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Measurement of Iminodipeptides in the Serum of Patients with Prolidase Deficiency Using Liquid Chromatography-Mass Spectrometry

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Summary: Iminodipeptides containing C-terminal proline or hydroxyproline were determined in sera from patients with prolidase deficiency, in their mother's serum, and in the sera of unrelated controls, using liquid chromatography-mass spectrometry with an atmospheric pressure ionization interface system. The separation was carried out on a reversed phase column using 1 g/l aqueous trifluoroacetic acid-methanol (75 + 25, by vol.). The quasi-molecular ions $([M + H])^+$ of various iminodipeptides containing C-terminal proline and hydroxyproline were observed in the sera of patients with prolidase deficiency, using selected ion monitoring. The quasi-molecular ions $([M + H])^+$ of iminodipeptides containing C-terminal proline were not observed in the sera of normal subjects or the patients' mother, but the latter did contain various iminodipeptides with C-terminal hydroxyproline. This method proved useful for the determination of iminodipeptides in the sera 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). These patients also excrete large amounts of iminodipeptides in the urine (1–3).

The iminodipeptides excreted by patients have been demonstrated by amino acid analysis (1–3) and by isotachophoresis (4). However, these methods are unsuitable for the exact identification of urinary iminodipeptides.

The analyses of mixtures of non-volatile compounds using liquid chromatography combined with atmospheric pressure ionization mass spectrometry (LC-APCI-MS) has recently produced promising results in different fields (5).

We previously reported the identification of a series of synthetic iminodipeptides containing a C-terminal proline or hydroxyproline, as well as various endogenous iminodipeptides, in the urine of patients with iminodipeptiduria, using LC-APCI-MS (6).

The iminodipeptides in the sera of patients with prolidase deficiency could not be detected by scan mode, because the concentration of iminodipeptides in sera is much lower than that in urine.

The present paper describes the identification of various iminodipeptides containing C-terminal proline or hydroxyproline in the sera of patients with prolidase deficiency, in the serum of their mother, and in normal subjects, using selected ion monitoring.

Experimental

Materials

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

Serum samples

The samples of normal human serum were obtained from laboratory personnel. The samples from patients with prolidase deficiency and their mother were obtained from two girls (sisters) and their mother, as reported previously (3, 4).

Instrumentation (mass spectrometry)

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 APCI interface (7, 8). The nebulizer and vaporizer temperatures were 320 and 380 °C, respectively. Analyses of standard and serum in iminodipeptides were carried out with a mobile phase of 1 g/l trifluoroacetic acid-methanol (75 + 25, by vol.) at a flow-rate of 0.9 ml/min.

Preparation of samples

Six ml of 20 g/l sulphosalicylic acid were added to 2 ml of serum, stirred and centrifuged at 3000 min^{-1} for 10 min. The supernatant 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 iminodipeptides were then eluted with 2 mol/l ammonia. The eluate was evaporated to dryness in vacuo. The residue was dissolved in 0.2 ml of water, then subjected to LC-APCI-MS.

Assay of prolidase activity

Erythrocyte lysates were prepared from the heparinized blood of patients and normal volunteers according to *Umemura*'s method (9). A mixture containing 10 µl of diluted erythrocyte lysate, 10 µl of 10 mmol/l $MnCl_2$ and 80 µl of 50 mmol/l Tris-HCl (pH 7.8) was preincubated for 10 min at 37 °C, then incubated with 100 µl of 10 mmol/l substrate (X-Pro) in 1 mmol/l $MnCl_2$ for 30 min at 37 °C. The reaction was terminated by the addition of 200 µl of 100 g/l trichloroacetic acid solution. After 10 min at 90 °C with *Chinard*'s reagent (10), which consisted of ninhydrin, phosphoric acid and acetic acid, liberated proline was estimated by spectrophotometry (wavelength 515 nm). The activity towards X-Hyp was assayed according to the method reported previously (11).

Results and Discussion

The various iminodipeptides in the urine of patients with prolidase deficiency were easily observed by scan mode using LC-APCI-MS, but since the concentration of iminodipeptides is much lower in serum than in urine, the scan mode was of no use for the analysis of serum iminodipeptides.

Therefore, iminodipeptides were analysed in serum using selected ion monitoring which is more sensitive than the scan mode.

Selected ion monitoring chromatograms of quasi molecular ions $[M + H]^+$ of the standard iminodipeptides, Gly-Pro, Ala-Pro, Val-Pro, Leu-Pro, Ser-Pro, Pro-Pro, Gly-Hyp and Pro-Hyp, obtained using the LC-APCI-MS system are shown in figure 1. These iminodipeptides were easily observed by scanning their quasi-molecular ions $[M + H]^+$.

Selected ion monitoring chromatograms of quasi-molecular ions [M + H] of various iminodipeptides in sera from patients (sisters) with prolidase deficiency and their mother are shown in figure 2. The quasi-molecular ions $[M + H]^+$ of Gly-Pro, Ala-Pro, Val-Pro, Leu-Pro,

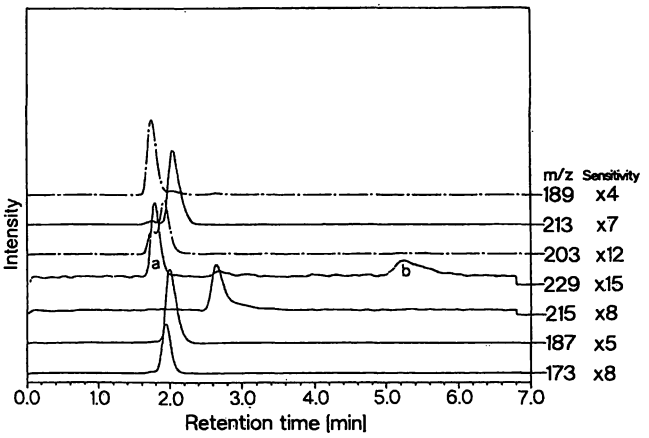

Fig. 1 Selected ion monitoring chromatogram of authentic iminodipeptides, Gly-Pro ([M + H] 173), Ala-Pro (187), Val-Pro (215), Pro-Hyp (229-a), Leu-Pro (229-b), Ser-Pro (203), Pro-Pro (213) and Gly-Hyp (189).

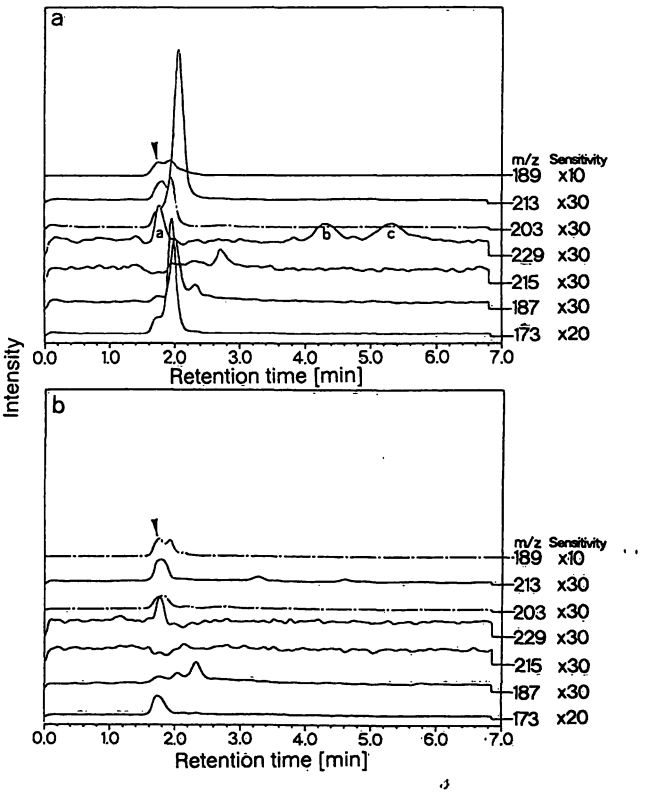

Fig. 2 Selected ion monitoring chromatograms of iminodipeptides in the serum samples from a patient with prolidase deficiency (A) and her mother (B).
Gly-Pro ([M + H] 173), Ala-Pro (187), Val-Pro (215), Pro-Hyp (229-a), Ile-Pro (229-b), Leu-Pro (229-c), Ser-Pro (203), Pro-Pro (213), Gly-Hyp (189).

Ile-Pro, Ser-Pro, Pro-Pro, Gly-Hyp and Pro-Hyp were observed in the serum of a patient with prolidase deficiency (fig. 2a). The quasi-molecular ions $[M + H]^+$ of iminodipeptides containing C-terminal proline were not observed in the serum of the patient's mother (fig. 2b) or normal subjects, but the quasi-molecular ions $[M + H]^+$ of iminodipeptides containing C-terminal hydroxyproline, Gly-Hyp and Pro-Hyp were clearly observed.

In the LC-APCI-MS system, quasi-molecular ions $[M + H]^+$, $[M + H]\text{-}H_2O$, and proline residue + 2H (m/z 116) were commonly observed in the mass spectra of iminodipeptides as reported previously (6, 7).

Selected ion monitoring chromatograms of the simultaneous scanning of the $[M + H]^+$ ion $- H_2O$ and proline residue + 2H (m/z 116) are shown in figures 3a, b, c for Gly-Pro, for Pro-Pro and for a mixture of these two dipeptides.

The peaks of the $[M + H]^+$ ion, $[M + H]^+ - H_2O$, and proline residue + 2H of Gly-Pro or Pro-Pro have the same retention time (figs. 3a and b).

On the other hand, in a chromatogram of a mixture of these two compounds, the $[M + H]^+$ ion and $[M + H]^+ - H_2O$ ion of Gly-Pro or Pro-Pro have the same retention time, but the peak of the proline residue + 2H overlaps and is unclear (fig. 3c).

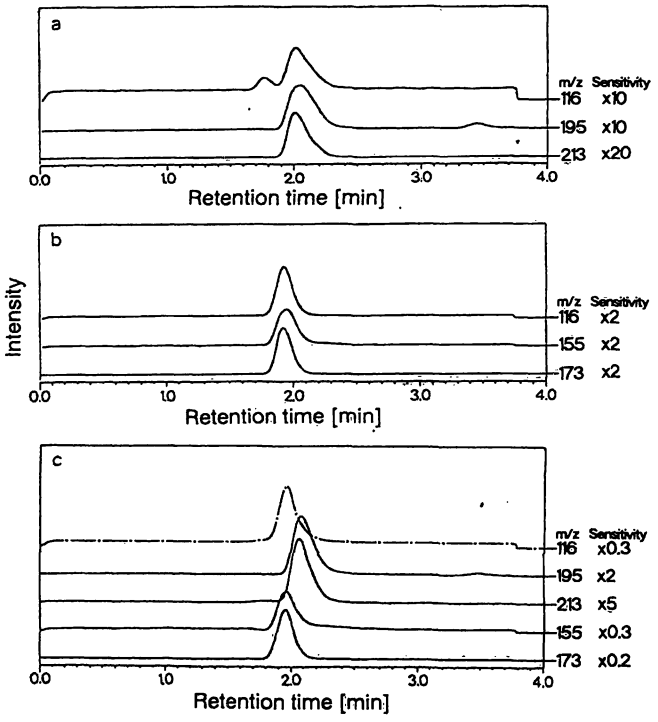

Fig. 3 Selected ion monitoring chromatograms of the $[M + H]$ ion (213), $[M + H] - H_2O$ ion (195) and proline residue + 2H ion (116) of Pro-Pro (a); the $[M + H]$ ion (173), $[M + H] - H_2O$ ion (155) and proline residue + 2H ion (116) of Gly-Pro (b); the same ions in a mixture of these two dipeptides (c).

Therefore, the identification of iminodipeptides was based on the simultaneous scanning of $[M + H]^+$ ion and $[M + H]^+ - H_2O$, and their retention time.

Selected ion monitoring chromatograms from the simultaneous scanning of the $[M + H]^+$ ion and $[M + H]^+ - H_2O$ ion (Gly-Pro and Pro-Pro) in the serum of a patient with prolidase deficiency are shown in figure 4.

In each chromatogram, the peaks of the $[M + H]^+$ and $[M + H]^+ - H_2O$ ions for Gly-Pro and Pro-Pro have the same retention times as those of standard Gly-Pro and Pro-Pro. These results indicate that the various iminodipeptides in the serum of a patient with iminodipeptiduria can be identified by selected ion monitoring.

The standard curve for different concentrations of Gly-Pro is shown in figure 5. The slope of the curve was linear over the concentration ranges from 100 nmol to 500 nmol.

The recovery of standard iminodipeptides after ion exchange resin treatment was approximately 94%, ranging from 80 to 103%, which indicates that the method is reliable for the measurement of iminodipeptides in biological samples.

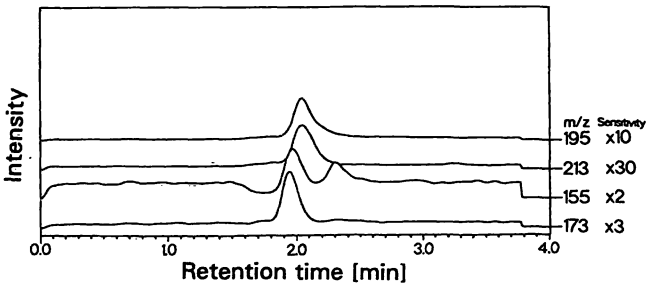

Fig. 4 Selected ion monitoring chromatogram of the $[M + H]$ ion (173) and $[M + H] - H_2O$ ion (155) from Gly-Pro, and the $[M + H]$ ion (213) and $[M + H] - H_2O$ (195) of Pro-Pro in the serum from a patient with prolidase deficiency.

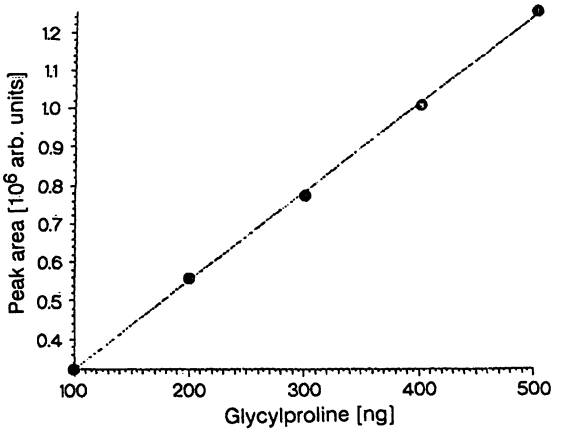

Fig. 5 Calibration curve for Gly-Pro, showing concentrations plotted against peak areas.

Several iminodipeptides (Gly-Pro; m/z 173, Ser-Pro; m/z 203, Pro-Pro; m/z 213, Gly-Hyp(a), Ile-Pro(b), Leu-Pro(c); m/z 229) were found in the serum of a patient with prolidase deficiency, using selected ion monitoring (fig. 6). The resulting chromatogram showed clear peaks of iminodipeptides with no interference from other peaks.

Quantification of various iminodipeptides in sera from patients with prolidase deficiency, their mother and normal subjects, based on peak areas, is shown in table 1.

The iminodipeptides containing C-terminal proline or hydroxyproline (Pro-Hyp) were increased in serum from patients with prolidase deficiency.

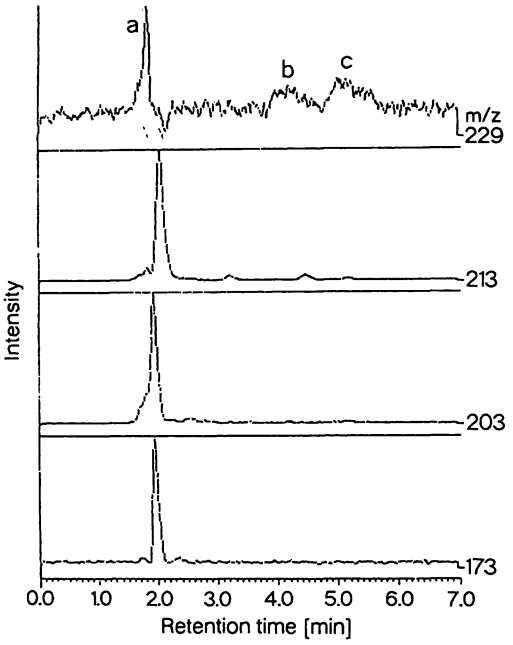

Fig. 6 Selected ion monitoring normalized chromatograms of Gly-Pro (173), Ser-Pro (203), Pro-Pro (213), Pro-Hyp (229-a), Ile-Pro (229-b) and Leu-Pro (229-c) in the serum from a patient with prolidase deficiency.

Tab. 1 Concentrations of various iminodipeptides in the sera of patients with prolidase deficiency, their mother and a control (mean ± SD (n = 3), mg/l).

	Patient A	Patient B	Mother	Control
Gly-Pro	2.77 ± 0.09	2.95 ± 0.22	ND	ND
Ala-Pro	1.56 ± 0.22	1.58 ± 0.17	ND	ND
Val-Pro	2.02 ± 1.38	1.57 ± 0.71	ND	ND
Leu-Pro	2.12 ± 0.53	3.49 ± 0.44	ND	ND
Ile-Pro	1.98 ± 0.55	2.43 ± 0.14	ND	ND
Ser-Pro	2.09 ± 0.28	1.76 ± 0.36	ND	ND
Pro-Pro	5.10 ± 0.79	5.27 ± 0.32	ND	ND
Gly-Hyp	+	+	+	+
Pro-Hyp	1.27 ± 0.32	1.35 ± 0.73	+	+
Met-Pro	ND	ND	ND	ND

ND: not detectable

The X-Pro dipeptides could not be detected in sera from the patients' mother and from normal subjects, but the peaks corresponding to Gly-Hyp and Pro-Hyp were detected on selected ion monitoring chromatograms (fig. 7).

Serum Pro-Hyp in siblings with prolidase deficiency was much higher than in either their mother or normal subjects.

The amounts of Gly-Hyp in serum from patients with prolidase deficiency, and of Gly-Hyp and Pro-Hyp in sera from their mother and normal subjects could not be exactly determined due to interference from other peaks.

Prolidase activities against X-Pro and X-Hyp were determined in erythrocytes from patients, their mother and normal subjects, using the method described previously (11). The prolidase activities of patients towards Gly-Pro and Pro-Pro were very low compared with controls, and the mother had approximately half as much activity as normal subjects, which agreed with a previous report (12). The prolidase activities of patients and normal subjects towards Gly-Hyp and Pro-Hyp were much lower than those towards Gly-Pro and Pro-Pro.

In patients with prolidase deficiency there was a complete deficiency of prolidase I, which shows the highest activity towards Gly-Pro.

Prolidase II, which is active mainly against Met-Pro, shows the same activity in patients as in normal subjects.

Complete digestion of Met-Pro by prolidase II seems to be the reason why Met-Pro cannot be detected at all in serum samples from patients with prolidase deficiency (tab. 1).

The present investigations showed the presence of serum Gly-Hyp and Pro-Hyp not only in the patients, but also in their mother and normal subjects. The concentration of Pro-Hyp in the serum of patients was much higher than in either their mother or controls.

These results suggest that the mother's halved activities can hydrolyse all the iminodipeptides containing a C-terminal proline, which are produced by the metabolism of collagen, so that X-Pro cannot be detected in her urine. However, low prolidase activities towards X-Hyp in the mother and normal volunteers were not sufficient for the hydrolysis of all the endogenous iminodipeptides containing a C-terminal hydroxyproline, so that small amounts of unhydrolysed Gly-Hyp and Pro-Hyp are found in their urines.

This report indicates that LC-APCI-MS (selected ion monitoring mode) is very useful for the detection and

quantification of iminodipeptides in serum, even in very small amounts, and for studying the relationships between clinical symptoms and collagen metabolism in patients with prolidase deficiency.

Acknowledgement

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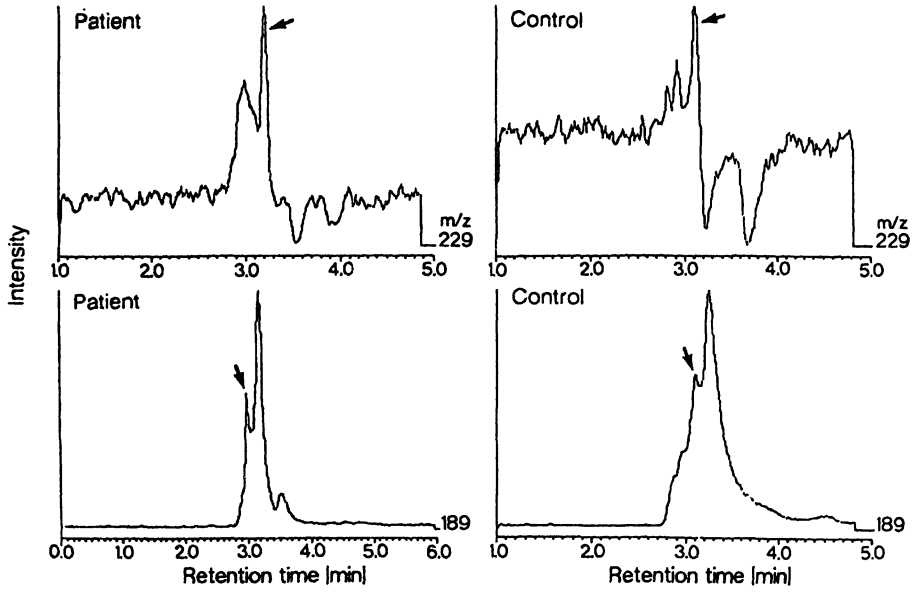

Fig. 7 Selected ion monitoring normalized chromatograms of Gly-Hyp (189) and Pro-Hyp (229) in the serum samples from a patient with prolidase deficiency, and in a control.

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