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Liquid chromatography–mass spectrometry for the qualitative analyses of iminodipeptides in the urine of patients with prolidase deficiency

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SUMMARY

Analyses of standard iminodipeptides and iminodipeptides in the urine of patients with prolidase deficiency have been demonstrated using liquid chromatography–mass spectrometry with an atmospheric pressure ionization interface system. The separation was carried out on a reversed-phase column using 0.1% aqueous trifluoroacetic acid–methanol (70:30 or 80:20). Very intense quasi-molecular ions ($[M+H]^+$) of various standard iminodipeptides were observed by this method. The quasi-molecular ions $[M+H]^+$ of various iminodipeptides were also observed in the urine samples of patients with prolidase deficiency, and Gly-Pro, Ala-Pro, Val-Pro, Leu-Pro, Ile-Pro, Ser-Pro, Thr-Pro, Glu-Pro, Asp-Pro, His-Pro, Lys-Pro, Pro-Pro and Tyr-Pro as iminodipeptides containing proline with C-terminal residue and Glu-Hyp, Pro-Hyp, Ile-Hyp and Gly-Hyp as iminodipeptides containing hydroxyproline with C-terminal residue were identified in the urine of patients with prolidase deficiency.

INTRODUCTION

Prolidase (EC 3.4.13.9) deficiency is a rare autosomal recessive disease characterized by chronic ulcerative dermatitis and mental retardation [1,2]. It has been known that these patients also excrete large amounts of iminodipeptides in the urine due to hereditary prolidase deficiency [1–3].

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The iminodipeptides excreted in the urine of patients with prolidase deficiency have been identified by amino acid analysis [1–3] and by isotachophoresis [4]. However, it was impossible to identify exactly the iminodipeptides in the urine using these methods.

High-performance liquid chromatography (HPLC) is one of the most widely used methods used for the analysis of peptide mixtures [5]. Recently, analyses of mixtures of non-volatile compounds has become increasingly important, and liquid chromatography–atmospheric pressure ionization mass spectrometry (LC–API-MS) shows promise as a new analytical method in various fields [6]. We have succeeded in recording the quasi-molecular ions $[M+H]^+$ of a series of standard iminodipeptides using this technique. The aim of this experiment was to demonstrate the utility of this approach in qualitative analyses of iminodipeptides in biological samples.

EXPERIMENTAL

Reagents

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

Instrumentation

The apparatus used was an Hitachi L-6200 HPLC instrument, equipped with a 5-μm Inertsil ODS-2 packed column (150 mm × 4.6 mm I.D.) from Gasukuro Kogyo (Tokyo, Japan), connected to a Hitachi M80B mass spectrometer–computer system, through the API interface [7]. The nebulizer and vaporizer temperatures were 320 and 380°C, respectively. Analyses of standard and urinary iminodipeptides were carried out with a mobile phase of 0.1% trifluoroacetic acid–methanol (70:30 or 80:20, v/v) at a flow-rate of 0.9 ml/min.

Urine samples

The samples of normal human urine were obtained from laboratory personnel. The samples from patients with prolidase deficiency were obtained from two girls (sisters), as reported previously [3,4].

Isolation of iminodipeptides from 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 200 ml of water, then 100 ml of the saturated copper sulphate were added to the column and allowed to stand overnight. The column was washed well with water, and then buffered with 0.01 *M* borate buffer (pH 11.0). Each 3-ml urine sample of two patients and of a normal human 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 in vacuo at 40°C. The residue containing peptides was dissolved in water, made weakly acidic with 2 *M* hydrochloric acid and filtered. The filtrate was transferred to a column containing 10 ml of Diaion SK-1 (H^+ form, 100 mesh, Mitsubishi Kasei, Tokyo, Japan) and washed with 50 ml of water. The peptides were then eluted with 2 *M* ammonia. The eluate was evaporated to dryness in vacuo. The residue was dissolved in a small amount of water and then subjected to LC–API-MS.

RESULTS AND DISCUSSION

Mass chromatograms and mass spectra of standard iminodipeptides Gly-Pro, Ala-Pro, Val-Pro and Leu-Pro obtained using the LC–API-MS system are shown in Fig. 1. In the LC–API-MS system, quasi-molecular ions $[M+H]^+$ of these iminodipeptides were observed as base peaks, in addition to the ions

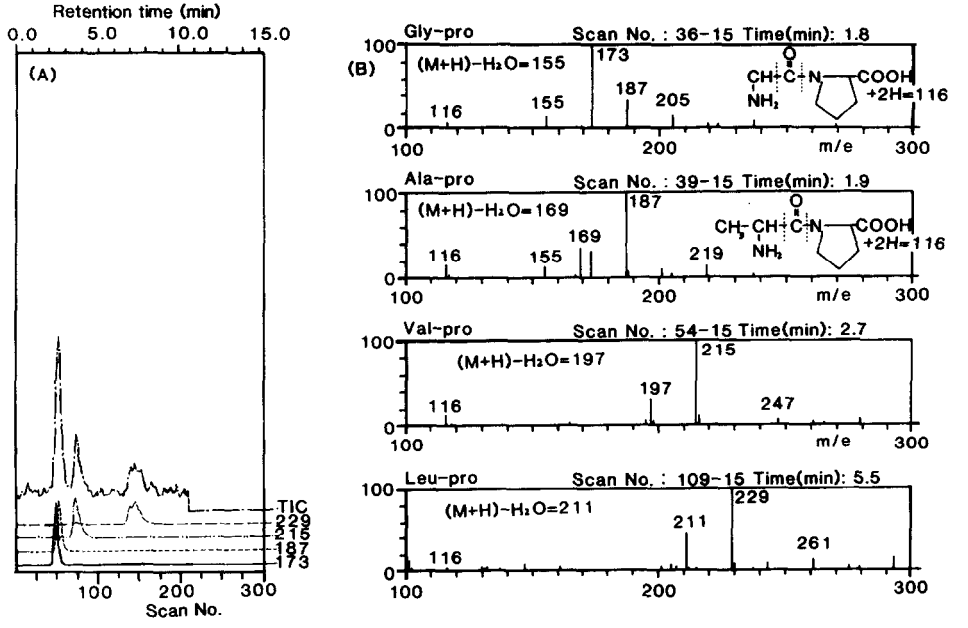

Fig. 1. (A) Mass chromatograms of standard iminodipeptides: Gly-Pro (173), Ala-Pro (187), Val-Pro (215) and Leu-Pro (229). (B) Mass spectra scanned at the peak tops of mass chromatograms in (A). The chromatographic conditions were: mobile phase, 0.1% trifluoroacetic acid–methanol (80:20, v/v); flow-rate, 0.9 ml/min. The mass spectrometer was scanned from *m/z* 100 to 300 at a rate of 4 s per scan.

$[M+H]^+ - H_2O$, C-terminal residue +2H, and N-terminal residue (Fig. 1B). The HPLC separation of Gly-Pro and Ala-Pro was incomplete, but mass chromatograms of both were clearly distinguished (Fig. 1A).

Mass chromatograms of peptide fractions of the urine samples from patients (A and B) with prolidase deficiency and from a normal human are shown in Fig. 2. Peaks of the quasi-molecular ions of Gly-Pro (173), Ala-Pro (187), Val-Pro (215) and Leu-Pro (229) appeared in the chromatograms obtained from the urine samples from the patients, but not in the chromatograms obtained from normal urine samples. The retention times of ion peaks of *m/e* 173, 189, 215 and 227 in the urine of patient B were the same as those of authentic Gly-Pro, Ala-Pro, Val-Pro and Leu-Pro, and the ions of quasi-molecule $[M+H]^+$, $[M+H]^+ - H_2O$ and proline residue +2H of iminodipeptides Gly-Pro (215, 197, 116), Ala-Pro (187, 116), Val-Pro (215, 197, 116) and Leu-Pro (229, 211, 116) were observed in mass spectra scanned at the peak top of each mass chromatogram (Fig. 2B).

These results indicate the presence of iminodipeptides of Gly-Pro, Ala-Pro, Val-Pro and Leu-Pro in the urine of patients with prolidase deficiency. How-

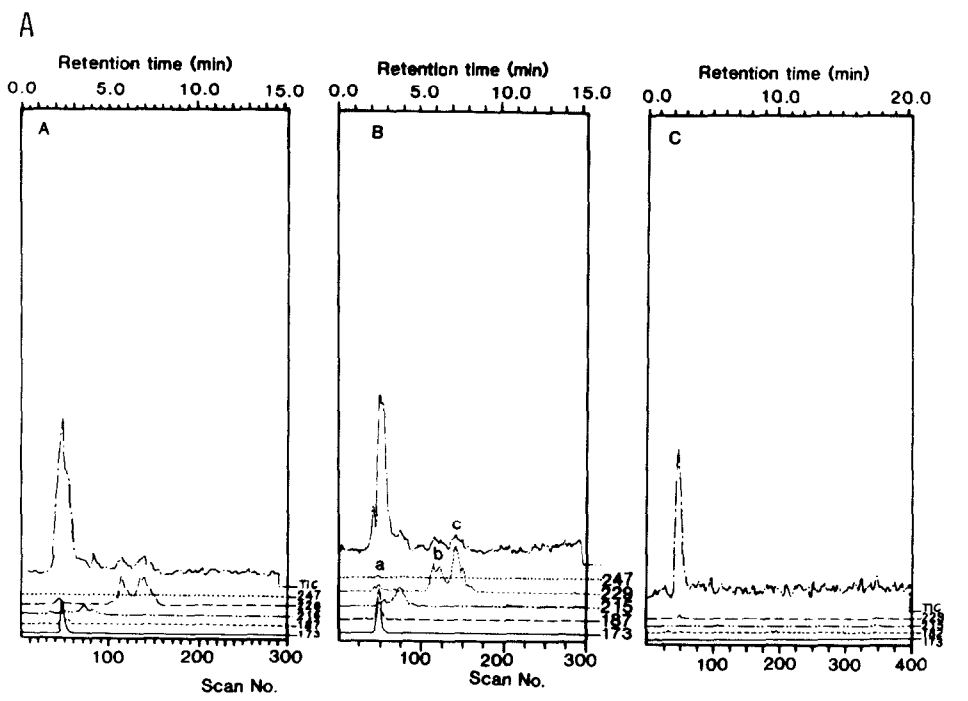

Fig. 2.

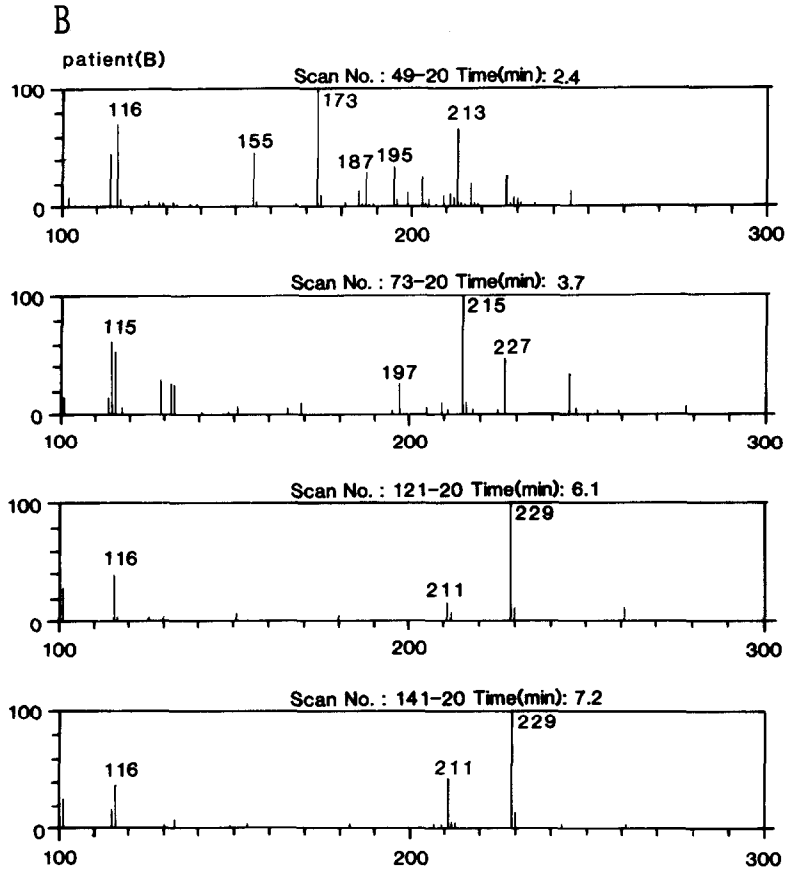

Fig. 2. (A) Mass chromatograms of iminodipeptides (*m/e* 173, 187, 215, 229) in the urine from patients with prolidase deficiency (A and B) and a normal human (C). (B) Mass spectra scanned at the peak tops of the mass chromatograms in (A). The chromatographic and MS conditions were the same as in Fig. 1.

ever, three ion peaks in the mass chromatograms of $[M+H]^+$ (229) were observed as shown in Fig. 2A and B.

The identification of those ion peaks was based on the retention times on the mass chromatogram and on the mass spectra of standard iminodipeptides. The retention times of a and c (Fig. 3A) on the mass chromatograms of $[M+H]^+$ (229) in the urine from patient B were the same as those on the mass chromatograms of Pro-Hyp (a) and Leu-Pro (b), as shown in Fig. 3B. Mass spectra of a and c (Fig. 3A) of urine samples from the patients were similar to those of standard Pro-Hyp and Leu-Pro, and iminodipeptides of

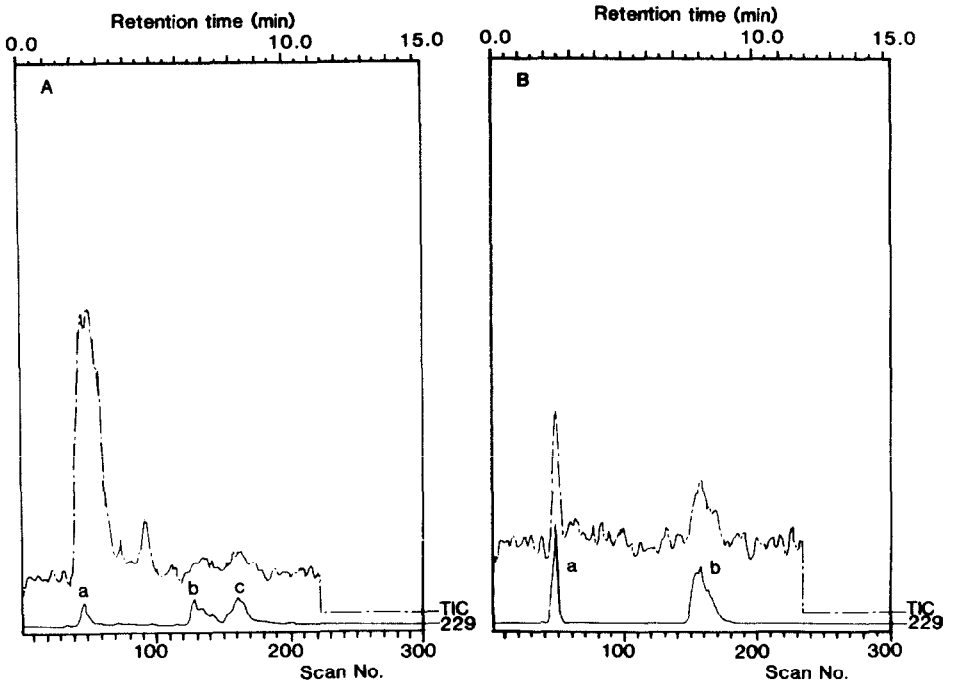

Fig. 3. Mass chromatograms of (A) *m/e* 229 in the urine from patient B and (B) standard iminodipeptides Pro-Hyp (a) and Leu-Pro (b). The chromatographic and MS conditions were the same as in Fig. 1.

$[M+H]^+$ (229) were thought to be Pro-Hypro, Ile-Pro and Leu-Pro. The mass spectrum of b in Fig. 3A was also the same as that of Leu-Pro, as shown in Fig. 2B, but the retention time of the peak was different from that of Leu-Pro (Fig. 4).

From these results, the three peaks (a, b, and c) on the mass chromatograms were identified as Pro-Hyp (a), Ile-Pro (b) and Leu-Pro (c). Two peaks of $[M+H]^+$ (245) on the mass chromatograms of urine samples from patient B were observed, as shown in Fig. 4. Iminodipeptides of $[M+H]^+$ (245) were thought to be Glu-Pro, Leu-Hyp and Ile-Hyp. Ions of *m/e* 245, 227, 116, 130 were observed in peak a of the mass chromatogram, and ions of *m/e* 245, 227, 132, 86 in peak b (Fig. 4). From these observations, peaks a and b were identified as Glu-Pro and Leu-Hypro or Ile-Hypro, respectively.

The mass chromatograms of various iminodipeptides containing proline with C-terminal residues, and containing hydroxyproline with C-terminal residues, from the urine sample of patient B are shown in Figs. 5 and 6. From these

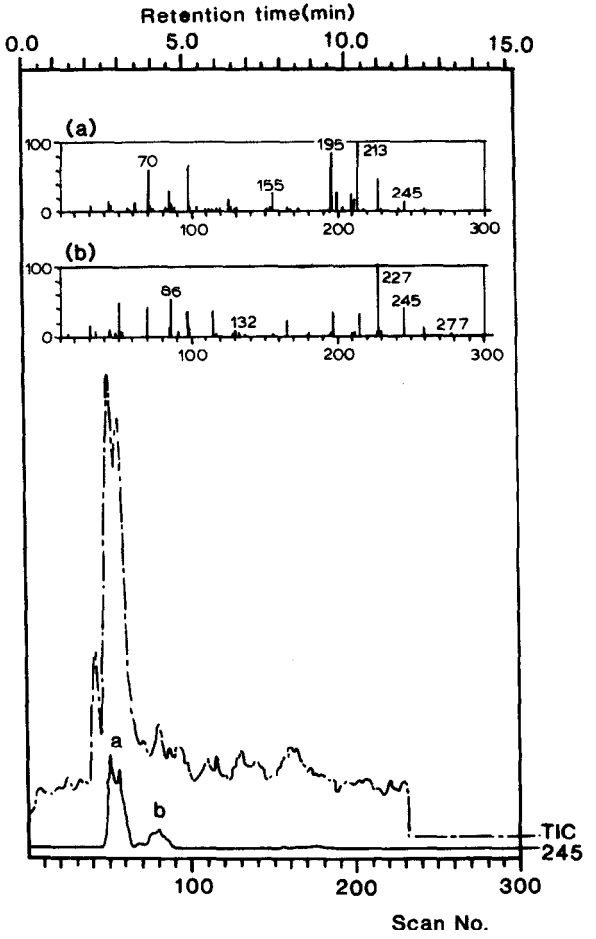

Fig. 4. Mass chromatogram of *m/e* 245 in the urine from patient B, and mass spectra scanned at the peak tops of the mass chromatogram. The chromatographic and MS conditions were the same as in Fig. 1.

chromatograms we identified Gly-Pro, Ala-Pro, Ser-Pro, Thr-Pro, Val-Pro, Leu-Pro, Ile-Pro, Phe-Pro, Tyr-Pro, Glu-Pro, Asp-Pro, Cys-Pro, His-Pro, Lys-Pro and Pro-Pro as iminodipeptides containing proline, and Gly-Hyp, Glu-Hyp, Pro-Hyp and Leu-Hyp or Ile-Hyp as iminodipeptides containing hydroxyproline.

The contents of free amino acids in the hydrolysates of the peptide fraction of urine samples from the two patients are shown in Table I.

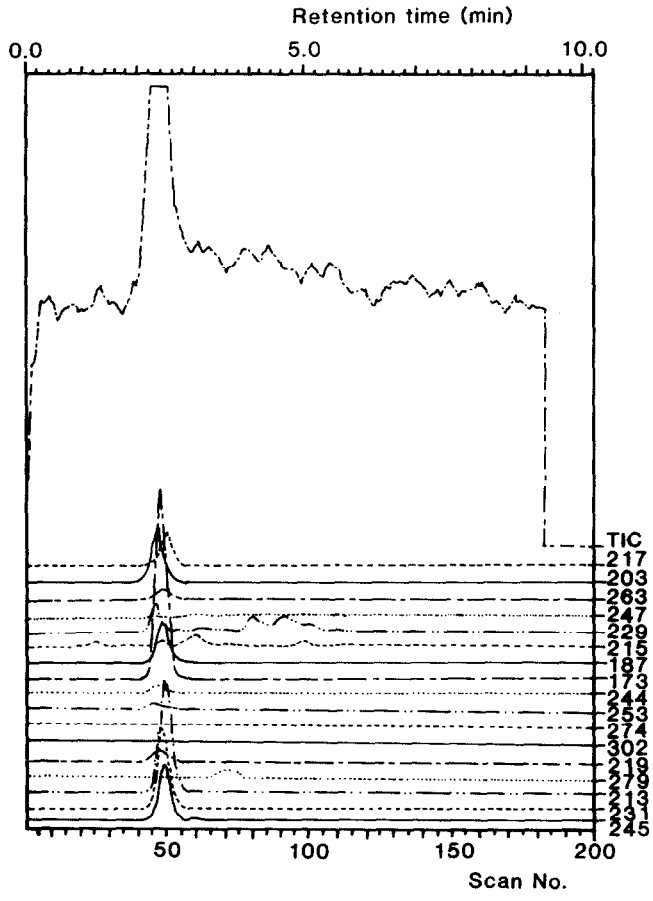

Fig. 5. Mass chromatograms of iminodipeptides (M+H) containing proline in the urine from patient A: Gly-Pro (173), Ala-Pro (187), Val-Pro (215), Leu-Pro (229), Met-Pro (247), Phe-Pro (263), Ser-Pro (203), Thr-Pro (217), Glu-Pro (245), Asp-Pro (231), Pro-Pro (213), Tyr-Pro (279), Cys-Pro (219), Tyr-Pro (302), Arg-Pro (274), His-Pro (253), and Lys-Pro (244). The chromatographic conditions were: mobile phase, 0.1% trifluoroacetic acid–methanol (70:30, v/v); flow-rate, 0.9 ml/min. The mass spectrometer was scanned from *m/z* 50 to 300 at a rate of 4 s per scan.

Methionine, arginine and tryptophan could not be detected in the urine samples. These results also agree with the absence of ion peaks of Met-Pro, Arg-Pro and Trp-Pro in the urine samples from the patients.

We have thus demonstrated that this method is very useful for the identification of iminodipeptides in biological samples, such as urine samples of patients with prolidase deficiency.

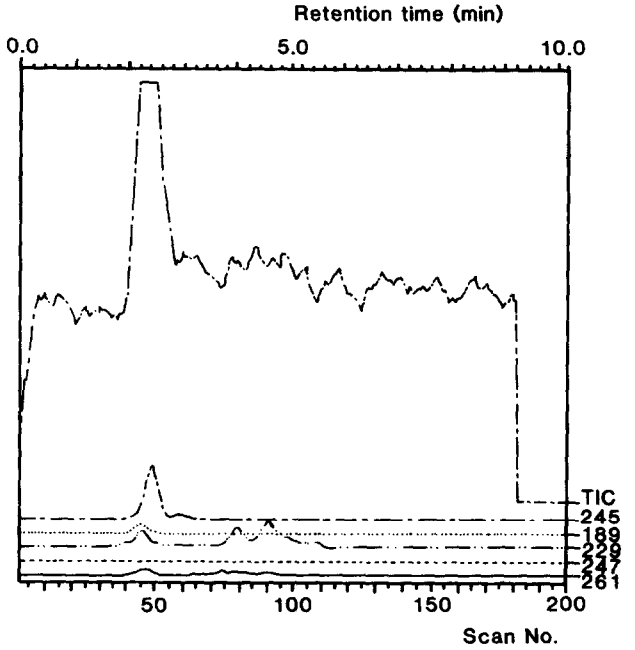

Fig. 6. Mass chromatograms of iminodipeptides containing hydroxyproline in the urine from patient A: Gly-Hyp (189), Leu-Hyp or Ile-Hyp (245), Glu-Hyp (261), Asp-Hyp (247), and Pro-hyp (229). The chromatographic and MS conditions were the same as in Fig. 5.

TABLE I

CONTENT OF AMINO ACIDS IN THE HYDROLYSATES OF PEPTIDE FRACTIONS OF URINE SAMPLES FROM PATIENTS WITH PROLIDASE DEFICIENCY

The peptide fractions obtained from the urine samples of patients A and B were hydrolysed in 6 *M* hydrochloric aid and 2 *M* sodium hydroxide at 110°C for 24 h (for tryptophan). Amino acids of the hydrolysates were determined by an amino acid analyser.

Amino acid	Concentration (μmol/ml)		Amino acid	Concentration (μmol/ml)	
	Patient A	Patient B		Patient A	Patient B
Asp	1.739	1.975	Leu	0.934	1.237
Thr	0.771	0.826	Tyr	0.233	0.307
Ser	0.721	0.866	Phe	0.588	0.648
Glu	1.707	2.297	Lys	0.477	0.443
Gly	1.836	2.713	His	0.077	0.076
Ala	0.659	0.798	Arg	N.D.	N.D.
Val	0.474	0.599	Trp	N.D.	N.D.
Cys	0.072	0.077	Pro	8.410	10.910
Met	N.D.	N.D.	Hyp	0.627	1.505
Ile	0.741	0.846			

REFERENCES

1 S.I. Goodman, C.C. Solomons, F. Muschenheim, C.A. McIntyre, B. Miles and D. O'Brien, Am. J. Med., 45 (1969) 152.
2 G.F. Powell, N.A. Rasco and R.M. Maniscalco, Metabolism, 23 (1974) 505.
3 H. Kodama, S. Umemura, M. Shimomura, S. Mizuhara, J. Arata, Y. Yamamoto, A. Yasutake and N. Izumiya, Physiol. Chem. Phys., 8 (1976) 463.
4 H. Mikasa, K. Sasaki and H. Kodama, J. Chromatogr., 305 (1984) 204.
5 V.R. Perkins, J.V. Nau and A. McPartland, LC·GC, Mag. Liq. Gas Chromatogr., 5 (1987) 419.
6 E.C. Horning, D.I. Carroll, I. Dzidic, K.D. Haegele, M.G. Horning and R.N. Stillwell, J. Chromatogr., 99 (1974) 13.
7 M. Sakairi and H. Kambara, Anal. Chem., 60 (1988) 774.