms of the mixtures of the synthetic N-terminal proline iminodipeptides Pro-Gly (173), Pro-Hyp (229a), Pro-Ile (229c), Pro-Leu (229d), Pro-Val (215), Pro-Met (247), Pro-Pro (213) and Pro-Ala (187) obtained using the LC–API-MS system are shown in Fig. 1.

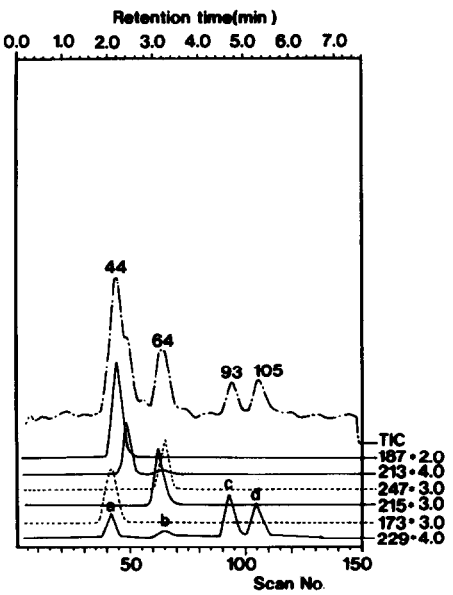

Fig. 1. Mass chromatograms of synthetic N-terminal proline iminodipeptides Pro-Gly (173), Pro-Ala (187), Pro-Val (215), Pro-Met (247), Pro-Pro (213), Pro-Hyp (229:a), Pro-Ile (229:c) and Pro-Leu (229:d). Chromatographic conditions: mobile phase, 0.1% trifluoroacetic acid–methanol (75:25, v/v); flow-rate, 0.9 ml/min. The mass spectrometer was scanned from m/z 100 to 300 at a rate of 4 s per scan.

In the LC–API-MS system, the protonated molecular ions $[M + H]^+$ of these iminodipeptides were observed as the base peaks. The HPLC separations of Pro-Gly, Pro-Ala, Pro-Pro and Pro-Hyp, and Pro-Met and Pro-Val were incomplete, but the mass chromatograms of these iminodipeptides clearly distinguished each compound.

The protonated molecular ions $[M + H]^+$ of Pro-Hyp, Pro-Ile and Pro-Leu had the same m/z value of 229: four ion peaks (a, b, c, d) were observed in the mass chromatogram (Fig. 1). The identification of those ion peaks was based on

the retention times on the mass chromatogram and on the mass spectra of those iminodipeptides. The retention times of peaks a, c and d on the mass chromatogram were the same as those on the mass chromatograms of Pro-Hyp (a), Pro-Ile (c) and Pro-Leu (d). In the mass spectra of Pro-Met, $[M + H]^+ - H_2O$ (229) was observed, in addition to the protonated molecular ion $[M + H]^+$ (247), and the retention time of the ion peak (b) was the same as that of Pro-Met. The ion peak (b) at 229 was thus identified as $[M + H]^+ - H_2O$ of peak of Pro-Met.

Mass chromatograms and spectra of the mixture of Gly-Pro, Ala-Pro, Val-Pro and Leu-Pro are shown in Fig. 2A and B (a, b, c, d). These iminodipeptides, which contain a C-terminal proline, were separated by scanning the $[M + H]^+$ ions.

The ions $[M + H]^+$, $[M + H]^+ - H_2O$ and proline residue + 2H of the iminodipeptides Gly-Pro (173, 155 and 116), Ala-Pro (187, 169 and 116) and Val-Pro (215, 197 and 116) were observed in mass spectra scanned at the peak top of each mass chromatogram (Fig. 2B).

Mass chromatograms and spectra of the mixture of Pro-Gly, Pro-Ala, Pro-Val

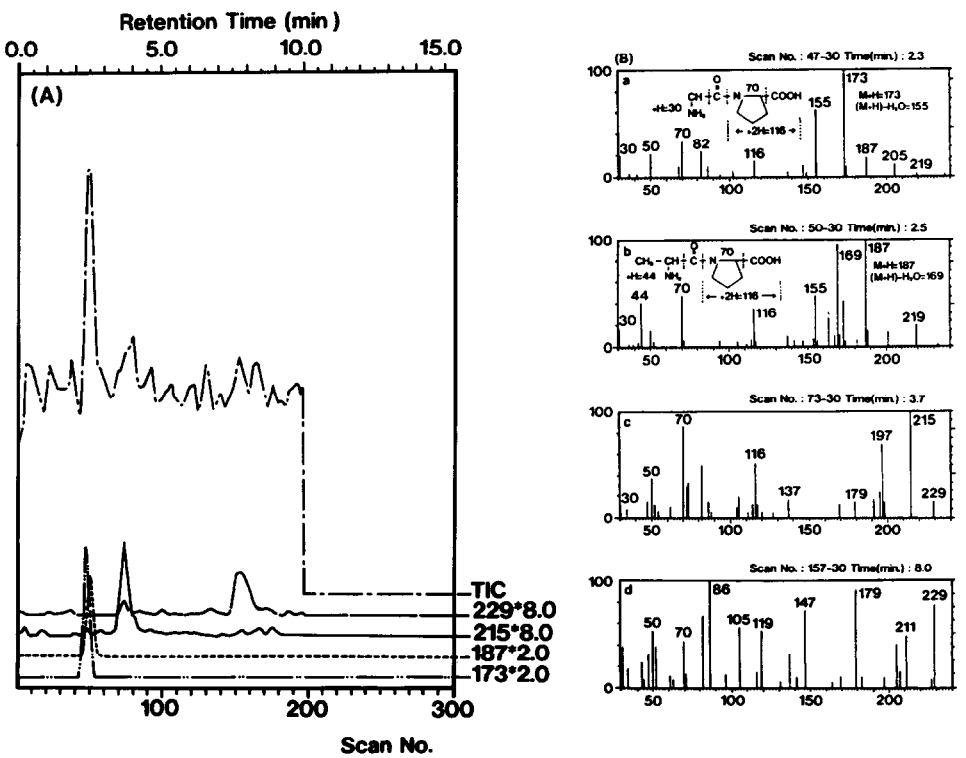

Fig. 2. Mass chromatograms (A) and spectra (B) of the mixture of Gly-Pro (173), Ala-Pro (187), Val-Pro (215) and Leu-Pro (229). The chromatographic and MS conditions were the same as in Fig.1.

and Pro-Leu are shown in Fig. 3A and B (a, b, c, d). These iminodipeptides, which contain N-terminal proline, were separated by scanning the $[M + H]^+$ ions. The ions $[M + H]^+$ and proline residue of the iminodipeptides Pro-Gly (173 and 70), Pro-Ala (187 and 70), Pro-Val (215 and 70) and Pro-Leu (229 and 70) were mainly observed in mass spectra scanned at the peak top of each mass chromatogram. The retention times of Pro-Gly and Pro-Ala were shorter than those of Gly-Pro and Ala-Pro. The retention times of Pro-Val and Pro-Leu were longer than those of Val-Pro and Leu-Pro.

The retention times on mass chromatograms and the mass spectra of the iminodipeptide pairs Pro-Gly/Gly-Pro, Pro-Ala/Ala-Pro, Pro-Val/Val-Pro and Pro-Leu/Leu-Pro, which have the same molecular mass, were different as shown in Figs. 2 and 3, respectively. Therefore, the mixture of iminodipeptides containing an N-terminal and a C-terminal proline, Pro-Gly/Gly-Pro (173), Pro-Ala/Ala-Pro (187), Pro-Val/Val-Pro (215) and Pro-Leu/Leu-Pro (229), could be identified by scanning the $[M + H]^+$ ions of iminodipeptides on the mass chromatograms (Fig. 4).

Mass chromatograms of a control human urine and a sample to which iminodipeptides containing an N-terminal proline were added to a normal human urine

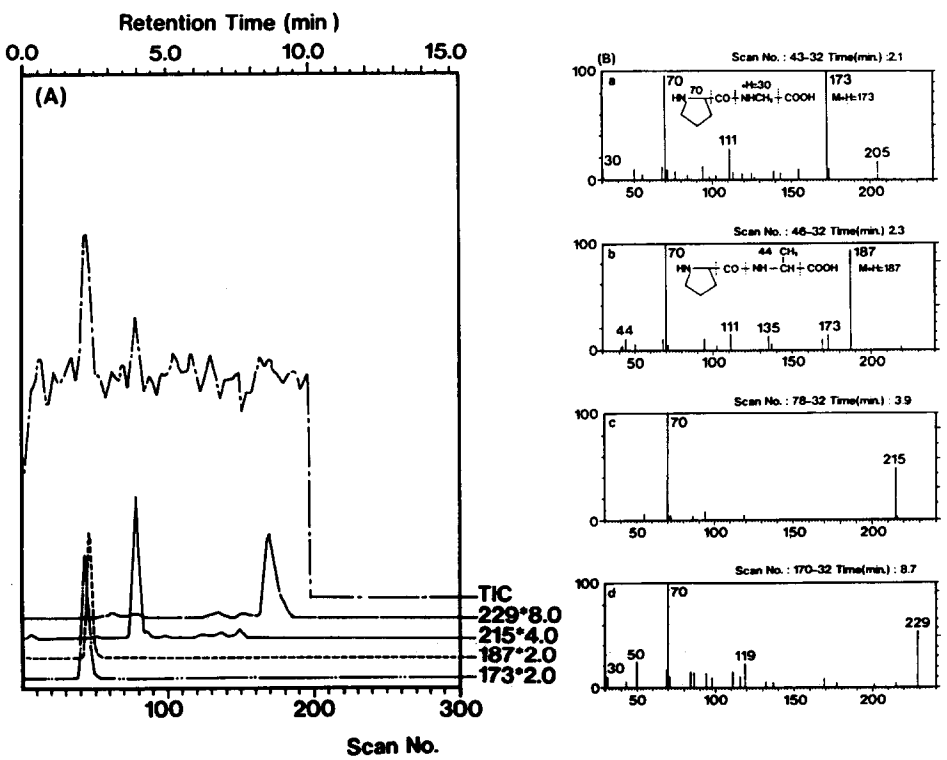

Fig. 3. Mass chromatograms (A) and spectra (B) of the mixture of Pro-Gly (173), Pro-Ala (187), Pro-Val (215) and Pro-Leu (229). The chromatographic and MS conditions were the same as in Fig. 1.

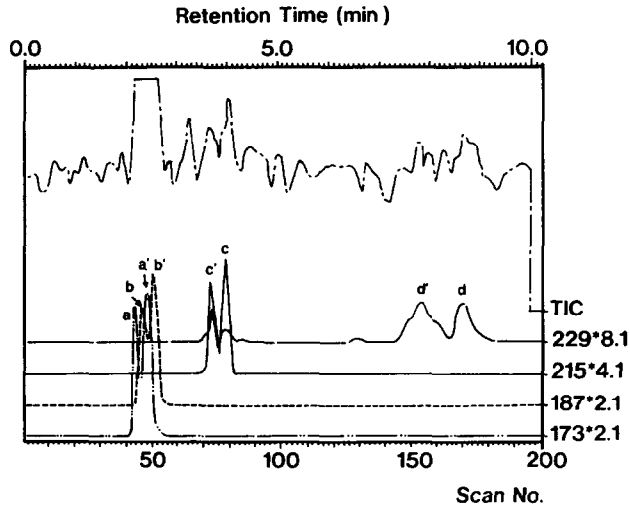

Fig. 4. Mass chromatograms of the mixture of Pro-Gly (a)/Gly-Pro (a') (173), Pro-Ala (b)/Ala-Pro (b') (187), Pro-Val (c)/Val-Pro (c') (215) and Pro-Leu (d)/Leu-Pro (d') (229). The chromatographic and MS conditions were the same as in Fig. 1.

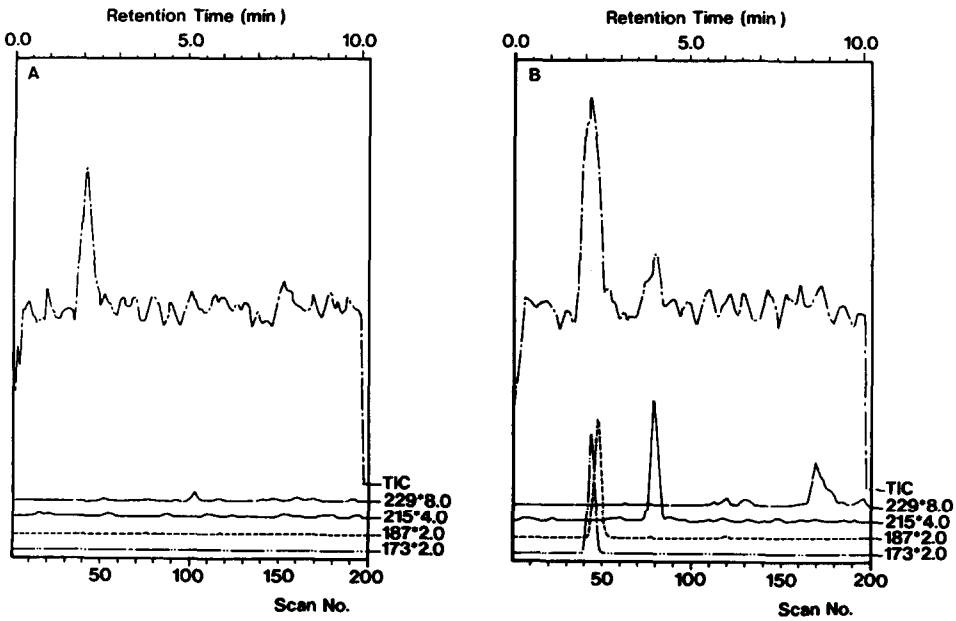

Fig. 5. Mass chromatograms of iminodipeptides in a sample of control human urine (A) and in a sample to which synthetic iminodipeptides were added (B). The chromatographic and MS conditions were the same as in Fig. 1.

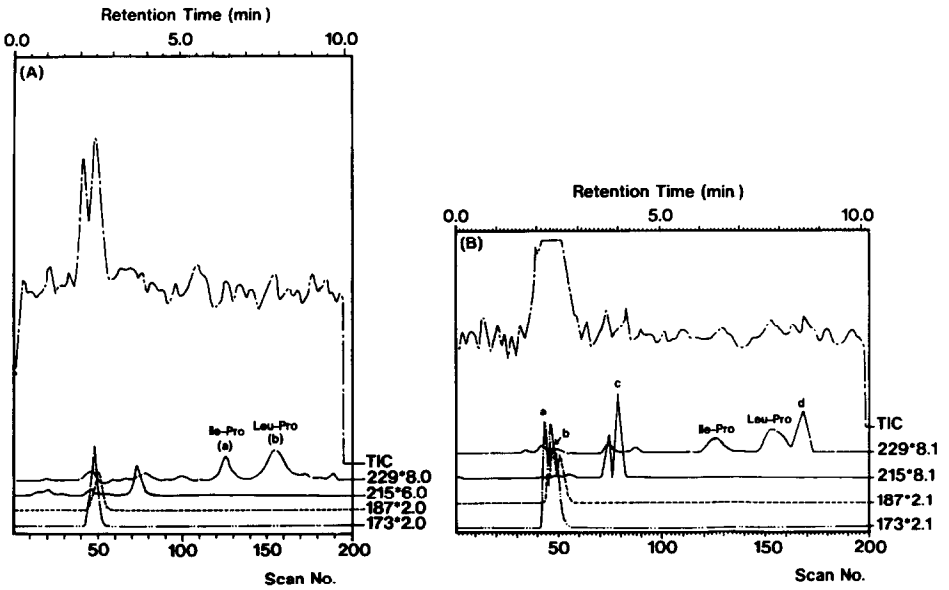

Fig. 6. Mass chromatograms of iminodipeptides (Gly-Pro, 173; Ala-Pro, 187; Val-Pro, 215; Ile-Pro, 229-a; Leu-Pro, 229-b) containing a C-terminal proline in the urine from a patient with prolidase deficiency (A) and in a patient's urine to which synthetic iminodipeptides [Pro-Gly (a), Pro-Ala (b), Pro-Val (c) and Pro-Leu (d)] containing an N-terminal proline were added (B). The chromatographic and MS conditions were the same as in Fig. 1.

are shown in Fig. 5A and B. $[M + H]^+$ ions of iminodipeptides containing an N-terminal proline could not be detected in a normal human urine, but $[M + H]^+$ ions of iminodipeptides added to a normal human urine could be clearly detected.

Mass chromatograms of a urine sample of a patient with prolidase deficiency and a sample to which iminodipeptides containing an N-terminal proline were added to a patient's urine, are shown in Fig. 6A and B. $[M + H]^+$ ion peaks of Gly-Pro, Ala-Pro, Val-Pro and Leu-Pro were observed in the urine of a patient with prolidase deficiency, as reported in a previous paper [6]. $[M + H]^+$ ion peaks of Pro-Gly/Gly-Pro, Pro-Ala/Ala-Pro, Pro-Val/Val-Pro and Pro-Leu/Leu-Pro in a patient urine to which Pro-Gly, Pro-Ala, Pro-Val and Pro-Leu were added were also identified by scanning the $[M + H]^+$ ion of each iminodipeptide (Fig. 6B).

These results demonstrate that this method can be used for the simultaneous analysis of various iminodipeptides containing an N-terminal or a C-terminal proline, for the screening of patients with prolidase deficiency or prolinase deficiency, and for the identification of various iminodipeptides in biological samples.

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