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Optimization of a capillary electrophoretic method to detect and quantify the Gly–Pro dipeptide in complex matrices from long term cultured prolidase deficiency fibroblasts

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Abstract

A capillary electrophoresis (CE) method has been developed and optimized for the detection of Gly–Pro dipeptide in complex biological samples: medium, cell layer and matrix obtained from long term cultured human fibroblasts of control and prolidase deficiency patients. The influence of different detergents in the sample preparation and electrophoretic conditions were investigated. The method was validated for cellular extracts with respect to limits of detection and quantitation, precision, linearity, accuracy and robustness. The optimized method was applied to real samples and revealed a significant increase of intracellular Gly–Pro dipeptide in prolidase deficiency fibroblasts with respect to the control.

Keywords: Prolidase deficiency; Dipeptide

1. Introduction

Prolidase (E.C. 3.4.13.9) is a ubiquitous enzyme, which hydrolyses the di- and tripeptides containing C-terminal prolyl or hydroxyprolyl residues. Thus it is involved in the terminal stages of proteins catabolism. Mutations in the prolidase gene had been demonstrated to cause prolidase deficiency (PD), a rare autosomal recessive inherited connective tissue disorder characterized mainly by chronic skin ulceration, recurrent respiratory infections, mental retardation and dysmorphic facies [1].

The affected patients have severe imidodipeptiduria and the diagnosis of prolidase deficiency is based on the determination of urine imidodipeptides by capillary isotachophoresis, chromatography and more recently by capillary zone electrophoresis and micellar electrokinetic chromatography [2–5]. The measure of prolidase activity in erythrocytes, leukocytes or fibroblasts is necessary to validate the diagnosis since individuals with massive imidodipeptiduria do not necessarily have PD and heterozygotes often do not have increased dipeptides in urine [1,6,7].

Although a limited number of mutations: five point mutations, four exon splicing, a trinucleotides deletion and a large deletion, has so far been characterized, it is clear that PD patients present a

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wide range of clinical outcomes not only among subjects with different molecular defects but also in the presence of an identical mutation [1].

The bases of this phenotypic variability as well as the mechanism involved in the pathogenesis of the disease are still poorly understood.

Gly–Pro is the more abundant dipeptide generated during the final stage of degradation of collagen type I, the major protein synthesized by skin fibroblasts. In order to contribute in the understanding of the pathophysiology of the disease the purpose of the present study was to provide an efficient and rapid method to investigate the effect of the lack of prolidase activity in the skin, which is the mostly affected tissue in PD, by using, as in vitro model, long term cultured fibroblasts. Capillary electrophoresis (CE) appears the ideal instrument for this research since it is one of the suitable separation techniques used to analyse metabolites in complicated sample matrices such as plasma, serum and urine. It allows high separation efficiencies of small amount of analytes over a short period of time, thus resulting an attractive separation technique in clinical applications due to the complexity of the samples that often contain multiple endogenous components, which have to be resolved from the solute of interest. Furthermore, the possibility to rinse and clean the capillary between injections with relatively harsh solutions, such as NaOH, allows direct injection of biofluids with possible reduction in samples pretreatment requirements, which is attractive in clinical chemistry where sample throughput is high [8]. Finally the limited sample volume required for CE analysis makes it particularly suitable for the resolution of components in small samples as cellular extracts. In conclusion, CE minimizes costs, errors and artefacts. In the present study we develop a fast and sensible assay, based on a CE technique, to detect and quantify the Gly–Pro dipeptides in medium, cellular extract and matrix deposited by long term cultured fibroblasts obtained from PD patients. The accumulation site and/or the quantity of the Gly–Pro dipeptide in five pathological fibroblast lines have also been evaluated to verify a possible correlation with the severity of the disease in order to further investigate the clinical variability characteristic of PD.

2. Experimental

2.1. Chemicals

Gly–Pro dipeptide, Dulbecco's modified Eagle's medium (DMEM), the serum substitute ITS+3, Dulbecco's phosphate buffered saline (PBS), sodium deoxycholate (DOC), α-cyclodextrin, Nonidet (NP-40) and sodium dodecylsulfate (SDS) were purchased from Sigma (Milan, Italy). Tris, Triton-X-100, acetone, trichloroacetic acid (TCA), KCl, were from BDH (Milan, Italy). Ascorbic acid was from Fluka (Milan, Italy), sodium tetraborate was from Carlo Erba (Milan, Italy). All the reagents were of analytical grade and were used without further purification. Sodium phosphate buffer solution 100 m*M* was purchased from Bio-Rad (Milan, Italy). All solutions were made up with distilled water. Ultrafiltration system Biomax-5K was obtained from Millipore (Bedford, MA, USA).

2.2. Cell culture

Dermal fibroblasts of five PD patients (P1–P5) were grown from a skin biopsy obtained after informed consent [1,9–12]. Control cell lines (2056.CRL and 2127.CRL) were purchased from American Type Culture Collection (ATCC, Bethesda, MD, USA). For all experiments patient and control fibroblasts were used between the 8th and 14th passages, plated at $8{\cdot}10^5$ in T75 tissue culture flasks and grown for 8 days at 37 °C in the presence of 5% CO_2 in DMEM, supplemented with a serum substitute which does not contain prolidase (1% v/v ITS+3). Ascorbic acid at final concentration of 100 μg/ml was added every third day to the culture and no medium change was performed [13].

2.3. Medium, cell layer and matrix harvesting conditions

Medium, cell layer and extracellular matrix fractions obtained from 8 days cultured fibroblasts were collected as follows: the medium fraction were denatured at 80 °C and concentrated by ultrafiltration

using Biomax-5k, (Millipore) centrifuged at 3000 *g* for 5 min at 4 °C. The cell layer was dissolved in 2 ml of 1% (w/v) Triton-X-100, 0.6 *M* KCl in PBS [14] or 1% (w/v) Nonidet in 50 m*M* Tris, pH 7.8, adjusted by HCl [15], by shaking for 30 min at room temperature. Also a buffer containing 0.5% (w/v) sodium deoxycholate (DOC) in 10 m*M* Tris buffered saline phosphate, pH 8.0 adjusted by HCl, was used for cell layer solubilization by shaking for 30 min on ice [16]. All the buffers contained protease inhibitors [2 m*M* *N*-ethylmaleimide (NEM), 7.5 m*M* benzamidine, 4 m*M* EDTA, 0.1 μ*M* phenylmethanesulfonylfluoride (PMSF)]. The cell layer samples collected with the three different methods were denatured at 80 °C for 15 min. Extracellular matrix was dissolved in 2 ml of PBS or 50 m*M* Tris pH 7.8, adjusted by HCl upon denaturation at 80 °C for 15 min. The reduced volume of 2 ml for cell layer and extracellular matrix fractions allowed to avoid concentration steps.

2.4. Preparation of standard curves

Gly–Pro dipeptide standard solutions were prepared as follows: to obtain concentrations in the range of 0.1–1.5 m*M*, Gly–Pro dipeptide was dissolved in 9 ml of DMEM 1% (v/v) ITS to mimic medium fraction; in 2 ml of 50 m*M* Tris, pH 7.8, adjusted by HCl, containing 1% (w/v) Nonidet or in 2 ml of PBS 1% (w/v) Triton-X-100, 0.6 *M* KCl or in 2 ml of PBS 0.5% (w/v) DOC for cell layer fraction; in 2 ml 50 m*M* Tris pH 7.8, adjusted by HCl, or 2 ml of PBS for matrix fraction, all containing protease inhibitors (2 m*M* NE*M*, 7.5 m*M* benzamidine, 4 m*M* EDTA, 0.1 μ*M* PMSF).

2.5. Instrumentation

Beckman (Palo Alto, CA, USA) P/ACE 2100 instrument equipped with a UV detector and external nitrogen pressure was used throughout this study. The Beckman P/ACE STATION software was used for data analysis.

Gly–Pro identification in real samples and standards was obtained by sequence analysis of the collected peaks. Sequence was performed on the HP G1000A protein sequencing system (Hewlett-Packard, Palo Alto, CA, USA).

2.6. Initial experimental conditions

A 75-μm uncoated fused-silica capillary was used throughout the study with a total length of 57 cm (50 cm effective length), kept at a temperature of 25±0.1 °C by means of a cooling liquid circulating continuously through the cartridge. Samples were injected by hydrodynamic pressure (3.4 kPa) for 2 s. A range of voltages, between 7 and 15 kV, was tried. The electric current ranged between 50 and 150 μA. All runs were performed with injections at anodic side. The running time was 25 min for medium fraction, 20 min for cell layer in buffer with Triton -X-100 and 40 min for cell layer in buffer containing Nonidet and for matrix fraction.

Based on our previous experience [4] imidopeptides present in urine samples can be better analyzed in CE at basic pH. Therefore, preliminary experiments on medium, cell layer and matrix fractions were performed using 50 m*M* sodium tetraborate buffer (pH 9.3), containing 50 m*M* SDS. UV detection was used at the wavelength of 200 nm.

3. Results and discussion

3.1. Method development of analysis conditions for standard solutions

Our goal was the determination of the amount of imidodipeptide Gly–Pro by CE in medium, cell extract and matrix fractions obtained from long term cultured fibroblasts from PD patients and controls. The experimental conditions were first set up on the standard solutions described in Experimental.

The proteins were removed from the medium fraction by precipitation. Two different methods were tested: the addition of 50% (v/v) of acetone or the addition of TCA at a 10% (v/v) final concentration. The latter reagent was preferred because no time-consuming evaporation was required. Due to the large medium volume (9 ml) necessary for growing cells in T75 flasks, to maximize the Gly–Pro detection, a concentration step was necessary. Initially lyophylization was attempted, but the more rapid ultrafiltration system was preferred. Furthermore, the ultrafiltration with an appropriate cut-off

(M_r 5000) allowed us to avoid the precipitation step (data not shown).

We tested Nonidet, Triton-X-100 and DOC as detergents for the plasmatic membrane. A detergent was required to collect the cell layers separately from the matrix fractions. No electrophoretic separation was obtained in presence of DOC with the CE experimental conditions described previously.

The resolution was more encouraging with Nonidet and Triton-X-100 solutions, the Gly–Pro peak was detectable at all the tested concentrations (data not shown).

The choice to solubilize Gly–Pro in 50 m*M* Tris, pH 7.8, adjusted by HCl, or PBS buffers followed by a denaturation step for matrix standard solutions was determined by the advantage to be able to apply the same electrophoretic conditions used for cell layer standard solutions.

3.2. Method optimisation: determination of Gly–Pro in real matrices

Initial CE experimental conditions failed on medium real samples. No Gly–Pro was detectable in this matrix even the co-injected commercial standard at concentration of 1 m*M*. Probably long term culture caused the secretion of multiple catabolites of low molecular mass from the cells that complicated the electrophoretic pattern.

The effect of lower pH buffers such as 50 m*M* sodium citrate, pH 5.0, adjusted by HCl, and 100 m*M* sodium phosphate buffer, pH 2.5, adjusted by phosphoric acid, were investigated. The pK_a values of Gly–Pro are 2.81 and 8.65, so a variation in pH buffer will determine different ionisation state of the molecule and consequently a variation in its electrophoretic mobility. In particular, at acidic pH ($\cong$2.5) the carboxyl end of the peptide Gly–Pro is protonated and the amino terminal is fully charged. Although sticking of Gly–Pro peptide had been reported under this condition [17], we obtained better results.

With the aim of optimizing the method efficiency and sensitivity for Gly–Pro dipeptide other factors were improved, in particular: (1) the capillary I.D. was decreased from 75 μm to 50 μm; (2) the injection time was increased from 2 to 30 s (a significant improvement, but lower than the overloading limit fixed in 10% of the capillary total volume); and (3) voltage was increased to 30 kV, maintaining the injection at anodic side, in order to obtain separation in short analysis time (15 min).

The optimised method with the capacity to determine the concentration range from 0.1 to 0.8 m*M* was applied to the real sample: the new conditions were found to have significant effects on electrophoretic patterns and commercial standard detectability (Fig. 1), but no endogenous Gly–Pro was detectable in the medium fraction.

Also for cell layer real samples, the initial CE conditions described (50 mM sodium tetraborate buffer pH 9.3, 50 m*M* SDS) were not effective. No peak corresponding to Gly–Pro was identifiable and the electrophoretic conditions applied allowed only the detection of the Gly–Pro standard coinjected both with Nonidet and Triton-X-100 at a concentration of 1 m*M*. So optimisation was necessary.

The real cell layer samples harvested in the buffer containing 1% Nonidet were concentrated by

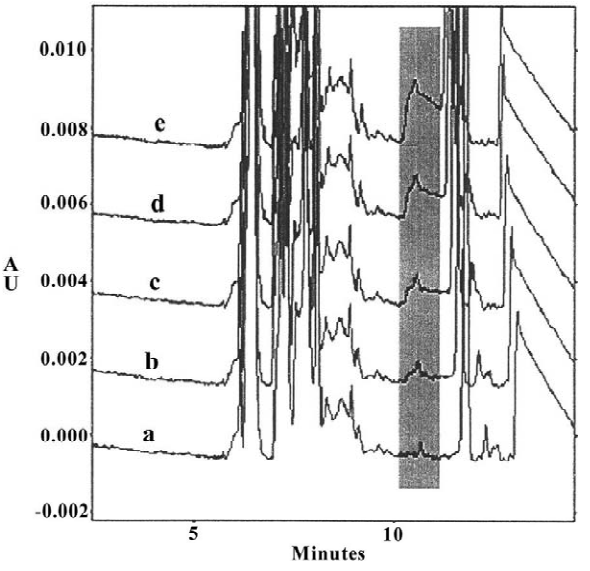

Fig. 1. Electropherograms of medium fractions from real samples with increasing concentrations of Gly–Pro standard: 0 (a), 0.2 m*M* (b), 0.4 m*M* (c), 0.6 m*M* (d), 1 m*M* (e). Samples were injected for 30 s in a 50 μm I.D., 57 cm total length uncoated fused-silica capillary with an applied voltage of 30 kV. All runs were done with normal polarity using 100 m*M* sodium phosphate buffer (pH 2.5), at the wavelength of 200 nm. The Gly–Pro peak is highlighted.

lyophylization and analysed using a reduced 50 μm I.D. capillary and a prolonged injection of 10 s, but no progress was obtained. Even the change from micellar electrokinetic chromatography (MEKC) to capillary zone electrophoresis (CZE) did not improve separations, analysis were performed using 100 m*M* sodium phosphate buffer, pH 2.5, adjusted by phosphoric acid, or 50 m*M* sodium borate pH 9.3 always with injection at anodic side.

So 1% Nonidet solution was discarded and cell layer fractions, solubilized in the buffer containing 1% Triton-X-100, were tested thoroughly.

The samples were directly injected for 60 s (below of overloading limit) in a 50 μm I.D. fused-silica capillary and separated at 30 kV in sodium tetraborate, pH 9.3. SDS was abandoned because of the falling down of the current due to its increment above the limit tolerated by the instrument (250 μA). With these new conditions the Gly–Pro was detectable in 15 min.

Nevertheless another setback invalidated the analysis: the capillary was blocked after about ten electrophoretic runs and various washing procedures did not solve the problem.

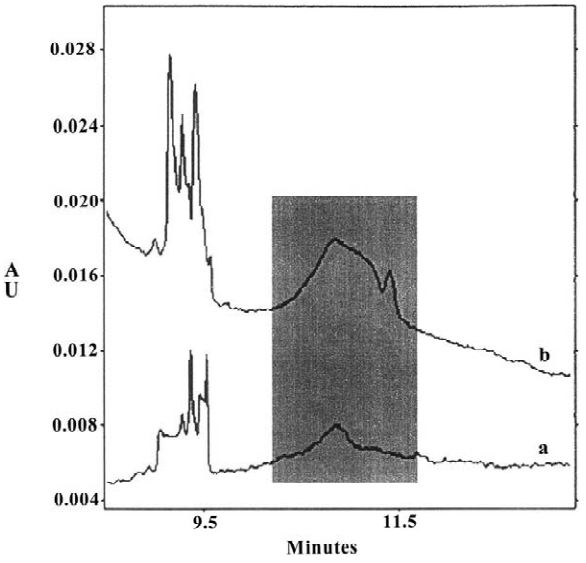

Fig. 2. Electropherograms of cell layer fractions from real samples: a control (a) and a patient (b) electrophoretic patterns. Samples were injected for 60 s in a 50 μm I.D., 57 cm total length uncoated fused-silica capillary with an applied voltage of 30 kV. All runs were done with normal polarity using 50 m*M* sodium tetraborate buffer (pH 9.3) 30 m*M* α-cyclodextrin, at the wavelength of 200 nm. The Gly–Pro peak is highlighted.

Cyclodextrins, either native or derivatized, have been introduced to affect the separation selectivity in many cases [18,19].

The α-cyclodextrin introduction in our method (30 m*M* final concentration) improved electrophoretic analysis allowing the detectability of Gly–Pro at a concentration range of 5–20 μ*M* and resulting also in capillary protection (Fig. 2).

The determination of Gly–Pro intracellular content with this method in five fibroblast lines from PD patients and two control lines revealed a significant increase of Gly–Pro in all PD patients with respect to the controls (Table 1). The Gly–Pro amount had been expressed as mean±standard error mean (S.E.M.) of a minimum of four experiments for each case and three electrophoretic runs for each sample.

A reliable identification of the peak as Gly–Pro was performed by automated *N*-terminal Edman degradation of the sample on a sequencer. Microprereparative CE was performed for samples collection using a fused-silica capillary of 100 μm I.D., 50 cm effective length and the best conditions described above: 50 m*M* sodium tetraborate buffer, pH 9.3, containing 30 m*M* α-cyclodextrin. Samples were injected for 20 s and 8 kV voltage was applied.

The real extracellular matrix fractions, obtained after cell extract removal, were analysed upon solubilization in PBS solution and the method applied was the one optimised for cellular extracts and described above.

In this fraction no endogenous Gly–Pro was detectable in PD patients as well as in controls (Fig. 3).

To ensure run-to-run reproducibility of separation, the capillary column was purged with 0.1 *M* sodium hydroxide and fresh buffer each for 5 min before each injection.

3.3. Method validation

The method used for Gly–Pro cellular extract detection and quantitation was validated with respect to sensitivity, linearity, accuracy, precision and robustness [20].

The limit of detection (LOD) [21] was defined as the lowest drug concentration, which can be clearly

Table 1
Intracellular Gly–Pro content in control and pathological cultured fibroblasts

Cases	nmol Gly–Pro/ mg proteins±S.E.M.
Controls	4.3±0.5
P1	7.9±0.5
P2	8.8±1.1
P3	8.5±1.7
P4	11.2±0.6
P5	12.1±1.7

detected above the baseline signal. The LOD, with a signal-to-noise ratio of 3, was calculated to be 5 μ*M*.

The limit of quantitation (LOQ) was defined as the lowest drug concentration, which can be reproducibly quantified above the baseline signal [21]. The LOQ, with a signal-to-noise ratio of 10, resulted in 6 μ*M*.

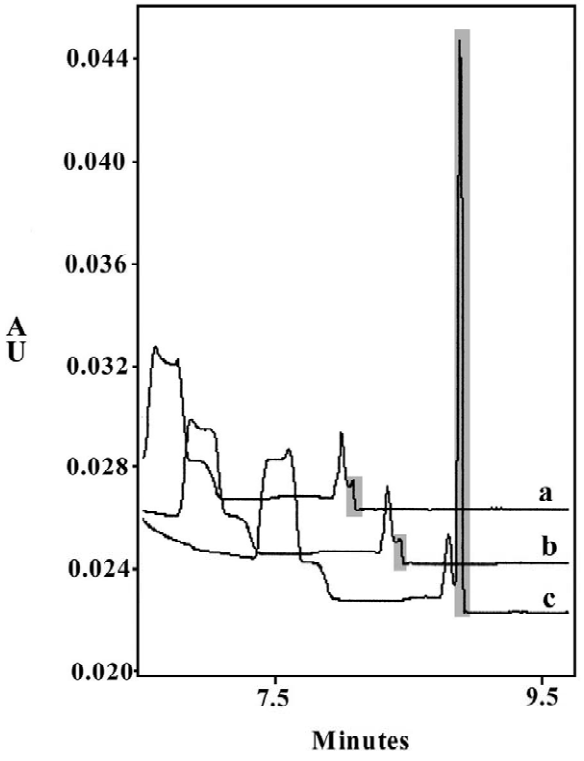

Fig. 3. Electropherograms of matrix fraction from real samples: a control electrophoretic pattern (a), a patient analysis (b) and a patient Gly–Pro coinjected run (c). Samples were injected for 60 s in a 50 μm ID, 57 cm total length uncoated fused-silica capillary with an applied voltage of 30 kV. All runs were done with normal polarity using 50 m*M* sodium tetraborate buffer (pH 9.3) 30 m*M* α-cyclodextrin, at the wavelength of 200 nm. The Gly–Pro peak is highlighted. The electropherograms have been shifted to make the peaks heights clearly visible.

Linearity was determined over a wide range of sample concentrations by plotting a standard curve of Gly–Pro solutions ranging from 10 to 300 μ*M* and in a separate experiment from 5 to 20 μ*M* in triplicate. A linear response was obtained in both cases, with an $R^2 = 0.9948$ and $R^2 = 0.9800$, respectively. The lower end was placed around the LOQ of the method and the upper level was selected to permit the measurement of the increase in pathological samples. Linearity of response for samples was tested in the same way but replacing standard solutions with cellular extracts ($R^2 = 0.9959$).

The average accuracy over the concentration range studied was 109% [22].

Analytical precision in terms of both peak areas and migrations times was evaluated day-to-day. The precision calculated over a minimum of four experiments for each patient and control and three electrophoretical runs for each sample gave a 5% RSD value. It is noticeable that the area of 1 m*M* creatinine used as internal standard in all real matrices studied (patients and controls) gave 11% RSD, this value is considered acceptable in analysis performed on samples from complex matrices such as cell extracts. The migration time RSD on Gly–Pro peak over 84 analyses was 2%.

The assay was run with a separation buffer adjusted to pH 9.25±0.25 with no significant effect on the performance parameters.

4. Conclusion

A new CE approach has been developed for the rapid determination of Gly–Pro dipeptide in medium, cellular extract and matrix obtained from long term human fibroblasts.

(a) The variation of pH (from 9.3 to 2.5) and ionic strength (from 50 to 100 m*M*), a decrease in capillary diameter (from 75 to 50 μm), an increase in injection time (from 2 to 30 s) and in voltage (from 7–15 to 30 kV) allowed a good sensitivity and timing for analysis of medium samples.

(b) The decrease in capillary I.D. (from 75 to 50

μm), the increase in injection time (from 2 to 60 s) and the addition of 30 m*M* α-cyclodextrins improved electrophoretic analysis resolution, sensitivity and reproducibility in cell layer and matrix samples.

(c) Sensitivity, linearity, precision and robustness were evaluated to validate the method for Gly–Pro detection and quantitation in cell layer samples.

(d) The intracellular Gly–Pro content in patients fibroblasts was significantly higher ($P<0.05$) than that found in control cells. But no significant difference was detected among the five different examined patients. Due to the low number of the analysed cases we cannot draw any definitive conclusion but our preliminary data suggest no correlation between Gly–Pro intracellular content in cultured cells and clinical outcome. Further cases are under investigation.

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