Journal of Chromatography, 343 (1985) 179—185
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2677

Note

Simultaneous measurement of prolidase and prolinase activities in erythrocytes using an isotachophoretic analyser

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(First received February 5th, 1985; revised manuscript received April 2nd, 1985)

Iminopeptiduria is a relatively rare inherited disorder characterized by clinical features such as chronic recurrent infection, mental retardation, splenomegaly and skin lesions. Since 1968, general cases of prolidase deficiency have been described [1—12]. We have also reported [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 owing to hereditary prolidase deficiency. Measurements of prolidase and prolinase activities have been carried out by several methods including the amount of bound peptide which disappeared at 220 nm [13], and spectrophotometric determination of glycine [11] and proline by Chinard's method [8, 14, 15]. However, it was impossible to measure simultaneously both prolidase and prolinase activities by using the above methods.

In a recent paper, we reported that prolidase activity in human erythrocytes

0378-4347/85/$03.30

could be determined by using an isotachophoretic analyser [16]. In this paper, a method is described for the simultaneous measurement of prolidase and prolinase activities in erythrocytes from a patient with iminopeptiduria and her mother involving the use of an isotachophoretic analyser [17—20]. The measurement of prolidase and prolinase activities was carried out by measuring simultaneously the lengths of two zones of H-Gly—Pro-OH and H-Pro- Gly-OH on the isotachopherogram. The sum of the decrease in the two zones on the isotachopherogram agreed well with the increase in the zone of Gly that appeared after the enzymatic reactions.

This method was also compared with Chinard's method [15], in which the enzyme activity was measured by determination of proline in the reaction mixture. The results obtained by the two methods agreed well.

Prolidase activity in erythrocytes from the patient with iminopeptiduria could not be detected using isotachophoresis owing to prolidase deficiency, but prolinase activity could easily be measured. On the other hand, prolidase and prolinase activities in erythrocytes from the patient's mother could be measured simultaneously.

The isotachophoretic method presented here is the first description of the use of isotachophoresis for the simultaneous measurement of prolidase and prolinase activities in erythrocytes.

EXPERIMENTAL

Materials

H-Gly—Pro-OH, H-Pro—Gly-OH and Gly were obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical-reagent grade.

Venous blood was taken into heparinized tubes from a patient with iminopeptiduria and her mother. 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 lower layer of erythrocytes. Erythrocytes were washed three times with two volumes of physiological saline.

The washed erythrocytes were haemolysed by repeated freezing and thawing. The lysate was dialysed overnight at 4°C against 0.05 *M* Tris—HCl buffer (pH 7.4).

Enzymatic reactions

Erythrocyte lysate was diluted 1:10 with 0.05 *M* Tris—HCl buffer (pH 7.4) containing 1 m*M* manganese chloride and preincubated for 1 h at 37°C. A 500-μl volume of substrate solution (H-Gly- Pro-OH and H-Pro—Gly-OH) containing 1 m*M* manganese chloride and 10 m*M* of iminopeptide in 0.05 *M* Tris—HCl buffer (pH 7.4) was added to an aliquot of 0.5 ml of preincubated lysate containing 60—70 mg of protein and incubated at 37°C for 10, 20, 40 and 60 min for the measurement of prolidase activity and for 60 and 120 min for the measurement of prolinase activity. The reaction was stopped by heating for 2 min in boiling water. The reaction solution was centrifuged for 10 min at 1600 *g* and H-Gly—Pro-OH, H-Pro—Gly-OH and Gly in the supernatant were

simultaneously determined using an isotachophoretic analyser. Proline in the supernatant was determined by Chinard's method.

Instrumentation

The capillary apparatus was a Shimadzu IP-1B isotachophoretic analyser (Shimadzu Seisakusho, Kyoto, Japan). The separations were carried out in a capillary tube (200 × 0.5 mm I.D.), which was maintained at a constant temperature of 20°C. The detector cell had an I.D. of 0.5 mm and a length of 0.05 mm. The leading electrolyte was 10 m*M* HCl [containing 0.05% of poly(vinyl alcohol)] adjusted to pH 6.0 with 2-amino-2-methyl-1-propanol. The terminating electrolyte was 10 m*M* γ-aminobutyric acid adjusted to pH 10.9 with $Ba(OH)_2$. The chart speed was 10 mm/min and the migration current was 75 μA.

RESULTS AND DISCUSSION

The measurement of prolidase activity in erythrocytes from the patient with iminopeptiduria and her mother was reported in a previous paper [16].

Isotachophoretic determinations of prolinase activity in erythrocytes from the patient with iminopeptiduria are shown in Fig. 1. These figures indicate that the substrate, H-Pro–Gly-OH, is degraded by prolinase with increasing incubation time, giving Gly.

A comparison of prolinase activity in erythrocytes from the patient with iminopeptiduria and her mother determined by Chinard's method and deter-

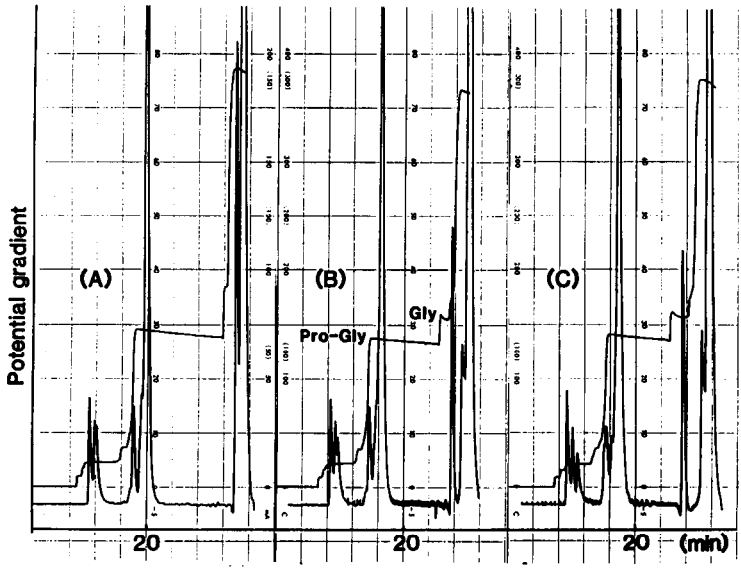

Fig. 1. Isotachophoretic runs for the determination of prolinase activity in erythrocytes from the patient with iminopeptiduria at (A) 0 min, (B) 60 min and (C) 120 min. The initial concentration of H-Gly–Pro-OH was 10 m*M*. The leading electrolyte was 0.01 *M* HCl containing 0.05% of poly(vinyl alcohol)] adjusted to pH 6.0 with 2-amino-2-methyl-1-propanol. The terminating electrolyte was 10 m*M* GABA adjusted to pH 10.9 with $Ba(OH)_2$. The chart speed was 10 mm/min and the migration current was 75 μA.

TABLE I

COMPARISON OF PROLINASE ACTIVITY IN ERYTHROCYTES OF THE PATIENT WITH IMINOPEPTIDURIA AND HER MOTHER DETERMINED BY CHINARD'S METHOD AND USING THE ISOTACHOPHORETIC ANALYSER

The proline, glycine and Pro—Gly values are means ± S.D. (μmol/ml) obtained in each experiment (n = 5).

Incubation time (min)	Chinard's method		Isotachophoresis			
	Proline values		Glycine values		Pro—Gly values	
	Patient	Mother	Patient	Mother	Patient	Mother
0	0.00	0.00	0.00	0.00	5.00	5.00
5	0.19 ± 0.04	0.17 ± 0.02	0.20 ± 0.03	0.14 ± 0.06	4.80 ± 0.04	4.85 ± 0.03
10	0.40 ± 0.03	0.29 ± 0.03	0.35 ± 0.05	0.24 ± 0.04	4.66 ± 0.05	4.75 ± 0.08
20	0.59 ± 0.03	0.40 ± 0.08	0.55 ± 0.04	0.32 ± 0.07	4.48 ± 0.04	4.65 ± 0.09
30	0.69 ± 0.04	0.45 ± 0.06	0.66 ± 0.07	0.35 ± 0.08	4.38 ± 0.04	4.62 ± 0.04

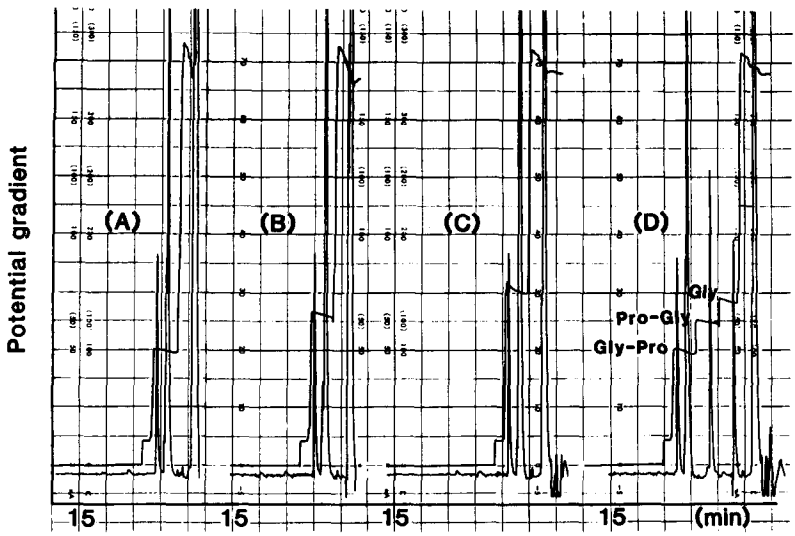

Fig. 2. Isotachophoretic runs on (A) authentic H-Gly—Pro-OH, (B) H-Pro—Gly-OH, (C) Gly and (D) a mixture of H-Gly—Pro-OH, H-Pro—Gly-OH and Gly. Analytical conditions as in Fig. 1.

mined with the isotachophoretic analyser is shown in Table I. The two methods gave almost identical values, and the increase in glycine and the decrease in H-Pro—Gly-OH measured by isotachophoresis also agreed well. These results indicate that the proposed method is suitable for the measurement of enzyme activity such as prolinase in erythrocytes.

Isotachophoresis of the substrates of prolidase and prolinase, H-Gly—Pro-OH and H-Pro—Gly-OH, and the product, Gly, was carried out under the conditions described under Experimental. When each of H-Gly—Pro-OH, H-Pro—Gly-OH and Gly were subjected separately to isotachophoresis, good separations were obtained (Fig. 2A—C), and analysis of the mixture of H-Gly—Pro-OH,

H-Pro—Gly-OH and Gly also gave a good separation (Fig. 2D). However, proline could not be detected, as shown in Fig. 2. The values of potential units of the three compounds on the isotachopherogram were H-Gly—Pro-OH 0.30, H-Pro—Gly-OH 0.38 and Gly 0.44. These results suggest that the simultaneous measurement of prolidase and prolinase activities in erythrocytes using isotachophoresis could be carried out by determining simultaneously the amounts of H-Gly—Pro-OH and H-Pro—Gly-OH in the incubation mixture.

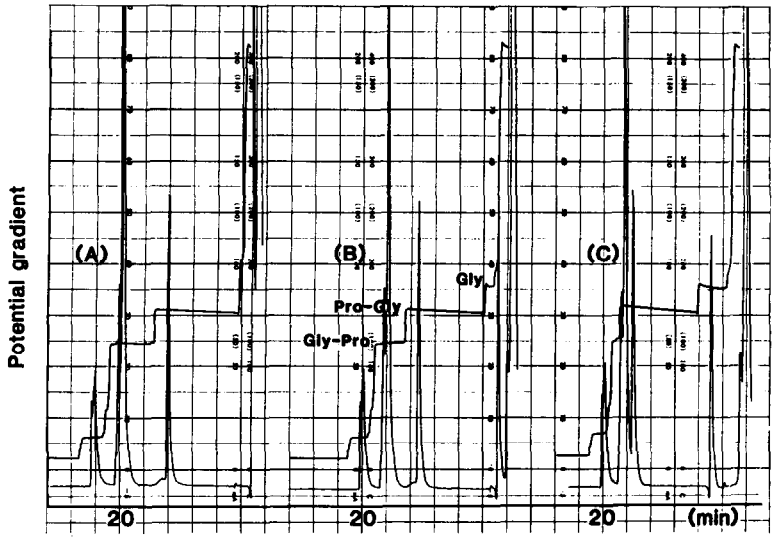

Fig. 3. Isotachophoretic runs for the simultaneous determination of prolidase and prolinase activities in erythrocytes from the mother of the patient with iminopeptiduria at (A) 0 min, (B) 30 min and (C) 60 min. The initial concentrations of H-Gly—Pro-OH and H-Pro- Gly-OH were 5 and 10 m*M*, respectively. Analytical conditions as in Fig. 1.

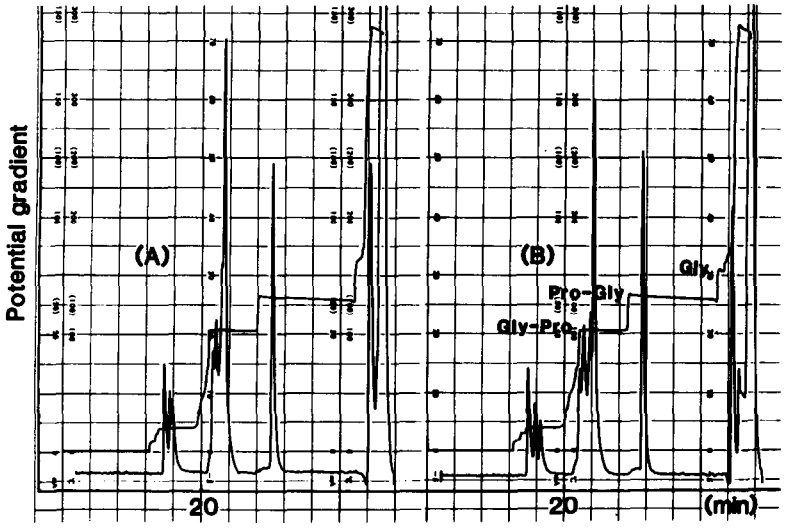

Fig. 4. Isotachophoretic runs for the simultaneous determination of prolidase and prolinase activities in erythrocytes from the patient with iminopeptiduria at (A) 0 min and (B) 60 min. Analytical conditions as in Fig. 1.

Isotachophoretic runs for the simultaneous measurement of prolidase and prolinase activities in erythrocytes from the mother of the patient with iminopeptiduria are shown in Fig. 3. The initial concentrations of H-Gly—Pro-OH and H-Pro—Gly-OH in the reaction mixture were 5 and 10 m*M*, respectively (Fig. 3A). The zone lengths of H-Gly—Pro-OH and H-Pro—Gly-OH on the isotachopherogram decreased with increasing incubation time, and an increase in the zone length of Gly was observed (Fig. 3B and C), with a decrease in H-Pro—Gly-OH.

Isotachophoretic runs for the simultaneous measurement of prolidase and prolinase activities in erythrocytes from the patient with iminopeptiduria are shown in Fig. 4. Together with the decrease in the zone length of H-Pro—Gly-OH, a zone of Gly appeared at an incubation time of 60 min, but the zone length of H-Gly—Pro-OH did not show the significant change due to prolidase deficiency.

The concentrations of H-Gly—Pro-OH, H-Pro—Gly-OH and Gly in the reaction mixtures of erythrocytes from the patient with iminopeptiduria and from her mother determined by using the isotachophoretic analyser are shown in Table II. No decrease in H-Gly—Pro-OH in the reaction mixture of erythrocytes from the patient with iminopeptiduria could be detected, but the content of H-Pro—Gly-OH decreased with increasing incubation time. On the

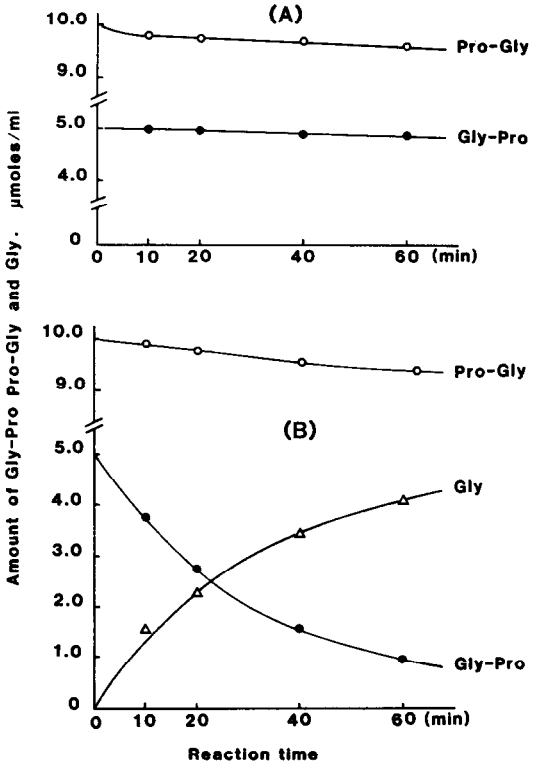

Fig. 5. Relationship between the incubation time and prolidase and prolinase activities in erythrocytes from (A) the patient with iminopeptiduria and (B) her mother. The initial concentrations of H-Gly—Pro-OH and H-Pro—Gly-OH and the analytical conditions are as in Fig. 4.

TABLE II

SIMULTANEOUS MEASUREMENT OF PROLIDASE AND PROLINASE ACTIVITIES IN ERYTHROCYTES FROM THE PATIENT WITH IMINOPEPTIDURIA AND HER MOTHER DETERMINED USING THE ISOTACHOPHORETIC ANALYSER

The Gly-Pro, Pro-Gly and Gly values are means ± S.D. (μmol/ml) obtained in each experiment (n = 4).

Incubation time (min)	Gly-Pro values		Pro-Gly values		Gly values	
	Patient	Mother	Patient	Mother	Patient	Mother
0	5.00	5.00	10.00	10.00	0.00	0.00
10	4.95 ± 0.04	3.76 ± 0.15	9.84 ± 0.01	9.84 ± 0.08	0.15 ± 0.03	1.31 ± 0.07
20	4.93 ± 0.03	2.90 ± 0.48	9.79 ± 0.15	9.80 ± 0.15	0.20 ± 0.08	2.24 ± 0.22
40	4.90 ± 0.02	1.75 ± 0.24	9.74 ± 0.24	9.76 ± 0.09	0.25 ± 0.06	3.51 ± 0.43
60	4.90 ± 0.02	1.15 ± 0.21	9.68 ± 0.27	9.70 ± 0.12	0.35 ± 0.04	4.25 ± 0.46

other hand, both H-Gly—Pro-OH and H-Pro—Gly-OH in the reaction mixture of erythrocytes from the mother decreased with increasing incubation time, and the sum of the decreases in H-Gly—Pro-OH and H-Pro—Gly-OH degraded by the enzyme reactions was almost the same as the increase in Gly. The results given in Table II are shown in Fig. 5A and B.

The results of the enzymatic reactions in the patient with iminopeptiduria and her mother presented above indicate that the simultaneous measurement of prolidase and prolinase activities in erythrocytes could be measured by using an isotachophoretic analyser. The isotachophoretic method presented here can simultaneously determine both prolidase and prolinase activities, and is more sensitive and simpler than the methods described previously.

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