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Note

Isotachophoretic analysis of iminopeptides in the urine of patients with iminopeptiduria

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Prolidase deficiency is a rare disorder characterized by clinical features such as chronic recurrent infections, mental retardation, splenomegaly, and skin lesions [1]. It has been known that these patients also excrete large amounts of iminopeptides in the urine due to hereditary prolidase deficiency [2—4].

We also reported in a previous paper [4] that a patient with mental retardation and chronic recurrent ulcers on the legs and soles of the feet excreted massive amounts of iminopeptides in her urine. The detection of these iminopeptides has been previously carried out either using an amino acid analyzer, or by paper or thin-layer chromatography [4]. The analysis of some peptides using an isotachophoretic analyzer has been previously reported [5—7], but analysis of iminopeptides has not yet been reported.

It is the purpose of this paper to describe a new, simple and rapid method using isotachophoresis for detecting urinary iminopeptides. The isotachophoretic method presented here has several advantages over previously described techniques. This method can be applied to the screening of patients with prolidase deficiency.

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MATERIALS AND METHODS

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) who were admitted to Okayama University Hospital several years ago. Each of the normal urines and the urines of the patients with prolidase deficiency was directly analyzed by an amino acid analyzer and an isotachophoretic analyzer.

Identification of iminopeptides

A column containing 40 ml of Chelex 100 (Na^+, 100—200 mesh, Bio-Rad) was prepared for the collection of urinary peptides according to the following method. After washing the column with 200 ml of water, 100 ml of a saturated copper sulphate solution were added to the column and allowed to stand overnight. The column was washed well with water and buffered with 0.01 *M* borate buffer (pH 11).

Each 100 ml of the urine samples of two patients and that of a normal human was adjusted to pH 11 with 1 *M* sodium hydroxide, transferred to the buffered Chelex column prepared as above and washed with 100 ml of 0.01 *M* borate buffer (pH 11).

The effluent and washings were combined and evaporated in vacuo below 40°C. The residue containing peptides was dissolved in water, made weakly acidic with 6 *M* hydrochloric acid and filtered. The filtrate was transferred to a column (25 × 2.2 cm) containing 90 ml of Diaion Sk-1 (H^+, 100 mesh, Mitsubishi Kasei Co., Tokyo, Japan), washed with 500 ml of water, and the peptides were then eluted with 2 *M* ammonia. The eluate was evaporated to dryness in vacuo. An aliquot of the residue was analyzed using an amino acid analyzer and an isotachophoretic analyzer.

The rest of the residue was transferred to a column (40 × 1.8 cm) containing 100 ml of Diaion SK-1 (H^+, 100 mesh), washed with water, fractionated with 1100 ml of 0.5 *M* hydrochloric acid and 700 ml of 1 *M* hydrochloric acid, and then washed with water and eluted with 500 ml of 2 *M* ammonia. Each 100 ml of the 0.5 *M* hydrochloric acid and 1 *M* hydrochloric acid eluates, and of the ammonia eluate were evaporated in vacuo below 40°C. The peptides in each fraction were detected by reaction with ninhydrin on paper chromatography. Each of the fractions containing peptides was separated from the others by large-scale paper chromatography (Toyo-Roshi No. 50, 40 × 40 cm) in *n*-butanol—acetic acid—water (4:1:4). All peptides showing the same R_F value were combined, extracted with water, and then evaporated in vacuo.

The peptides contained in the residue were identified according to the following methods. (1) Comparison with authentic samples on isotachophoresis and paper chromatography. (2) Identification of the components of amino acids after hydrolysis of each peptide. (3) Identification the of N-terminal amino acid by dinitrophenylation.

Instrumentation

The capillary apparatus was a Shimadzu IP-1B isotachophoretic analyzer

(Shimadzu Seisakusho, Kyoto, Japan) [8]. The separations were carried out in a capillary tube, 20 × 0.5 cm I.D., which was maintained at a constant temperature of 20°C. The detector cell was 0.5 mm I.D. and 0.05 mm long. The leading electrolyte consisted of 10 m*M* hydrochloric acid and 2-amino-2-methyl-1-propanol (pH 7.50). The terminal electrolyte was 10 m*M* γ-aminobutyric acid and barium hydroxide (pH 10.90). The chart speed was 10 mm/min, migration current was 75 μA.

Reagents

Glycylproline (Gly-Pro) was purchased from Protein Research Foundation, Osaka, Japan. All other chemicals were of analytical grade.

RESULTS AND DISCUSSION

Isotachophoretic analysis of the original urine samples of a normal human and a patient with prolidase deficiency is shown in Fig. 1. The patterns of zones were obviously different between the two samples. The peptide fractions of urine samples of a normal human and of two patients with prolidase deficiency were obtained as described under Materials and methods; isotachophoresis of these peptide fractions is shown in Fig. 2. The isotachophoretic zones detected in the urine of a normal human disappeared after treatment with Chelex-100 and SK-1 (Fig. 2A), but the zones in the urine samples of patients with prolidase deficiency did not disappear (Fig. 2B and C).

The results of analyzing urinary peptides with an amino acid analyzer were also in good agreement with the results obtained using an isotachophoretic analyzer [4]. The results described above indicate that a normal human excretes a very low amount of peptides in the urine; however, patients with

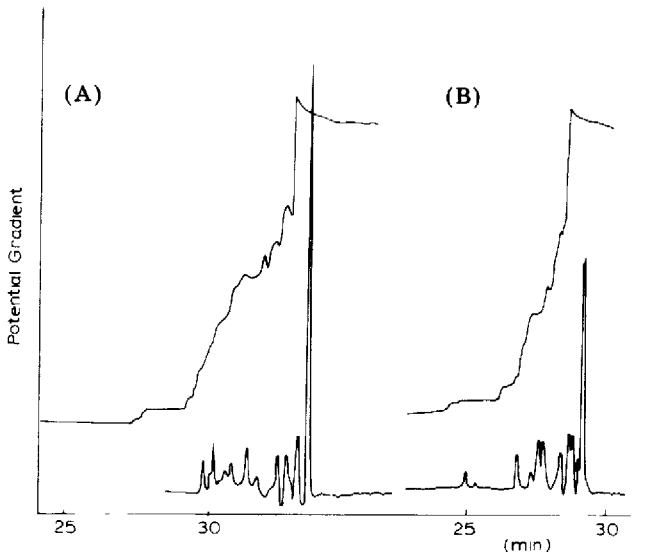

Fig. 1. Isotachophoretic runs of the original urine samples of a patient with prolidase deficiency (A) and of a normal human (B). The leading electrolyte was 0.01 *M* hydrochloric acid and 2-amino-2-methyl-1-propanol (pH 7.50) and the terminal electrolyte was 0.01 *M* γ-aminobutyric acid and barium hydroxide (pH 10.90).

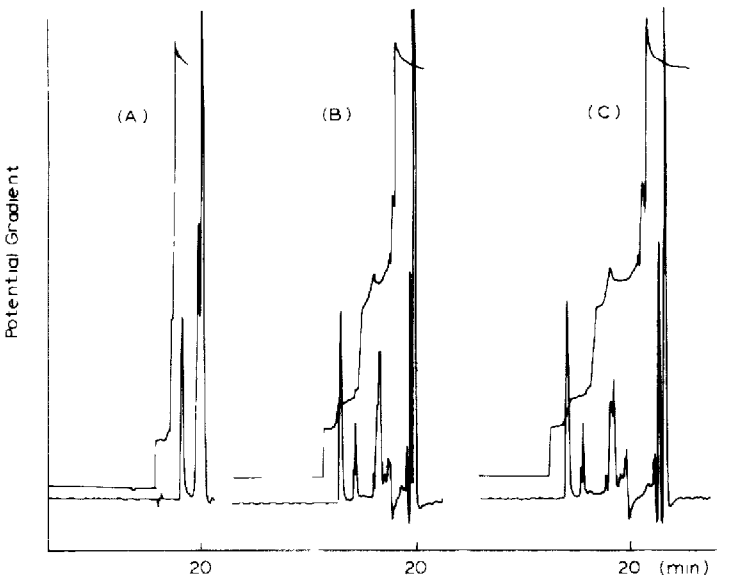

Fig. 2 Isotachophoretic runs of the peptide fractions from the urine of a normal human (A) and patients with prolidase deficiency (B, C). Analytical conditions were as in Fig. 1.

prolidase deficiency excrete massive amounts of peptides in their urine [3, 4].

In order to check the recovery of Gly-Pro during treatment with Chelex-100 and Diaion SK-1, an internal standard of authentic Gly-Pro (10 μmol) was added to the urine samples (2 ml) before chromatography on Chelex-100 and Diaion SK-1. The urine without any addition of Gly-Pro was also processed in parallel. The results indicate a recovery of the added Gly-Pro of about 89.2 ± 6.55% (n = 5).

Each peptide zone from the isotachophoresis was isolated from the urine of the patients, as described under Materials and methods, and identified. The peptides with the same potential gradient as zone c were mainly eluted between 700 and 900 ml of 0.5 M hydrochloric acid and then each peptide in the peptide zone was isolated by paper chromatography. A peptide in zone c was hydrolyzed in 6 M hydrochloric acid, and the hydrolysate dried under reduced pressure. Two amino acids, glycine and proline, were detected in the hydrolysate by paper chromatography and an amino acid analyzer. This peptide also gave the same R_F value as authentic Gly-Pro, and the same potential gradient as Gly-Pro on isotachophoresis, as shown in Fig. 3A and C. This peptide zone on the isotachophoretic chart disappeared upon hydrolysis, and a zone of glycine was newly detected.

Isotachophoretic runs of peptide fractions obtained from the urine of a patient with iminopeptiduria and with Gly-Pro added to the peptide fractions are shown in Fig. 4. Zone c in Fig. 4A and authentic Gly-Pro were made to overlap by adding authentic Gly-Pro to the peptide fractions, resulting in the elongation of zone c as shown in Fig. 4B. These results indicate strongly that zone c of the peptide fractions contains Gly-Pro.

Peptides other than Gly-Pro were also identified by comparison with authentic samples, the determination of the component of amino acids after

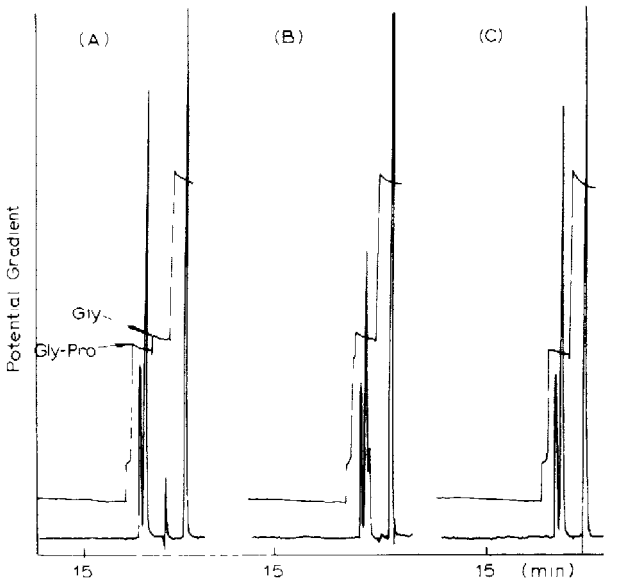

Fig. 3. Isotachophoretic runs of authentic Gly-Pro and Gly (A), the hydrolysate of C (B), and a peptide isolated from peptide fraction (C). Analytical conditions were as in Fig. 1.

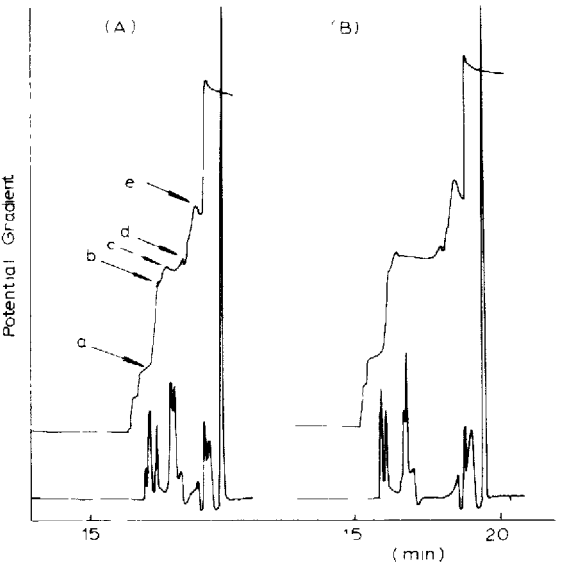

Fig. 4. Isotachophoretic runs of peptide fractions from the urine of a patient with prolidase deficiency (A) and with added authentic Gly-Pro (B). Analytical conditions were as in Fig.1.

hydrolysis of each peptide, and identification of the N-terminal amino acid by dinitrophenylation. It was demonstrated that zone a is a mixture of Glu-Pro and Asp-Pro; zone b, Thr-Pro; zone c, a mixture of Gly-Pro, Leu-Pro, Ileu-Pro, Ala-Pro, Val-Pro, Phe-Pro, Tyr-Pro; zone d, Ser-Pro; and zone e, Pro-Pro. These results indicate that the determination of peptides in each peptide fraction can not be carried out using the isotachophoresis conditions presented

here, but this simple and rapid isotachophoretic method can certainly be applied for the screening of patients with iminopeptiduria.

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