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Direct monitoring of prolidase activity in cultured skin fibroblasts using capillary electrophoresis

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Abstract

Capillary electrophoresis (CE) was used as an alternative to current analysis schemes for detecting prolidase activity in erythrocytes and skin fibroblast cultures because of its unique selectivity and high resolving power. Kinetic measurement of peptide bond hydrolysis was performed using porcine kidney prolidase on different substrates (Gly–Pro, Leu–Pro and Ala–Pro) and by following the disappearance of the peptide–substrate's peak. The K_m values obtained were in agreement with those previously reported. Interestingly, in the case of Phe–Pro as the substrate, simultaneous analysis of the product and parent peptide was possible, thus showing the superiority of the capillary electrophoresis (CE) assay with respect to the standard spectrophotometric method. The application of the CE technique to the characterization of prolidase activity in control and prolidase-deficient skin cultured fibroblasts was successful. Enzyme activity was easily calculated in all controls tested and the K_m values determined were slightly lower than those obtained with the colorimetric reaction, thus confirming our assumption that the CE assay shows higher specificity than the ninhydrin technique. Our results demonstrate the feasibility of using CE as a simple and reliable technique for determining prolidase activity.

Keywords: Prolidase; Enzymes

1. Introduction

Prolidase (EC 3.4.13.9) is an ubiquitous mammalian dipeptidase that plays a role in the final stages of the catabolism of both exogenous and endogenous protein [1–4]. The enzyme specifically hydrolyses aminoacylprolines and hydroxyprolines to their constituent amino acids [5]. Its essential role in protein metabolism is shown by the dramatic clinical consequences affecting patients with prolidase deficiency (PD), a severe disorder in which deficient activity of prolidase results in a toxic build-up of the C-terminal proline- and hydroxyproline-containing dipeptides [6–8].

Chronic, recurrent leg ulceration is the main clinical symptom associated with the disorder, while massive imidodipeptiduria is the most important biological symptom [9–14]. High-performance liquid chromatography (HPLC) [15] or capillary zone electrophoresis (CZE) [16], used for determination and identification of urinary imidodipeptides in PD patients, are powerful methods for diagnostically screening for this disease. However, since individuals with massive imidodipeptiduria do not necessarily have PD [17] and as heterozygotes do not

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appear to have increased imidodipeptiduria [18,19], more reliable diagnosis may be obtained by assaying prolidase activity in erythrocytes, leukocytes or in skin fibroblast cultures and the plasma of patients [20,21]. Several techniques are currently available for detecting prolidase activity, including high-voltage electrophoresis [13,22] and quantitative ion-exchange chromatography [14,23]. The determination of prolidase activity, however, is mainly performed using methods based on the colorimetric reaction of proline using Chinard's reagent [24], following hydrolysis of X–Pro peptide substrate, and continuous spectrophotometric analysis, by following the decrease in light absorption at low wavelength as the peptide bond is hydrolysed [25,26]. Chinard's original method [24], modified by Myara et al. [27] to increase its sensitivity, was later successfully applied to the determination of prolidase activity in erythrocyte haemolysates and dermal fibroblasts.

However, although the sensitivity of the assay was improved, the method continues to be time-consuming and the presence of other amino acids and of the reduced form of glutathione can interfere with the colorimetric reaction. Conversely, the spectrophotometric method cannot be applied to substrates such as Phe–Pro because of interference by phenylalanine absorption [25].

The need for greater specificity and sensitivity led us to develop a capillary electrophoretic assay as an attractive alternative to current analysis schemes. Due to the low amount of sample required and the short migration times, CE appeared to be ideally suited to monitoring small peptides such as the prolidase substrates, X–Pro. The purpose of this paper is to demonstrate the feasibility of CE as a technique for the characterization of activity of this particular proteolytic enzyme, and we show that CE can be used to obtain activity profiles for prolidase.

Kinetic measurements of peptide bond hydrolysis were performed on different substrates by following the disappearance of the peptide–substrate's peak and, in the case of Phe–Pro, simultaneous analysis of the product and parent peptide was possible. Comparison of the results obtained using the standard colorimetric assay permits us to establish that CE is superior to other methods, in terms of accuracy, speed and specificity.

2. Experimental

2.1. Chemicals

Porcine kidney prolidase was obtained as an ammonium sulfate suspension from Sigma (St. Louis, MO, USA). Manganese chloride ($MnCl_2$) and glutathione, reduced form (GSH), were purchased from Merck (Darmstadt, Germany). All X–Pro peptides were purchased from Sigma.

The distilled water that was used in the preparation of all solutions was purified in a Milli-Q apparatus (Millipore, Bedford, MA, USA). All buffers and samples were filtered through 0.45-μm filters (Millipore) before use.

2.2. Preparation of standard curves

X–Pro dipeptide–substrate standard solutions were prepared from 50 m*M* stock solutions by diluting with water to give concentrations in the range 0.1–30 m*M* for CE analyses and 20–40 m*M* for the colorimetric assay. The conditions of analysis for CE experiments are described below.

2.3. Activation of porcine kidney prolidase and kinetic analyses

Porcine kidney prolidase, which was used to determine the peptide bond cleavage rate, was activated using the following procedure. The enzyme (50 μl of a suspension containing 12.7 mg/ml, 170 U/mg) was added to 1.45 ml of a solution (freshly prepared) containing 1.2 ml of 50 m*M* Tris at pH 8.00, 0.2 ml of 200 m*M* $MnCl_2$ and 0.05 ml of 30 m*M* GSH. The mixture was incubated for 45 min at 37°C, after which time, aliquots (300 μl) were taken and added to 100 μl of a peptide–substrate solution (final concentration of X–Pro substrate, 20 m*M*). At various time points, 100-μl volumes were withdrawn, the reaction was stopped by addition of 0.45 *M* trichloroacetic acid (5 μl), and then the reaction mixtures were centrifuged at 5000 *g* for 5 min. Supernatants were submitted to capillary electrophoretic analysis.

2.4. Preparation of cultured skin fibroblasts

Skin biopsies from five controls and four PD patients (two siblings C.M. and C.F., aged fourteen and eighteen years, and two unrelated individuals C.Ma. and G.D., aged 31 and 55 years, respectively) were obtained after informed consent. Fibroblast cultures were established and grown as previously reported [28]. At confluence, media were removed, cell layers were washed with phosphate buffered saline (PBS) and then scraped into a small volume of 0.05 *M* Tris–HCl, pH 7.8. After centrifugation at 2000 *g* for 5 min, the pellet was resuspended in the same buffer. The cell extract obtained after centrifugation at 15 000 *g* for 5 min was then activated as already reported for porcine kidney prolidase (Section 2.3) and finally incubated with the various peptide substrates. Protein concentration was determined by the method of Lowry et al. [29].

2.5. Colorimetric assay of prolidase activity

The content of proline released by dipeptide hydrolysis was determined using Chinard's method [24], measuring the absorbance at 515 nm. One unit of prolidase activity is defined as the amount of the enzyme that hydrolyses 1 μmol of X–Pro per min at 37°C.

2.6. Capillary electrophoresis equipment and running conditions

All experiments were performed with a P/ACE 2100 instrument (Beckman, Palo Alto, CA, USA). For these studies, the capillary cartridge used was fitted with a 75 μm I.D. untreated fused-silica column with a total length of 57 cm (50 cm from the inlet to the detector). The temperature was maintained at 25±0.1°C by means of a cooling liquid circulating continuously through the cartridge. Samples were injected by pressure, and the injected amounts were quantified using the Poiseuille equation. On-column detection was performed by measuring UV absorbance at 200 nm. Runs were carried out in 35 m*M* sodium tetraborate buffer (pH 9.3) containing 65 m*M* SDS, at a constant voltage of 20 kV. After every 5–10 injections, the capillary used was rinsed with water, 0.1 *M* NaOH, water and, finally, with the running buffer. Washing was applied for 5 min. Migration was positive to negative polarity.

2.7. Sequence analysis

Sequence analyses were performed on the HP G 1005 A protein sequencing system (Hewlett-Packard, Palo Alto, CA, USA) using the routine 3.0 chemistry, according to the manufacturer's protocol as previously described [16].

3. Results and discussion

The feasibility of using CE as a technique for the characterization of enzyme activity was first reported by Krueger et al. [30]. They used CZE to determine the action of endoproteinase Arg-C on adrenocorticotrophic hormone. Using the same CE mode, Mulholland et al. [31] monitored the activity of a tripeptidase from a crude extract of a strain of *Lactococcus lactis*, Okafo et al. [32] studied the mechanism of hydrolysis of a β-lactamase inhibitor and Bao and Regnier [33] and Wu and Regnier [34] used CE to perform enzyme micro- or ultramicro assays.

In all cases, CE proved to be superior to other methods in terms of sensitivity, specificity and speed and was ideal for the identification of the reaction products.

We were interested in finding a method to increase the sensitivity and to overcome the interferences caused by the ninhydrin reaction in the colorimetric prolidase assay. We believed that the peculiar properties of CE could also provide new opportunities for the kinetic analysis of prolidase activity in normal and prolidase-deficient skin fibroblast cultures.

The calibration curve for the substrate Gly–Pro was obtained by injecting different amounts of dipeptide onto a 75 μm I.D. uncoated fused-silica capillary, with a total length of 57 cm (50 cm from the inlet to the detector) using 35 m*M* sodium tetraborate containing 65 m*M* SDS as the run buffer. The response increased linearly with peptide concentration in the range 0.1–30 m*M* and r^2 for the

calibration curve was calculated to be 0.998. The same behaviour was shown by other commonly used dipeptides i.e. Leu–Pro, Ala–Pro and Phe–Pro (data not shown).

In an effort to establish a suitable CE assay for prolidase from skin fibroblast cultures we used commercial porcine kidney prolidase to verify the feasibility of using CE to measure the rates of imidodipeptide bond cleavage. The enzyme (170 U/mg) was activated as reported in Section 2 and was incubated at a concentration of 0.25 μ*M*, with saturating concentrations of the substrate Gly–Pro (the final concentration of substrate in the assay was 20 m*M*). At suitable time intervals, samples were analyzed (Fig. 1A) and plots of peptide concentration vs. reaction time allowed us to determine the rates of peptide cleavage. As shown in Fig. 1B, a linear, time-dependent decrease in concentration was evident for the dipeptide tested. From the time response curve, it was found that the half-life for the disappearance of Gly–Pro was 4.96 min. Peptide quantities in cleavage reactions were determined from peak areas that were converted to molar quantities by reference to peak areas obtained from injections of standard peptide solutions. The units of enzyme activity estimated from the plot of Fig. 1B correlated well with those actually used for the cleavage reaction of the substrate Gly–Pro in a range of enzyme concentrations between 50 n*M* and 2 μ*M*. The enzyme units calculated from the difference in

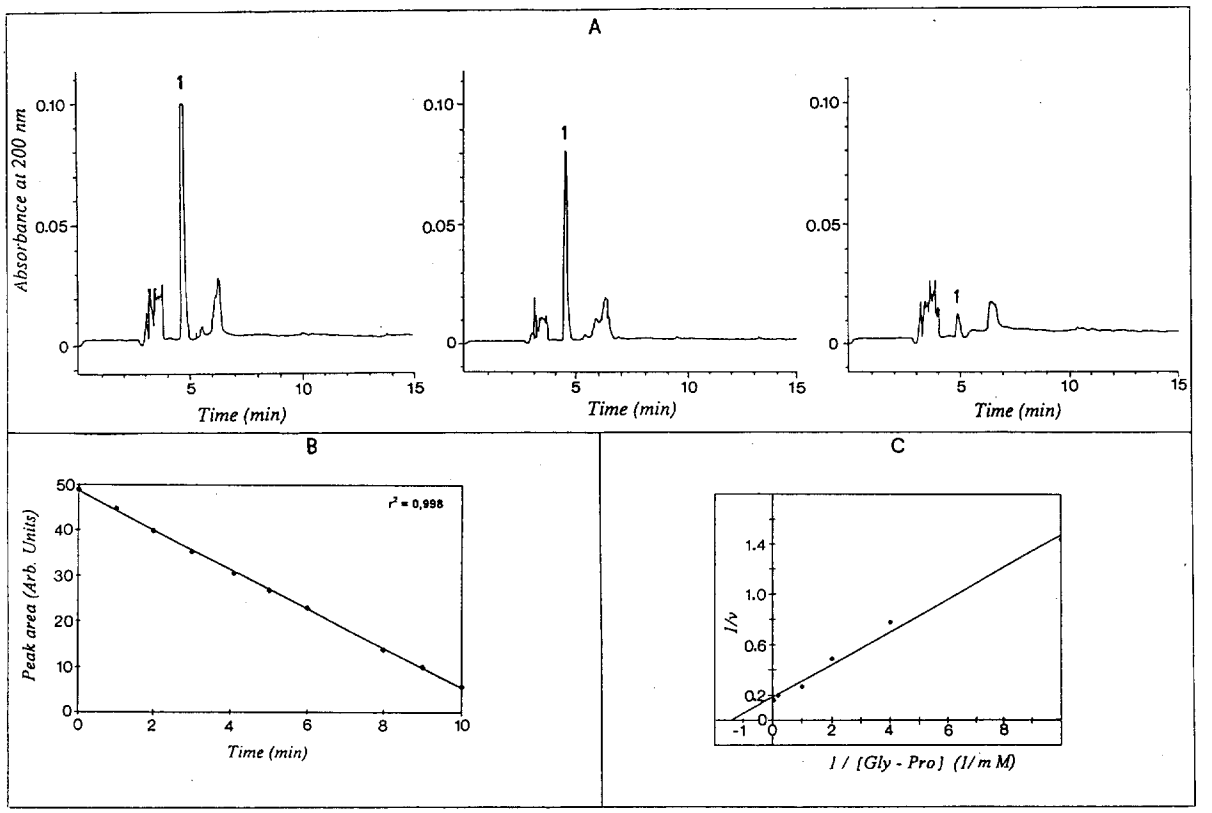

Fig. 1. (A) Electropherograms showing the proteolytic cleavage of the peptide Gly–Pro (peak 1) after treatment with porcine kidney prolidase for different times (0, 5 and 10 min, left to right). Capillary: untreated fused-silica column, 57 cm total length (50 cm effective length)×75 μm I.D. Peptide was analyzed using 35 m*M* sodium tetraborate (pH 9.3) containing 65 m*M* SDS as the electrolyte. The runs were carried out at 20 kV and the elution profile was monitored at 200 nm. Temperature: 25±0.1°C. Sample injection: 10-s hydrodynamic pressure (0.5 psi/s) injection corresponding to 53 nl of the peptide solution. (B) Kinetic profile for the proteolytic reaction described above. (C) Lineweaver–Burk plot of porcine kidney prolidase activity against Gly–Pro. V=μmol Gly–Pro; K_m=0.71 m*M*.

peak area/min were 0.21±0.05, in accordance with the theoretical value of 0.15 unit. Because of limitations in detector sensitivity, it was not possible to accurately detect prolidase activity at an enzyme concentration of less than 40 n*M*. The K_m value was determined by measuring the rate of Gly–Pro bond cleavage as a function of substrate concentration. The value obtained from the Lineweaver–Burk plot (Fig. 1C) was 0.71 m*M*, in agreement with the previously reported value [26].

Interestingly, the use of dipeptide Phe–Pro as a substrate was found to offer clear advantages over the other substrates. In fact, the inclusion of Phe, with its aromatic ring, meant that we could monitor both the intact peptide and the newly formed phenylalanine at 200 nm. It was thus possible to quantitatively assay the disappearance of substrate and the simultaneous appearance of product. The electropherograms obtained when the dipeptide Phe–Pro was incubated with porcine kidney prolidase under the experimental conditions previously mentioned are shown in Fig. 2. Peak 1 (retention time= 4.03±0.02 min) is that of the phenylalanine formed from peptide bond cleavage, while peak 2 (retention time=4.68±0.05 min) represents the intact substrate. Using the described CE mode of operation, it was possible to obtain baseline resolution of substrate and reaction product in a relatively short analysis time. This allowed a clear discrimination and an accurate quantification of the two peaks. However, as they were very close to each other, the reproducibility of the technique was evaluated from repeated injections of the mixture to be examined. After twenty injections, the retention times of the components remained almost the same, confirming that the methodology used was reliable. We also investigated the presence of other peaks already evidenced in the electropherograms of Fig. 1A. By injecting standard solutions of reduced glutathione, peak 3 (average retention time=5.80±0.03 min) was identified as GSH (contained in the incubation mixture of prolidase), while the small peaks that eluted immediately before phenylalanine were not identified. By plotting the concentrations of substrate and product vs. reaction time, it was possible to show that, as the reaction progressed, the peak corresponding to intact substrate diminished and the peak of phenylalanine increased proportionally (Fig. 3A). The amount of

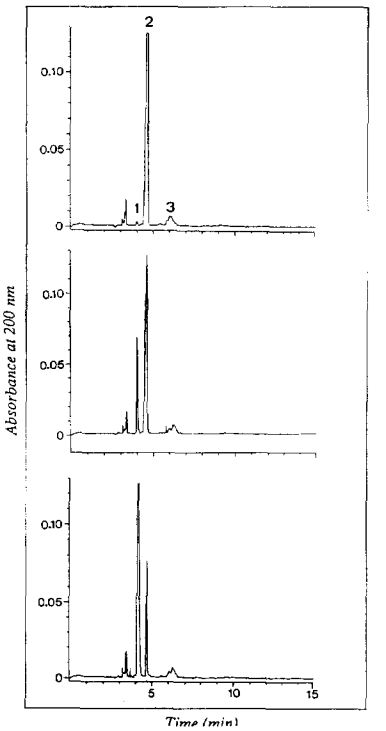

Fig. 2. Electropherograms showing proteolytic cleavage of the peptide Phe–Pro (peak 2) after treatment with porcine kidney prolidase for different times (0, 7 and 18 min, top to bottom). Peak 1 is free phenylalanine, while peak 3 was identified as GSH. All experimental conditions were the same as in (A).

free phenylalanine was calculated from peak areas by reference to peak areas obtained from injections of standard phenylalanine solutions (Fig. 3B). Although the calculated $t_{1/2}$ for Phe–Pro (12.05 min) was more than doubled in comparison with that reported for Gly–Pro, the possibility of confirming the rate of decrease of a substrate by simultaneously monitoring the formation of product is indeed the best way of establishing a suitable assay for the enzyme under investigation.

Because of the encouraging results obtained in evaluating the suitability of CE as a new method for assaying prolidase activity, considerable effort was devoted to the application of this technique for determination of prolidase in samples of cultured fibroblasts.

Skin fibroblasts from controls and PD patients were seeded into two 75 cm^2 flasks. At confluence, the medium was removed, the cell layer was washed,

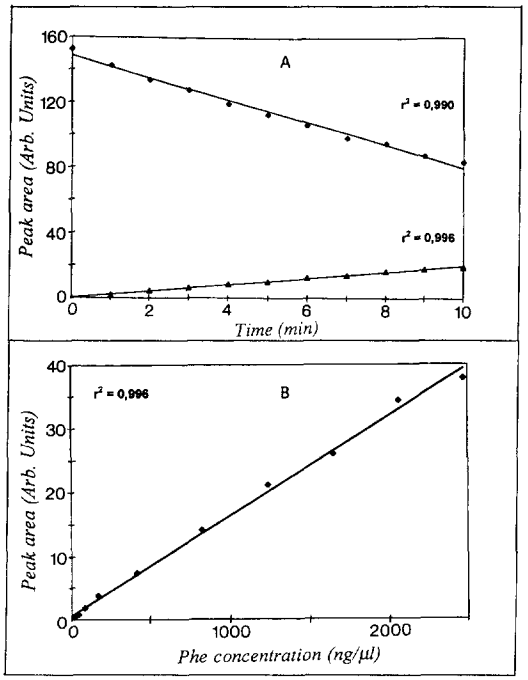

Fig. 3. (A) Kinetic profile of the proteolytic cleavage of the peptide Phe–Pro after treatment with porcine kidney prolidase (♦). Free phenylalanine formed from the reaction described above (▲). (B) Calibration curve for the determination of phenylalanine based on the peak area. The amino acid concentration was in the range 0.1–30 m*M*. All experimental conditions were the same as those in Fig. 2A.

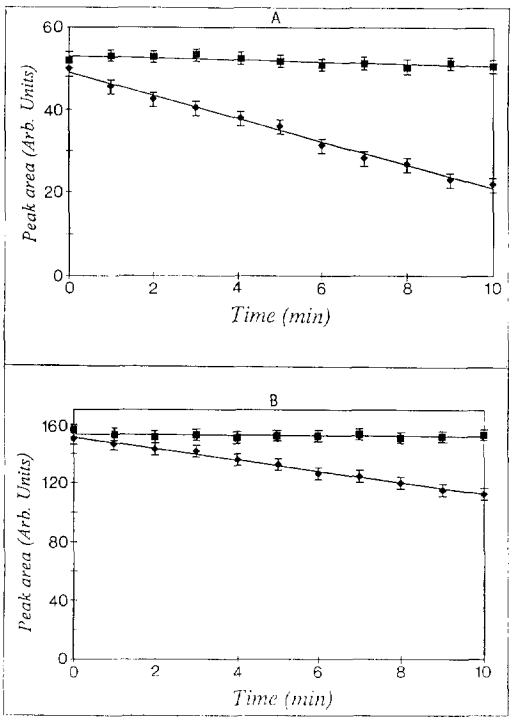

Fig. 4. (A) Kinetic profile of Gly–Pro cleavage by prolidase from cultured fibroblasts of PD patients (■) and of controls (♦). (B) Kinetic profile of Phe–Pro cleavage by prolidase from cultured fibroblasts of PD patients (■) and of controls (♦).

scraped, extracted and cell extracts were finally activated as described in Section 2. Individual extracts from controls and PD patients were then assayed for prolidase activity by both the newly developed CE technique and the colorimetric ninhydrin procedure. Aliquots were also withdrawn for measurement of protein content by the method of Lowry et al. [29]. Plots of Gly–Pro and Phe–Pro concentration vs. reaction time, obtained at saturating concentrations of peptides, using the electrophoretic conditions previously indicated are shown in Fig. 4 (panels A and B, respectively). The time-response curves indicated with (■) in both panels were obtained from the fibroblasts of PD patients, whilst curves with (♦) refer to controls. The values reported for the two substrates are the means of four independent determinations for PD patients and of five for controls. The rates of peptide bond cleavage for both substrates clearly show that almost no prolidase activity (<0.01 μmol of substrate hydrolysed/mg protein/min) is detectable in patients' fibroblasts, in agreement with the results already reported for these patients [35,36]. In contrast, enzyme activity was easily calculated in all of the controls tested (Table 1). The average values found with CE for Gly–Pro and Phe–Pro as substrates were 4.32±0.19 and 1.28±0.06 μmol hydrolysed/mg protein/min, respectively. Comparable results were obtained from samples that were assayed using Chinard's method [24]: average values of 3.84±0.15 and 1.12±0.05 μmol hydrolysed/mg protein/min were found for Gly–Pro and Phe–Pro, respectively. Discrepancies between the two methods were observed for the calculated K_m values; as shown in Table 1, the average K_m values obtained from CE experiments for the substrates Gly–Pro and Phe–Pro were 0.81±0.046 and 2.85±0.038 m*M*, respectively, compared to 2.88±0.053 and 7.18±0.06 m*M* obtained

Table 1
Prolidase activity of human fibroblast cell lines from controls and PD patients, determined using both CE and Chinard's method

	Prolidase activity (μmol Gly–Pro hydrolysed/mg protein/min)		Prolidase activity (μmol Phe–Pro hydrolysed/mg protein/min)		K_m Gly–Pro (m*M*)		K_m Phe–Pro (m*M*)	
	CE	Chinard's	CE	Chinard's	CE	Chinard's	CE	Chinard's
Control 1	4.60	4.00	1.27	1.10	0.84	2.82	2.80	7.10
Control 2	4.15	3.60	1.32	1.15	0.82	2.90	2.85	7.25
Control 3	4.20	3.80	1.20	1.05	0.76	2.85	2.90	7.15
Control 4	4.50	3.95	1.35	1.18	0.85	2.96	2.87	7.18
Control 5	4.35	3.83	1.28	1.14	0.80	2.89	2.83	7.23
Patient 1	<0.01	<0.01	<0.01	<0.01	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
Patient 2	<0.01	<0.01	<0.01	<0.01	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
Patient 3	<0.01	<0.01	<0.01	<0.01	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
Patient 4	<0.01	<0.01	<0.01	<0.01	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]

[a]Not detectable because the activity of the enzyme was too low.

using the ninhydrin reaction. Although only slightly lower than those determined with the colorimetric reaction, the K_m values obtained from CE experiments could, however, reflect an increased affinity of prolidase towards the substrates tested. These findings should confirm our assumption that the CE assay is more specific than the ninhydrin technique because the substrate peak area cannot be altered by any interference present in the assay. To this end, when the peak corresponding to the substrate Gly–Pro (the cleavage reaction in a control was considered) was collected from the CE system and submitted to three cycles of automated Edman degradation, the following results were obtained. The first cycle of sequencing resulted in the presence of only phenylthiohydantoin Gly (PTH–Gly), the signal of PTH–Pro was present in the second cycle and no signals were observed in the third cycle. No contaminants were detectable under the Gly–Pro peak. Moreover, a blank containing all of the components of the assay mixture, except the peptide–substrate Gly–Pro, was run and no peaks with a retention time 4.8±0.04 min were observed in the electropherogram (data not shown). Finally, since a mammalian endopeptidase that cleaves X–Pro bonds other than prolidase has not been observed to date, it may be concluded that the decrease in substrate area following CE can only be related to prolidase activity.

Experiments are in progress in our laboratory to calculate the K_m values of prolidase towards other X–Pro peptide–substrates, to definitively confirm the suitability and accuracy of CE as a method of assaying enzyme activity.

4. Conclusions

A new capillary electrophoretic approach has been developed for the rapid determination of prolidase activity in cultured fibroblasts. The method is simple and offers some advantages over the colorimetric techniques employed to date. Reproducible results were achieved from a set of determinations and, although excellent agreement was observed between the CE data and those obtained with the ninhydrin reaction, a comparison of results established that CE is superior to other methods in terms of accuracy, speed and sensitivity. Preliminary experiments performed on haemolysates were also possible; it may be concluded that the ability to assay prolidase activity, together with the ability to detect urinary imidodipeptides containing C-terminal prolyl or hydroxyprolyl residues [16], makes capillary electrophoresis a unique methodology in the study of prolidase deficiency.

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References

[1] T. Yoshimoto, F. Matsubara, E. Kawano and D. Tsuru, J. Biochem., 94 (1983) 1889.
[2] H. Sjöström and O. Noren, Biochim. Biophys. Acta, 359 (1974) 177.
[3] K.S. Hui and A. Lajtha, J. Neurochem., 35 (1980) 489.
[4] I. Myara, C. Charpentier and A. Lemonnier, Life Sci., 34 (1984) 1985.
[5] S.A. Bizzozero and B.O. Zweifel, FEBS Lett., 59 (1975) 105.
[6] V.M. Der Kaloustian, B.J. Freij and A.K. Kurban, Dermatologica, 164 (1982) 293.
[7] A. Yaron and F. Naider, Crit. Rev. Biochem. Mol. Biol., 28 (1993) 31.
[8] I. Myara, C. Cosson, N. Moatti and A. Lemonnier, Int. J. Biochem., 26 (1994) 207.
[9] P.M. Royce and B. Steinmann, in P.M. Royce and B. Steinmann (Editors), Connective Tissue and its Heritable Disorders, Wiley-Liss, New York, 1st ed., 1993, p. 533.
[10] S.I. Goodman, C.C. Salomons, F. Muschenheim, C.A. McIntyre, B. Miles and D.A. O'Brien, Am. J. Med., 45 (1968) 152.
[11] G.F. Powell, M.A. Rasco and R.M. Maniscalco, Metabolism, 23 (1974) 505.
[12] H. Kodama, S. Umemura, M. Shimomura, S. Mizuhara, J. Arata, Y. Yamamoto, A. Yusutake and N. Izumiya, Physiol. Chem. Phys., 8 (1976) 463.
[13] L.J. Sheffield, P. Schlesinger, K. Faull, B.J. Halpern, G.M. Schier, R.G.H. Cotton, J. Hammond and D.M. Danks, J. Pediatr., 91 (1977) 578.
[14] J. Arata, S. Umemura, Y. Yamamoto, M. Hagiyama and N. Nohara, Arch. Dermatol., 115 (1979) 62.
[15] H. Kodama, H. Nakamura, K. Sugahara and Y. Numajini, J. Chromatogr., 527 (1990) 279.
[16] G. Zanaboni, R. Grimm, K.M. Dyne, A. Rossi, G. Cetta and P. Iadarola, J. Chromatogr. B, 683 (1996) 97.
[17] C.R. Scriver, M.L. Efron and I.A. Schafer, J. Clin. Invest., 43 (1964) 374.
[18] A.P. Boright, F. Choy, G.A. Lancaster and C.R. Scriver, Am. J. Hum. Genet., 41 (1987) A4.
[19] M. Isemura, T. Hanyu, F. Gejyo, R. Nakazawa, R. Igarashi, S. Matsuo, K. Ikeda and Y. Sato, Clin. Chim. Acta, 93 (1979) 401.
[20] G. Zanaboni, K.M. Dyne, A. Rossi, V. Monafo and G. Cetta, Haematologica, 79 (1994) 13.
[21] J. Butterworth and D.A. Priestman, J. Inherit. Metab. Dis., 8 (1985) 193.
[22] G. Manao, P. Nassi, G. Capuggi, G. Camici and G. Ramponi, Physiol. Chem. Phys., 4 (1972) 75.
[23] S.H. Jackson, A.W. Dennis and M. Greenberg, Can. Med. Assoc., 113 (1975) 759.
[24] F.P. Chinard, J. Biol. Chem., 199 (1952) 91.
[25] L. Lin and J.F. Brandts, Biochemistry, 18 (1979) 43.
[26] W.L. Mock, P.C. Green and K.D. Boyer, J. Biol. Chem., 265 (1990) 19600.
[27] I. Myara, C. Charpentier and A. Lemonnier, Clin. Chim. Acta, 125 (1982) 193.
[28] M. Valli, M. Mottes, R. Tenni, A. Sangalli, M. Gomez Lira, F. Antoniazzi, G. Cetta and P.F. Pignatti, J. Biol. Chem., 266 (1991) 1872.
[29] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
[30] R.J. Krueger, T.R. Hobbs, K.A. Mihal, J. Tehrani and M.G. Zeece, J. Chromatogr., 543 (1991) 451.
[31] F. Mulholland, S. Movahedi, G.R. Hague and T. Kasumi, J. Chromatogr., 636 (1993) 63.
[32] G.N. Okafo, P. Cutler, D.J. Knowles and P. Camilleri, Anal. Chem., 67 (1995) 3697.
[33] J. Bao and F.E. Regnier, J. Chromatogr., 608 (1992) 217.
[34] D. Wu and F.E. Regnier, Anal. Chem., 65 (1993) 2029.
[35] J. Butterworth and D.A. Priestman, J. Inherit. Metab. Dis., 7 (1984) 32.
[36] M. Dolenga and P. Hechtman, Pediatr. Res., 32 (1992) 479.