Journal of Chromatography, 310 (1984) 401—406
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2197

Note

Measurement of prolidase activity in erythrocytes using isotachophoresis

HIROAKI MIKASA

Department of Chemistry, Kochi Medical School, Oko-cho, Nankoku-shi, Kochi 781-51 (Japan)

JIRO ARATA

Department of Dermatology, Kochi Medical School, Oko-cho, Nankoku-shi, Kochi 781-51 (Japan)

and

HIROYUKI KODAMA*

Department of Chemistry, Kochi Medical School, Oko-cho, Nankoku-shi, Kochi 781-51 (Japan)

(First received December 28th, 1983; revised manuscript received May 10th, 1984)

Prolidase deficiency with iminopeptiduria is a relatively rare inherited disorder characterized by clinical features such as chronic recurrent infections, mental retardation, splenomegaly, and skin lesion. Since 1968, several cases of prolidase deficiency have been described [1—12].

We also reported in previous papers [7—9] 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 due to hereditary prolidase deficiency.

The measurement of prolidase (EC 3.4.13.9) activity, which hydrolyses dipeptides containing proline or hydroxyproline as the C-terminal amino acid, has been carried out by several methods [8, 11, 13—15].

A new method for the measurement of prolidase activity has been developed using an isotachophoretic analyser [16—19], and carried out by measuring simultaneously the substrate, glycine-proline (Gly-Pro), and product, glycine

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(Gly), in the reaction mixture. This method was also compared with Chinard's method [15] in which he measured the enzyme activity by determining proline in the reaction mixture. The results obtained with isotachophoresis and Chinard's method agree well.

The isotachophoretic method presented here has several advantages over previously described techniques, and has been applied to the measurement of prolidase activity in erythrocytes from a patient with iminopeptiduria and her mother.

MATERIALS AND METHODS

Materials

Gly-Pro, Gly-Hyp, Val-Pro and Leu-Pro (Gly = glycine, Pro = proline, Hyp = hydroxyproline, Val = valine, Leu = leucine) were obtained from Sigma. All other chemicals used were of analytical grade. Venous blood was taken into heparinized tubes from a patient with iminopeptiduria, her mother, and rat. The heparinized blood was mixed with an equal volume of 6% dextran (molecular weight 200,000—300,000; Wako, Osaka, Japan) in saline. After standing for 60 min at room temperature, the upper suspension of leucocytes was separated carefully from the underlayer of erythrocytes. Erythrocytes were washed three times with 2 vols. of physiological saline. Washed erythrocytes were haemolysed by repeated freezing and thawing. The lysate was dialyzed overnight at 4° C against 0.05 *M* Tris—HCl buffer, pH 7.4.

Preincubation

Erythrocyte lysate was diluted 1:10 (human lysate) and 1:2 (rat lysate) with 0.05 *M* Tris—HCl buffer (pH 7.4) containing 1 m*M* manganese chloride and preincubated for 1 h at 37° C

Enzymatic reaction

A 0.5-ml volume of substrate solution containing 1 m*M* manganese chloride and 10 m*M* iminopeptide in 0.05 *M* Tris—HCl (pH 7.4) was added to an aliquot of 0.5 ml of preincubated lysate and incubated for 30 min at 37° C. The reaction was stopped by heating for 5 min in boiling water. After centrifugation the supernatant was used for estimation of Gly-Pro, glycine and proline. A blank was run under the same conditions. Proline was determined by Chinard's method. Gly-Pro and glycine were simultaneously determined using an isotachophoretic analyser.

Instrumentation

The capillary apparatus was a Shimadzu IP-1B isotachophoretic analyser (Shimadzu, Kyoto, Japan). 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 had an I.D. of 0.5 mm and length 0.05 mm. The leading electrolyte consisted of 10 m*M* hydrochloric acid and 2-amino-2-methyl-1-propranol (pH 7.5). 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.

RESULTS AND DISCUSSION

Isotachophoretic analyses of authentic Gly-Pro, Gly-Hyp, glycine and proline were carried out under the conditions described in Materials and methods. The two zones of Gly-Pro and Gly-Hyp overlapped under the analytical conditions and were detected as the same zone; proline could not be detected. However, it has been ascertained that Gly-Pro, glycine and hydroxyproline are easily

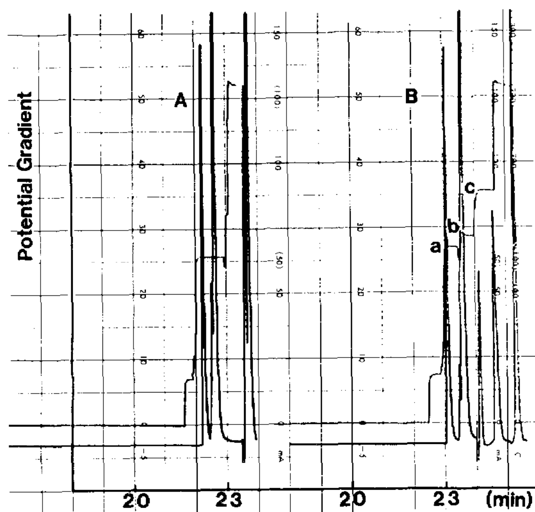

Fig. 1. Isotachophoretic runs of authentic Gly-Pro (A), Gly-Hyp (B, a), glycine (B, b) and Hyp (B, c). Analytical conditions were as follows. The leading electrolyte was 0.01 *M* hydrochloric acid and 2-amino-2-methyl-1-propanol (pH 7.5). The terminal electrolyte was 0.01 *M* γ-aminobutyric acid and barium hydroxide (pH 10.9). The chart speed was 10 mm/min. The migration current was 75 μA.

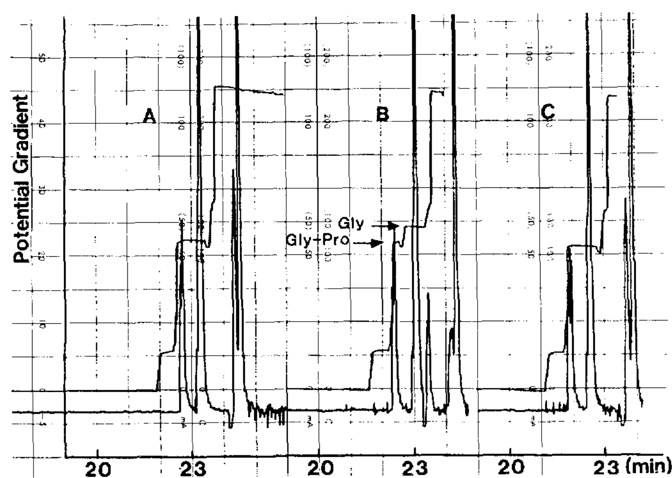

Fig. 2. Isotachophoretic runs of reaction mixture of the mother's erythrocyte lysate at 0 min (A) and 60 min (B) incubation time and of the erythrocyte lysate of a patient with iminopeptiduria at 60 min incubation time (C). A 4-μl volume of each reaction mixture was analysed using an isotachophoretic analyser under the same conditions as in Fig. 1.

separated as shown in Fig. 1. Therefore, prolidase activity in erythrocytes using isotachophoresis was measured by determining Gly-Pro as the substrate and glycine as the product in the reaction mixture.

Isotachophoretic runs of prolidase activity in erythrocytes of a patient with iminopeptiduria and her mother are shown in Fig. 2.

The rate of hydrolysis of Gly-Pro in erythrocyte lysate from the mother was about 70% with an incubation time of 60 min under our experimental conditions (Fig. 2B). On the other hand, no prolidase activity in the erythrocyte

TABLE I

COMPARISON OF PROLIDASE ACTIVITY IN RED CELLS OF A PATIENT WITH IMINOPEPTIDURIA AND OF HER MOTHER, DETERMINED BY CHINARD'S METHOD AND AN ISOTACHOPHORETIC ANALYSER

Values of proline, glycine and Gly-Pro represent mean ± S.D. (μmol/ml) obtained in each experiment (n = 3).

Incubation time (min)	Chinard's method		Isotachophoresis			
	Proline		Glycine		Gly-Pro	
	Patient	Mother	Patient	Mother	Patient	Mother
0	0	0	0	0	5.0	5.0
15	0	1.31 ± 0.14	0	1.43 ± 0.14	4.98 ± 0.01	3.31 ± 0.11
30	0	2.06 ± 0.12	0	2.05 ± 0.09	4.97 ± 0.01	2.73 ± 0.08
60	0	3.08 ± 0.08	0	3.31 ± 0.10	4.95 ± 0.01	1.56 ± 0.04

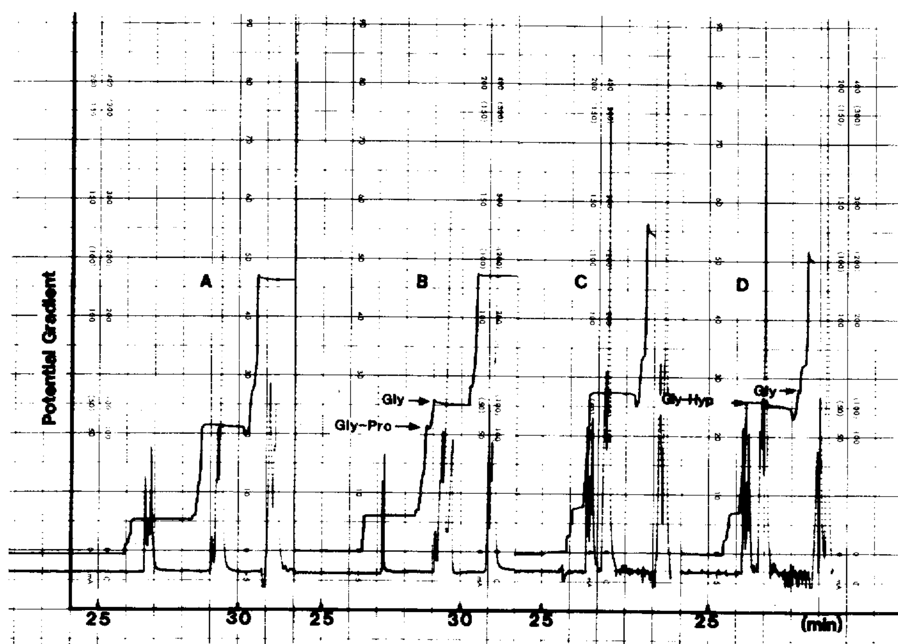

Fig. 3. Isotachophoretic runs of a reaction mixture of rat erythrocyte lysate. Gly-Pro (5 mM; A and B) and Gly-Hyp (5 mM; C and D) were used as substrates. Incubation times were 0 min (A and C) and 60 min (B and D).

lysate from the patient with iminopeptiduria was found (Fig. 2C). These results agree well with results reported previously by Umemura [8].

A comparison of the determination of prolidase activity in erythrocyte lysates from a patient with iminopeptiduria and from her mother using Chinard's method and the isotachophoretic analyser is shown in Table I. The two methods gave almost the same values, and the increase in glycine and the decrease in Gly-Pro determined by the isotachophoretic analyser also gave a good agreement. These results indicate that this method can be suitably utilized for the measurement of enzyme activity such as prolidase in erythrocytes.

In addition to Gly-Pro, several iminopeptides (Gly-Hyp, Leu-Pro, Val-Pro) were tested as substrate for prolidase in rat erythrocytes (Figs. 3 and 4).

Isotachophoretic runs of Gly-Pro and Gly-Hyp to determine prolidase activity in rat erythrocytes are shown in Fig. 3. When Gly-Pro was used as substrate, it was almost completely hydrolysed during the incubation time of 60 min, but hydrolysis of Gly-Hyp was about 6% that of Gly-Pro, as reported previously by Myara et al. [20].

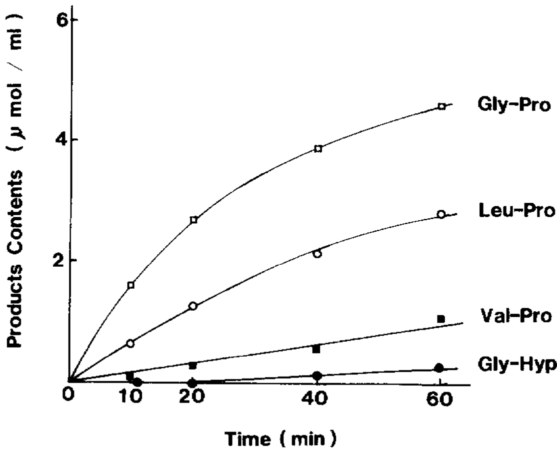

Fig. 4. The relationship between incubation time and several iminopeptides on prolidase of rat erythrocyte lysate. The reaction conditions are described in Materials and methods.

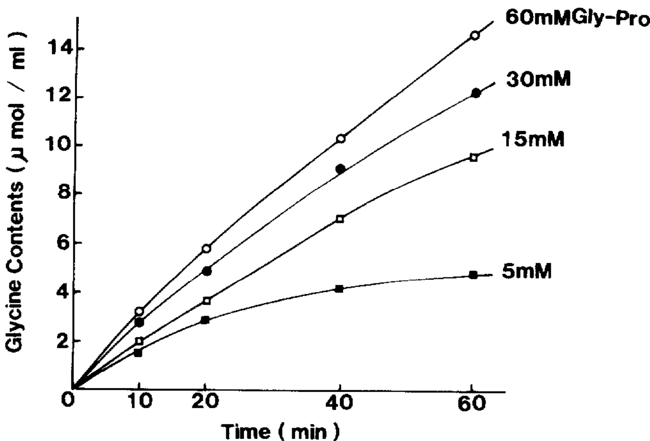

Fig. 5. Studies of incubation time at several Gly-Pro concentrations. The reaction conditions are described under Materials and methods.

The results of studies of incubation time at several concentrations of Gly-Pro are shown in Fig. 5. Under the conditions used, the release of glycine and proline by prolidase of rat erythrocytes, to a final Gly-Pro concentration of 60 m*M*, was linear up to 60 min incubation time.

The isotachophoresis presented here can simultaneously estimate both substrate and product, and was very useful in measuring prolidase activity in erythrocytes of a patient with iminopeptiduria and of a normal human.

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