

Determination of prolidase activity using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry[☆]

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Abstract

Proline-containing peptides of the X-proline type are cleaved by the dipeptidase prolidase. The classical method of prolidase assay relied on the colorimetric estimation of the liberated proline with ninhydrin using acidic media and heat. This method, however, gave inconsistent results due to the nonspecificity of the ninhydrin color reaction. We report here a method for the detection of the liberated proline using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Human sera were incubated with a mixture containing the dipeptide glycyl-proline in Tris-HCl supplemented with manganese at 37 °C for 24 h. The samples were precipitated with trifluoroacetic acid and centrifuged. An aliquot of the supernatant was mixed with an equal volume of ferulic acid solution. An aliquot from this mixture was spotted on a stainless steel mass spectrometry grid and analyzed using MALDI-TOF mass spectrometry. The activity of the enzyme was determined by the complete disappearance of the glycyl-proline peak with the concomitant appearance of the proline peak and can be expressed in terms of the ratio of the area beneath the proline to the area beneath the glycyl-proline peak. Subjects homozygous for prolidase deficiency had a ratio ranging from 0.006 to 0.04 while obligatory heterozygotes had a ratio ranging from around 1.1 to 2.4. Normal subjects had ratios ranging from 9 to 239. Using this method we have unambiguously identified subjects with homozygous or heterozygous prolidase deficiency. In addition to the advantage of rapid sample preparation time, this method is highly specific, reproducible, and sensitive.

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Prolidase (peptidase D, imidodipeptidase; EC 3.4.13.9) is an ubiquitous enzyme involved in collagen metabolism that was first described over 40 years ago. This enzyme, an iminodipeptidase, cleaves dipeptides that have a proline (or a hydroxyproline) in the C-terminal position and

any other amino acid in the N-terminal position. Small, noncharged amino acids such as glycine or leucine are preferred by the enzyme. The enzyme is a 110,000 MW homodimer and requires manganese. Prolidase is critical in the final stages of the metabolism of collagen molecules, which contain up to 25% proline or hydroxyproline. Collagenases degrade collagen such that dipeptides containing proline (or hydroxyproline) are produced. Then, prolidase cleaves such dipeptides into free amino acids. In this way proline can be reclaimed and recycled for use in synthesis of new collagen molecules. Normally, neither iminodipeptide nor free proline is excreted in the urine.

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Prolidase deficiency was first described in 1968 [1] but it was not until 1974 that the actual enzyme assay was performed [2]. By 2002 only 30 cases [3,4] have been described, and all of them have an absence of prolidase activity. A variety of symptoms of the disease have been described [4–8]. Onset has been in childhood mostly, but several patients with onset and diagnosis in adulthood have been described [5]. Skin manifestations include chronic lower extremity skin ulcers (a hallmark of the disease), telangiectasia, photosensitivity, edema, and an eczematouslike skin rash. Patients with prolidase deficiency have characteristic facies consisting of frontal bossing, low hairline, and saddle nose deformity. A few patients have mild mental retardation. Immune abnormalities frequently include increased serum immunoglobulin and abnormal C1q, which contains about 25% proline, similar to collagen. However, patients with prolidase deficiency but without any clinical symptoms have also been reported [9,10].

Several methods to assay the activity of the enzyme have been described. These methods include spectrometric detection of the disappearance of alanyl–proline at 220 nm [11], detection of the liberated glycine after glycy–proline hydrolysis using alcoholic potassium hydroxide [12], high-voltage electrophoresis [13,14], ion exchange chromatography [9,15,16], colorimetric assay of glycine [17] and proline [18,19], capillary electrophoresis [20,21] and isotachopheresis [22].

The most classic method of detection, the oldest and the most widely used, relies on the spectrophotometric monitoring of proline released from the X-proline dipeptide by the action of prolidase, following its reaction with ninhydrin in acidic media [23]. The liberated proline interacts with ninhydrin to form a water-insoluble red reaction product at a pH of approximately 1.0 that is read at 515 nm. However, this method is not very specific, since ninhydrin reacts with other amino acids such as lysine, ornithine, and others. Moreover it is not very useful in determining heterozygous activity of the enzyme.

We report here a new method to assay prolidase activity in serum. This method employs matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)² mass spectrometry to detect the appearance of proline from glycy–proline.

Materials and methods

Porcine prolidase, ferulic acid, α -cyano-4-hydroxycinnamic acid (CHCA), acetonitrile, trifluoroacetic acid (TFA), and glycy–proline were from Sigma Chemical

(St. Louis, MO, USA). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer, Voyager Elite BioSpectrometry Research Station (Serial No. 130), equipped with a delayed extraction option, was from PerSeptive Biosystems (Framingham, MA, USA).

Subjects

Human sera were collected under protocols approved by the OMRF Institutional Review Board after informed consent was obtained from participants/parents. Six subjects with prolidase deficiency, diagnosed primarily by urinary dipeptide excretion, were used in this experiment. Four of them were children, while two others were the parents of two of the children and, therefore, obligatory heterozygotes. Two others were adults, one having nonhealing lower extremity ulcers. In addition, 20 normals were used.

Preparation of ferulic acid and α -cyano-4-hydroxycinnamic acid solutions

Ferulic acid (10 mg/ml) is dissolved in 70% acetonitrile/5% formic acid. α -Cyano-4-hydroxycinnamic acid is prepared by dissolving 10 mg of CHCA in 1 ml of 50% acetonitrile/0.1% TFA.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometric assay for detection of prolidase activity

Human serum sample (25 μ l) was added to a microcentrifuge tube containing 100 μ l of Tris–HCl (50 mM, pH 7.4, containing 10 mM $MnCl_2$), 75 μ l of filtered dH_2O , and 50 μ l substrate (glycy–proline; 0.1 M). The tubes were incubated at 37 °C overnight for 24 h in the case of serum samples. After incubation, 250 μ l of 0.1% aqueous TFA was added to stop the reaction. Samples with purified prolidase were stopped after an incubation of 1 h. The samples were centrifuged at 10,000g for 10 min and the supernatants transferred to another set of microcentrifuge tubes. Then 0.5 μ l of the supernatant was mixed with 0.5 μ l of the ferulic acid matrix (ferulic acid was used as the matrix as it did not have any peak characteristic of glycy–proline or proline), and 0.5 μ l of this mixture was spotted on a stainless steel grid and vacuum dried. MALDI-TOF-MS of the dried mixture was used to detect the disappearance of glycy–proline and the appearance of proline to monitor the activity of prolidase. The MALDI spectra were obtained from a Voyager Elite BioSpectrometry Research Station, equipped with a delayed extraction option (Applied Biosystems, CA) operated at the accelerating voltage, 20 kV; grid voltage, 82%; guide wire voltage, 0.05%; pulse delay time, 75 ns; vertical scale, 1000 mV; vertical offset, 3%. A pulsed nitrogen laser operating at 337 nm was used as a

² Abbreviations used: MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; CHCA, α -cyano-4-hydroxycinnamic acid; TFA, trifluoroacetic acid.

desorption/ionization source. Mass spectrometry was performed in reflector mode with positive ion detection. The ion signal was recorded using a 500-MHz transient digitizer. The data were analyzed using GRAMS/386 (Galactic Industries Corp., Salem, NH, USA).

Determination of area beneath the monoisotopic proline ($m/z = 116$) and glycyl-proline ($m/z = 173$) peaks

The area beneath specific monoisotopic peaks ($m/z = 116$ and 173) were calculated using GRAMS/386 software obtained from Galactic Industries Corp. The ratio between the area under these two peaks was used to arrive at a range of values to denote normal, homozygous, or heterozygous prolidase activity.

Colorimetric assay for prolidase

Prolidase colorimetric assay was carried out essentially according to Chinard's [23] ninhydrin assay. Two sets of microcentrifuge tubes were used for the assay. The first set contained 25 μ l of sample (serum), 100 μ l of Tris-HCl (50 mM, pH 7.4, containing 10 mM $MnCl_2$), 75 μ l of filtered dH_2O , and 50 μ l substrate (glycyl-proline; 0.1 M). The second set contained all these ingredients except the substrate and this served as the control (50 μ l of milli Q water was added instead of the substrate). The control values were subtracted from the test value in each case to arrive at the prolidase activity of the sample. The samples were incubated at 37°C for 1 h and after incubation 250 μ l of 10% TCA were added to stop the reaction. The tubes were spun at 10,000g at 4°C for 10 min and the supernatants were transferred into separate glass tubes that could be capped. Aliquots (200 μ l) of 6 M phosphoric acid and acetic acid were then added followed by 200 μ l of ninhydrin solution (25 mg/ml in acetic acid/6 M phosphoric acid; heated at 90°C to dissolve). The bottles were capped tightly to prevent evaporation of acetic acid while the samples were being heated. The samples were heated at 90°C for 20 min and the color developed was read at 515 nm after the samples were cooled to room temperature.

Results

We have found that Chinard's ninhydrin colorimetric assay yielded ambiguous results due to the fact that ninhydrin reacted with several other amino acids. The sample had to be heated in the presence of acetic acid and phosphoric acid and often it was difficult to prevent the acetic acid from evaporating, despite our best efforts to contain it. Therefore we searched for an alternative assay that was reliable and reproducible. We used the reliability and reproducibility of MALDI-TOF-MS to develop an assay for prolidase.

First we searched for an appropriate matrix that did not produce background peaks that interfered with the glycyl-proline or proline peak. We found that we could not use α -cyano-4-hydroxycinnamic acid as the matrix since it produced a peak overlapping with the glycyl-proline peak. However, we found that ferulic acid did not yield any peaks resembling either proline or glycyl-proline and therefore chose to use ferulic acid as the matrix for our assays.

The prolidase activity was followed using the appearance of the $[M+H]^+$ ion of proline (m/z 116) and disappearance of the $[M+H]^+$ ion of glycyl-proline (m/z 173) in the mass spectra of the samples. We were able to identify four children homozygous for prolidase deficiency using this assay. Also, we were able to confirm total prolidase deficiency in an adult. This is revealed by the appearance of the m/z 173 peak, showing that the glycyl-proline is not hydrolyzed, and the lack of liberated proline (m/z 116 peak).

Fig. 1 shows the disappearance of the dipeptide peak (glycyl-proline; m/z 173) and the appearance of the liberated proline (m/z 116) catalyzed by purified prolidase bought commercially. The proline peak did not appear when the dipeptide incubated in the absence of the enzyme (control). However, the peak intensity increased with increasing amounts of the purified enzyme concomitant with corresponding decrease in glycyl-proline. As can be seen the dipeptide is completely hydrolyzed when 0.256 U (1 unit corresponds to 1 μ M glycyl-proline hydrolyzed in 1 min at pH 8.0) of purified prolidase was used (Fig. 1, Prolidase-5).

We were able to unambiguously identify a complete lack of prolidase activity in four subjects using this assay, the results for one of which are given in Fig. 2

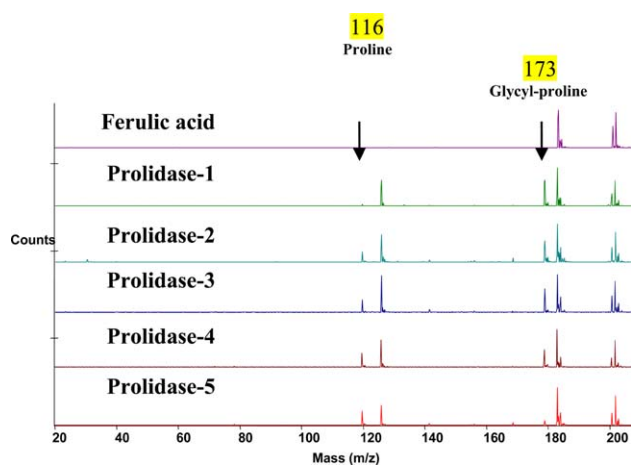


Fig. 1. The appearance of proline and disappearance of glycyl-proline using purified porcine prolidase. Ferulic acid shows the background peaks that are attributed to the ferulic acid matrix. Prolidase-1 refers to zero enzyme added. Prolidases 2–5 refer to increasing amounts of prolidase used (0.016–0.256 units; 1 unit corresponds to 1 μ M glycyl-proline hydrolyzed in 1 min at pH 8.0). X axis gives the mass to charge ratio (m/z). Y axis shows the intensity of the peaks in counts.

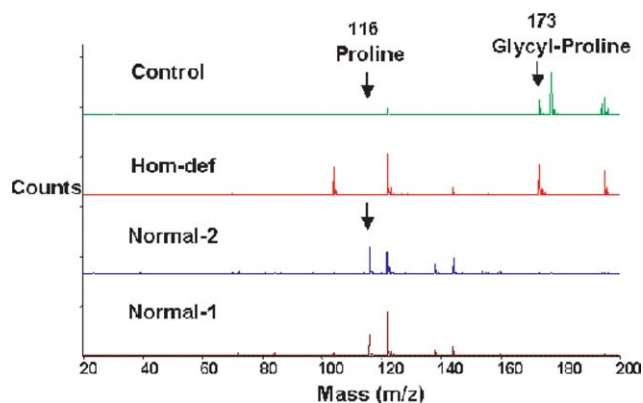


Fig. 2. The appearance of proline and disappearance of glycyL-proline using normal and prolidase-deficient human sera. Hom-def refers to sample that is homozygous for prolidase deficiency and m/z refers to mass/charge.

together with data from two normals. Three of them were infants less than 1 year old. In addition, we were able to obtain similar results using cord blood from a newborn baby. The proline peak failed to appear after incubation of the samples for 24 h (Fig. 2), concomitant with the nonutilization of the dipeptide. In addition, we were able to identify obligatory heterozygosity with respect to prolidase activity in the parents of two children born with complete lack of the prolidase enzyme and in another adult. It can be seen that the sera of normal subjects could hydrolyze the cleavage of the dipeptide (Figs. 2 and 3). The obligatory heterozygotes were able to hydrolyze only around half of the dipeptide when the samples were incubated with the dipeptide for 24 h while the normals hydrolyzed the dipeptide completely during that period.

Since the Y axis is arbitrary in this assay, we decided to compare the ratio of the areas beneath the peaks m/z 116 (proline) and m/z 173 (glycyL-proline) to calculate prolidase activity in the sera of the test subjects. Table 1 shows the results obtained using our method, analyzed

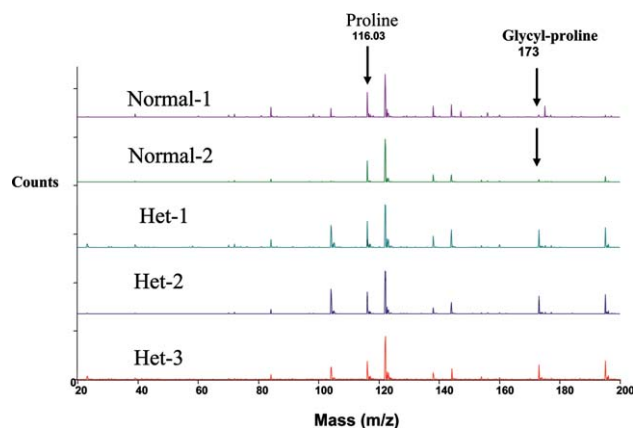


Fig. 3. The appearance of proline and disappearance of glycyL-proline using normal and prolidase-deficient human sera. Het refers to sample heterozygous for prolidase deficiency and m/z refers to mass/charge.

Table 1

Ratio of the area beneath the proline (m/z 116) and glycyL-proline (m/z 173) peaks for the samples that are homozygous and heterozygous for prolidase deficiency compared to activity for the same samples assayed according to Chinard's ninhydrin assay

No.	Ratio of area beneath m/z 116/173 peaks	Activity (Chinard's assay) (μmol of Gly-Pro/60 min/37 °C)
1	0.028 \pm 0.007	0.096
2	0.026 \pm 0.012	0.09
3	0.046 \pm 0.020	0.096
4	0.006 \pm 0.004	0.108
5	2.357 \pm 0.244	0.9

The assays were carried out, as mentioned under Materials and methods, to compare the activities of samples with homozygous and heterozygous prolidase activity. Values are expressed as means \pm standard deviation. Samples 1–4 are homozygous for prolidase deficiency while sample 5 is heterozygous for prolidase deficiency. Two other samples heterozygous for prolidase deficiency were also identified (m/z 116/173 ratio of 1.1 and 1.2). Chinard's assay was not carried out for these two samples. The mass spectrometry assays were carried out in triplicate. For the Chinard's assay the samples were assayed in duplicate, with the second set of tubes serving as the control (no dipeptide added). The control values were subtracted from the sample values to calculate prolidase activity.

as the ratio of the areas under the proline and glycyL-proline peaks, compared to results obtained using Chinard's ninhydrin assay. It can be seen that the patients homozygous for prolidase deficiency yielded ratios ranging from 0.006 to 0.04 using our method while obligatory heterozygotes yielded ratios ranging from 1.1 to

Table 2

Ratio of the area beneath the proline (m/z 116) and glycyL-proline (m/z 173) peaks for the samples that have normal prolidase activity compared to activity for the same samples assayed according to Chinard's ninhydrin assay

No.	Ratio of area beneath m/z 116/173 peaks	Activity (Chinard's assay) (μmol of Gly-Pro/60 min/37 °C)
1	102.28 \pm 4.66	2.06
2	49.36 \pm 2.05	1.92
3	114.06 \pm 32.70	1.58
4	101.63 \pm 17.71	0.91
5	239.07 \pm 43.87	0.11
6	67.82 \pm 8.84	1.5
7	106.82 \pm 15.35	1.44
8	96.07 \pm 3.81	0.38
9	128.04 \pm 7.87	0.43
10	67.60 \pm 3.26	1.92
11	30.89 \pm 9.7	2.26
12	14.65 \pm 2.53	1.65
13	26.65 \pm 5.73	1.38
14	97.55 \pm 5.43	1.49
15	54.68 \pm 2.93	1.95
16	111.66 \pm 0.71	1.54
17	50.93 \pm 11.04	2.02
18	39.42 \pm 4.36	1.68
19	9.03 \pm 0.98	0.36
20	26.32 \pm 5.83	ND

The assays were carried out as mentioned in the note to Table 1. Samples 1–20 are normal subjects. Samples with ambiguous results derived from Chinard's assay are given in boldface. ND refers to not determined.

Table 3

Means of the ratio of the area beneath the proline ($m/z = 116$) and glycyl-proline ($m/z = 173$) peaks for the samples that have normal prolidase activity compared to those with no prolidase activity

	<i>N</i>	Mean ratio \pm SD	Minimum value	Maximum value	Coeff. of variation
Homozygous PD	4	0.0262 \pm 0.016	0.006	0.045	61.034
Normal prolidase	20	76.68*** \pm 53.12	9.03	239.07	69.278

Values are expressed as means \pm standard deviation (SD). χ^2 9.6, $p < 0.0019$ (Kruskal–Wallis test); PD, prolidase deficient.

*** $p < 0.0028$.

2.4 (Table 1). Normals were found to yield ratios ranging from 9.0 to 239 (Table 2). In the subjects identified as prolidase deficient (homozygous deficient) using our method, prolidase activity was found to be around 0.1 μ mol of glycyl-proline hydrolyzed/60 min/37 °C using Chinard's assay. An obligatory heterozygote was found to have an activity of 0.9 μ mol of glycyl-proline/60 min/37 °C. Normals were found to have activities ranging from 0.1 to 2.26 μ mol of glycyl-proline hydrolyzed/60 min/37 °C. It can be seen that the Chinard assay could identify homozygous prolidase deficiency in known homozygous-deficient subjects. However, it identified one of the normal subjects as homozygous deficient and four other normals as having abnormally low prolidase levels (Table 2; given in boldface).

Table 3 shows the means of the ratio of the proline and glycyl-proline peaks of subjects with homozygous prolidase deficiency and normal activity assayed according to our method. Prolidase activity of the homozygous-deficient subjects was significantly lower than that of the normals ($p < 0.0028$). The coefficient of variation was found to be around 60 within the homozygotes and within the subjects with normal activity.

Discussion

Prolidase is an enzyme critical in the final stages of the metabolism of collagen which contains up to 25% proline or hydroxyproline. Collagenases degrade collagen such that dipeptides containing proline (or hydroxyproline) are produced. Prolidase then cleaves such dipeptides into free amino acids for proline to be reclaimed and recycled for use in synthesis of new collagen molecules. Normally, neither iminodipeptide nor free proline is excreted in the urine. Several assays that detect dipeptide in urine have been described for the detection of prolidase deficiency [24–26]. In addition, some assays have been used to detect dipeptides in human serum/plasma [27–29].

Our assay used MALDI-TOF-MS to detect the appearance of proline (m/z 116) with the concomitant disappearance of glycyl-proline (m/z 173) to detect prolidase deficiency. The liberated glycine did not appear as a peak in any of our mass spectra thus far, probably due to the fact that glycine cannot be ionized well by the laser beam. We had to incubate the sample for 24 h to

achieve complete hydrolysis of the dipeptide. We used ferulic acid as the matrix since it did not produce any background peaks corresponding to the peaks in which we were interested (m/z 116 and 173). We were unable to use α -cyano-4-hydroxycinnamic acid as the matrix since it had a peak at m/z 173 (data not shown).

Since the *Y* axis was arbitrary, we had to rely on comparing the area beneath the proline peak to the area beneath the glycyl-proline peak to arrive at a value to denote the state of prolidase deficiency (homozygous/heterozygous) or normalcy. The ratio for the normals showed a very wide fluctuation (9.0–239), suggesting that there appears to be a range of normal activity in these subjects.

Ninhydrin reacts with several amino acids such as lysine, proline, ornithine, glycine, arginine, histidine, and others [30,31]. Sugars have been shown to interfere with the color determinations of proline with ninhydrin [32]. We have found that Chinard's ninhydrin colorimetric assay yielded inconsistent results due to this nonspecificity of the ninhydrin color reaction. In our hands the assay identified one normal subject as being homozygous deficient, while it identified three other normal subjects as being nearly homozygous deficient. It was interesting to note that the subject identified with maximal activity with our method was identified as homozygous deficient using Chinard's assay. In addition, the Chinard assay identified one normal subject as being a heterozygote for prolidase activity.

The advantages of our assay over the classic Chinard assay are multifold. First, sample processing time has been reduced by a third. Second, caustic reagents such as phosphoric acid and acetic acid are not necessary. Third and most important, the results are unambiguous, allowing both homozygous and heterozygous deficiency to be identified.

Thus, the MALDI-TOF-MS assay described here for detecting prolidase activity is an useful tool that can assay for both homozygous and heterozygous deficiency reproducibly and efficiently without having to be exposed to the obnoxious fumes of acetic acid.

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