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Prolidase activity assays. A survey of the reported literature methodologies

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A B S T R A C T

Prolidase (EC.3.4.13.9) is a dipeptidase known nowadays to play a pivotal role in several physiological and pathological processes. More in particular, this enzyme is involved in the cleavage of proline- and hydroxyproline-containing dipeptides (imidodipeptides), thus finely regulating the homeostasis of free proline and hydroxyproline. Abnormally high or low levels of prolidase have been found in numerous acute and chronic syndromes affecting humans (chronic liver fibrosis, viral and acute hepatitis, cancer, neurological disorders, inflammation, skin diseases, intellectual disability, respiratory infection, and others) for which the content of proline is well recognized as a clinical marker. As a consequence, the accurate analytical determination of prolidase activity is of greatly significant importance in clinical diagnosis and therapy. Apart from the Chinard's assay, some other more sensitive and well validated methodologies have been published. These include colorimetric and spectrophotometric determinations of free proline produced by enzymatic reactions, capillary electrophoresis, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, electrochemoluminescence, thin layer chromatography, and HPLC. The aim of this comprehensive review is to make a detailed survey of the in so far reported analytical techniques, highlighting their general features, as well as their advantages and possible drawbacks, providing in the meantime suggestions to stimulate further research in this intriguing field.

1. Introduction

Prolidase (peptidase D, imidodipeptidase, EC.3.4.13.9) is a cytosolic dipeptidase found in all living organisms. Its main function is to hydrolyze imidodipeptides having proline (or hydroxyproline) at the carboxy terminus [1] (Fig. 1), thus finely regulating the homeostasis of free proline and hydroxyproline inside the body [2].

More in particular, prolidase promotes the last step of the degradation of procollagen, collagen, and other proline- and hydroxyproline-containing peptides into free amino acids making them available for cellular growth. Conversely, it also participates in the process of recycling proline from Xaa-Pro dipeptides for collagen resynthesis [3]. The only study about the kinetic characterizazion of prolidase has been reported by Besio and coworkers in 2011 [4]. This paper still represents a milestones in the field, being the only investigation related to the exact calculation of kinetic parameters of the subject enzyme. These Authors first evaluated numerous parameters influencing the rate and extent of the prolidase-catalyzed reaction, focusing in particular on Mn^{+2} and glutathione concentrations, temperature, and time. Thus, they found that optimal conditions for a reliable process were to employ $MnCl_2$ at a concentration of 1 mM, reduced glutathione at 0.75 mM for 20 min at 50 °C. Adopting these experimental conditions, Besio and coworkers accurately determined the V_{max}, K_m, and affinity for Mn^{+2} ions to be 489 U/mg, 5.4 mM, and 54 mM, respectively. Such values have been obtained in both the biological samples employed for the kinetic characterization, namely blood and fibroblasts lysates. Intra-assay coefficients of variability level in these two matrices were 8 % and 9 %, while the inter-assay 20 % and 21 %, respectively. These standardized and accurate measurement of prolidase activity provided by Besio and coworkers are essential for an effective and trustable diagnosis of disorders caused by prolidase malfunction. In fact, this enzyme is nowadays well known to be deeply involved in numerous physiological and pathological conditions [5,6]. Prolidase activity was found to be abnormally high respect to physiological levels in numerous acute and chronic syndromes like bipolar disorder, breast, endometrial, lung, and skin cancers, keloid scar formation, erectile dysfunction, liver diseases, hypertension, inflammation, and chronic pancreatitis. On the other hand, in case of prolidase deficiency, caused by congenital defects in the gene encoding for

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prolidase, collagen metabolism and proline circulation are partially or totally inhibited, resulting in a variety of disorders including chronic skin disease, intellectual disability, respiratory infection, splenomegaly, photosensitivity, and hyperkeratosis [7]. It is nowadays firmly accepted by the scientific community that prolidase can be included among the markers of all these syndromes and disorders [8–10]. Consequently, the accurate determination of prolidase activity is of great importance in clinical diagnosis, prevention, and therapy, not only to assess prolidase deficiency but also to elucidate the pathophysiology of other diseases. The oldest and most widely employed method for prolidase activity determination is the Chinard's reaction [11]. During recent years, some other more sophisticated, more sensitive, and well validated methodologies for the same purpose have been published in the literature. These include other suitably colorimetric and spectrophotometric determinations of free proline produced after the enzymatic reaction, capillary electrophoresis alone and/or coupled to electrochemoluminescence detection, fluorimetry, HPLC, thin-layer chromatography, isotachophoresis, kinetic characterization, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Thus, the aim of this comprehensive review is to make a detailed survey of the in so far reported techniques, highlighting their general features, as well as their advantages and possible drawbacks, providing in the meantime suggestions to stimulate further research in this intriguing field. Data have been acquired from the most popular web-based databases like PubMed, Google Scholar, Scopus, and WoS. The time interval considered covers the years from 1952 to the present day. The different analytical techniques other than the Chinard's reaction have been grouped in the present manuscript following the same order as mentioned above.

2. Analytical assays

2.1. The Chinard's reaction and other colorimetric detection methodologies

In 1952 Adams and Smith characterized for the first time prolidase in horse erythrocytes [1]. These Authors provided also details about the substrate specificity for this enzyme as well as the absolute need of Mn^{+2} ions as cofactors to effectively promote the cleavage of proline containing dipeptides. In the same year Chinard set up a fast and easy-to-handle procedure for the qualitative and quantitative determination of proline in a wide array of matrices, including biological ones [11]. Therefore, this last methodology has been followed in subsequent years to assess the activity of prolidase. The Chinard's reaction applied to this enzymatic assay consisted in the colorimetric quantification at (λ_{max} = 255 nm) after derivatization with ninhydrin of free proline in aqueous solution at pH 1 [11]. However, this method has some limitations: low specificity for proline and susceptibility to some colored interferences in the substrates. Therefore, in subsequent years some modifications to ameliorate the performance of this technique have been proposed. Among the most explicative examples to this aim are those reported by Myara and coworkers in 1982 who succeeded in achieving a > 3-fold increase in prolidase activity (thus increasing sensitivity of the assay) by adding Mn^{+2} ions 24 h prior performing the test [12] and by Torcin and coworkers in 2003, who found that the addition of reduced glutathione to the assay medium containing prolidase and its substrates greatly enhances the stability of this enzyme over time [13]. Both modifications have been successfully applied to determine the activity of prolidase in several cell lines and biological samples including plasma, skin fibroblast, liver collagen, and erythrocytes. The method modified by Myara has been successfully employed several times by Pałka and coworkers in numerous *in vitro* cultured human cells [14]. A substantial modification of the Chinard's reaction, both in its original form and in its variations, has been reported by Cauci and coworkers in 1998. These Authors employed L-proline-*p*-nitroanilide as the substrate instead of the "classic" glycyl-L-proline, finally quantifying the activity of prolidase in vaginal fluids recording the appearance of *p*-nitroaniline at 405 nm [15]. However, the applicability of this methodology has been very limited with only this example reported in the literature, also because of a poor reproducibility of results, as highlighted later by Kurien and coworkers [16]. Despite the numerous modifications of the original Chinard's experimental protocol and the fact that the direct spectrophotometric prolidase assays techniques are more practical and less time-consuming, higher accuracy is needed as quite different data have been obtained using the same method applied to the diagnosis of different syndromes caused by prolidase deficiency or hyperactivity. To this aim, very recently Kayadibi and coworkers described in sharp details the optimization of prolidase activity assays taking into proper account the influence of a series of parameters never considered before [17]. Thus, these Authors examined the efficiency and performance of the Chinard's reaction under the following points of view: a) enzyme-substrate incubation step, b) activation step, c) proline assay step. They finally validated their results in a single experimental model. More in particular, the best conditions for the enzyme-substrate incubation time and temperature were set for the Gly-Pro substrate in the presence or absence of human prolidase at 37 °C, 45 °C, 50 °C, and 55 °C for 5, 10, 15, 20, 25, 30, 40, 50, and 60 min, respectively and after activation with reduced glutathione 1 mM and $MnCl_2$ 50 mM at 37 °C for 30 min. To this context, the optimal parameters for which prolidase exhibited the best performance were shown to be incubation at 45 °C for 5 min. Applying this temperature, the activation step was then optimized using different concentrations of the activators $MnCl_2$, glutathione, and Triton X-100 in the time range 0–180 min. Under these experimental conditions, the best activation was achieved after 30 min of incubation with the addition of the combination 63 mM $MnCl_2$, 1 mM reduced glutathione, and 0.1 % Triton X-100. Finally, using different concentrations of ninhydrin dissolved in H_2O with the addition of different quantities of glacial acetic and orto-phosphoric acids and Tris-HCl buffer to reach a final pH value of 7.8, Kayadibi and coworkers firmly stated that incubating samples resulting from the prolidase action for 45 min at 80 °C with ninhydrin 15 g/L in a 6 : 4 glacial acetic acid/H_2O mixture and applying a reading interval up to 90 min for samples kept in ice water, represented the optimized conditions for the quantification of free proline. For the method validation, the recorded intra- and inter-assay precision values

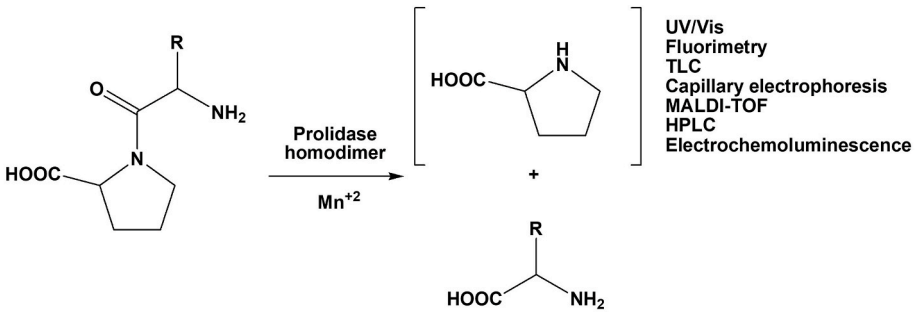

Fig. 1. Catalytic action of prolidase and strategies for the quantification of its effects.

were 7.4% and 6.2%, respectively, the correlation coefficient was 0.992, and the prolidase assay was linear in the range 150–4492 U/L. Taken together, these data clearly showed how the novel and alternative version set up and validated by Kayadibi and coworkers did follow the provisions given by the Clinical and Laboratory Standards Institute (CLSI) and could be considered as acceptable. Indeed, the technique rigorously elaborated by these Authors represents the best application of the Chinard's reaction for the quantification of prolidase activity in terms of easy-to-handle procedure, efficiency, and statistical significance.

2.2. Capillary electrophoresis and electrochemoluminescence

Capillary electrophoresis (CE) is among the most suitable separation techniques for the analysis of a wide panel of compounds in chemically complex matrices. It provides a very good degree of separation efficiencies even in the presence of small quantities of analytes over a short period of time [18]. The first application of CE for the determination of prolidase activity has been reported in the literature by Zanaboni and coworkers in 1997 [19]. These Authors employed different proline-containing dipeptides, but recorded the best data with the "classic" substrate Gly-Pro. They first recorded the calibration curve of this latter using a 75 μm internal diameter and 57 m total length uncoated-fused silica capillary and a run buffer composed by 35 mM sodium tetraborate and 65 mM SDS. Under these experimental conditions, the response was linear over the range 0.1–30 mM and an r^2 value of 0.998 was recorded. Subsequently, these Authors assayed prolidase activity by CE in human erythrocytes and skin fibroblast cultures comparing the values obtained with the ones recorded applying the Myara's version of the Chinard's reaction. The method set up and validated by Zanaboni and coworkers had undoubted advantages over any of the colorimetric and/or spectrophotometric techniques previously reported. In all cases CE revealed higher accuracy, speed, specifity, and sensitivity respect to the Chinard's reaction. Prolidase activity values recorded by CE were higher in the range of 0.40–0.60 μmol Gly-Pro hydrolyzed/mg protein/min. Furthermore, using CE, it was also possible to assess prolidase activity using the dipeptide Phe-Pro as the substrate. This was not allowed in the case of colorimetric and/or spectrophotometric methodologies as Phe represented in all cases an interference from the matrix that could not be eliminated. A few years later also the CE method was susceptible to improvements. In 2003 Lupi and coworkers applied CE for the detection of Gly-Pro in medium, cell layer, and matrix obtained from long term cultured human fibroblasts of control and prolidase deficiency patients [20]. In particular, these Authors investigated in detail the effects of modifications of some key experimental parameters on the efficiency and performance of the overall CE process. Thus, the best conditions to modify the original CE techniques reported by Zanaboni and coworkers were shown to be the following: pH was modified from 9.3 to 2.5 and ionic strength from 50 to 100 m*M*, capillary diameter was decrease from 75 μm to 50 μm, the injection time was increased from 2 s to 30 s and the same for the voltage from 7 kV to 30 kV. These modifications resulted in an appreciable increase of the sensitivity of the overall analytical method. Furthermore, the addition of 30 m*M* α-cyclodextrins improved resolution, sensitivity, and reproducibility in cell layer and matrix samples. The method set up by Lupi and coworkers was validated in terms of sensitivity, linearity, accuracy, precision, and robustness. Limit of detection (LOD) and limit of quantification (LOQ) values were 5.0 μM and 6.0 μM, respectively, the r^2 0.0059, and the average accuracy over the concentration range 1 μM–30 μM was 109.0 %. The versatility and general applicability of CE was subsequently and greatly enforced and underlined by its hyphenation to some other highly sensitive techniques and detection methodologies. Thus, in 2006 Quintana and coworkers showed how the coupling of CE to inductively coupled plasma mass spectrometry was fundamental for the characterization of Mn-containing enzymes in liver extracts [21]. These Authors in fact, after fractionation of the biological samples using preparative size exclusion chromatography, succeeded in determining the presence of prolidase by the application of the above-mentioned methodology in the subfraction corresponding to a mass range of 54–65 kDa. The paper by Quintana and coworkers clearly showed how the hyphenation of CE to inductively coupled plasma mass spectrometry represented a powerful tool for the assessment of prolidase in tissue extracts by a relatively easy-to-handle procedure. Quite surprisingly this technique was not followed up in subsequent years. In parallel and in the same year, the team by Yuan and coworkers reported the application of CE coupled to electrochemoluminescence detection with tris(2,2′-bipyridyl)ruthenium (II) [22,23]. These Authors provided evidence of a highly efficient and greatly sensitive method able to detect proline, as a result of prolidase catalytic activity, in the fmol scale. More in particular, they reported two different examples of this novel analytical technique. In the first one, CE coupled to electrochemoluminescence detection with tris(2,2′-bipyridyl)ruthenium(II) was employed to the kinetic study of the interaction between prolidase and four common non-steroidal anti-inflammatory drugs like aspirin, paracetamol, sodium salicylate, and phenacetin in cultured erythrocytes. By this way, it was possible to highlight how paracetamol behaved as an effective noncompetitive inhibitor of the enzyme under investigation. In this context the level of sensitivity of this novel CE-based methodology was at least 10-fold higher than the one recorded by Zanaboni's and coworkers, and hundreds of folds higher than the Chinard's reaction. Key parameters for a reliable detection of proline were found to be the concentration of $Ru(bpy)_3^{+2}$ and the pH of the assay medium. Under optimized experimental conditions, the first was set to 5 mM and pH to 7.5 providing for the detection of proline a linear range in the range 0.01 mM–3.75 mM ($r^2 = 0.999$) and a LOD of 5.0 μM. The second study reported by Yuan and coworkers in 2006 was focused on the study of collagen degradation as occurs during diabetes mellitus, following the prolidase promoted hydrolysis of the dipeptidide Gly-Pro in serum and plasma samples from clinical patients. After having sharply calibrated two other critical experimental parameters, namely the concentration of Mn^{+2} ions to 1 mM and the one of the substrate to 10 mM, these Authors provided clear evidence that detection limit for proline was 12.2 fmol (S/N = 3), corresponding to 1.22×10^{-8} U of prolidase catalyzing for 1 min. This acquisition, together with a wide linear range and good reproducibility, made the CE having the mentioned Ru (II) complex as the electrochemiluminescence detection mean an excellent protocol for the determination of prolidase activity in the clinical practice. Despite these favorable features, also this method experienced a scarce follow-up. The only example to this concern has reported very recently by Yang and coworkers [24]. These Authors developed and validated the performance of the first biosensor for the determination of prolidase activity based on the same Ru (II) complex mentioned above. Thus, $Ru(bpy)_3^{2+}$ was electrophoretically deposited within nanochannels of vertically-ordered mesoporous silica film on In Sn oxide electrodes. This new biosensor provided an excellent response towards proline attributable to the enhanced concentration of the reactants and improved electron transfer resulting from the nanoconfinement effect. Furthermore, the concentration of both Mn^{+2} and the dipeptide Gly-Pro as the substrate could be lowered to 0.1 mM and 1 mM, respectively, when compared to the previously mentioned Ru (II)-based electrochemiluminescence detection methods. Yang and coworkers recorded a linear response in the range 10–10000 U/L, with a detection limit of 1.98 U/L of prolidase. This recent discovery undoubtedly represents the most advanced and sensitive technique in the field of prolidase determination by CE. Having been reported in the literature very recently, it has had no other application examples in the literature. However, the feeling is that, since this same solid state biosensor technique is characterized by very high sensitivity and very favorable analytical parameters, it will be followed up by other research groups. If necessary, it could be further modified to increase analytical effectiveness, and will have notable applications in clinic and therapy for the diagnosis of all syndromes in which prolidase is involved.

2.3. Fluorimetry

The first fluorimetric measurement of prolidase activity has been reported in the literature in 1984 by Priestman and Butterworth [25]. However, the usefulness of this test was very limited as the fluorimetric detection was possible only using the dipeptide Phe-Pro as the substrate for prolidase and quantifying free Phe using the L-aminoacid oxidase assay. Using differently structured dipeptides prevented the use of such technique. Only several years later, namely in 2023, a much more versatile technique based on fluorimetric detection has been reported by Kabashima and coworkers [26]. The method set up and validated by these Authors is based on a fluorescence reaction selective for *N*-terminal glycine-containing peptides using 3,4-dihydroxyphenylacetic acid. In fact, this latter can selectively react with Gly–Pro, the substrate for prolidase, in the presence of borate and periodate anions. Under these experimental conditions unreacted dipeptide Gly-Pro form a 1:2 boron complex with 3,4-dihydroxyphenylacetic. The prolidase activity was determined by monitoring the decrease in fluorescence intensities at 370 nm excitation and 465 nm emission wavelengths. Using this method, prolidase activities were successfully measured in both human fibroblasts and cancer HeLa cells. The technique provided by Kabashima and coworkers is very simple and it did not require pre-incubation or deproteinization as necessary in all colorimetric and/or spectrophotometric methodologies. This fluorimetric assay is greatly versatile and may be applied to determine the prolidase activity in several mtrices deriving from human organs and tissues, both normal and cancer ones. As in the previous case, also this method has been described very recently, so it has so far, no other application examples in the literature, but it is hopeful that numerous applications will be reported in the next future.

2.4. HPLC

High performance liquid chromatography (HPLC) has been extensively used for the determination of prolidase activity in the 90s of the last century. The first applications to this concern has been reported in 1990 by Kodama and coworkers [27]. These Authors set up and validated an HPLC method coupled to mass spectrometry for the detection of a series of imidodipeptides in urine of patience affected by prolidase deficiency. The separation of these samples has been achieved on a reversed-phase column using 0.1% aqueous CF_3COOH – MeOH (70:30 or 80:20) as the mobile phase. The quantification of imidopeptides was accomplished by studying their fragmentation and comparing it with that recorded for a series of pure chemical standard. Very intensive molecular ion peaks have been obtained by this methodology. In the same year Harada and coworkers described a similar methodology but using only Gly-Pro as the substrate of the enzyme, a mobile phase consisting in an aqueous solution buffered at pH 2.1 and containing 0.5 mM octane-1-sulphonate, and finally serum from young adult patients affected by suspected prolidase deficiency as the biological matrix [28]. In subsequent years, the HPLC procedure was widened also to other biological samples like erythtocytes, leukocytes, and skin fibroblasts, providing in all cases appreciable results in terms of sensitivity and statistical significance [29]. A much more sensitive method has been reported in 2003 by Hu and coworkers [30]. These Authors investigated the distribution and functions of rat intestinal prolidase. They employed Phe-Pro as the substrate to maximize the response of the UV/Vis detection following the appearance of the chromatographic peak of Phe as a result of enzymatic catalysis and a mobile phase consisted in a 8:2 mixture of an aqueous solution of NaH_2PO_4 buffered at pH 5.0 and MeOH. Adopting these experimental conditions, Hu and coworkers succeeded in achieving a sensitivity of 100 pmol, a value never achieved before using HPLC methods. Thanks to this high sensitivity, these Authors sharply quantified the values of key parameters related to prolidase like K_m and V_{max}. Despite its versatility and ease of use, the application of HPLC-based methodologies, even in the form of hyphenated procedures, has seen a drastic decrease in the last two decades. This could be due to the appearance in the literature of more sophisticated techniques like CE and electroluminescence. However, it is hopeful that in the next future a renovated interest on HPLC as an analytical tecnhiques to record prolidase activity in biological matrices could be reported. The few examples cited herein and so far reported in the literature are perhaps too focused on the modification of columns and mobile phases to try to increase statistical significance and sensitivity of the method, while other key steps of the chromatographic analysis (e.g. sample preparation) could be taken in due count in the incoming years for the same purpose.

2.5. Thin-layer chromatography

Despite its low sensitivity, thin-layer chromatography (TLC) has been also proposed as a technique for the measurement of prolidase activity. This occurred practically parallel to the times of application of the Chinard's reaction with the scope of finding and validating an alternative route to this methodology. Thus, in 1979 Arata and coworkers tried to assess prolidase activity by cellulose TLC plates in cultured fibroblasts taken from a single subject suffering from its deficiency [31]. These Authors succeeded in confirming the total absence of prolidase in the selected biological matrix. However, most likely due to the very low sensitivity and statistical significance that characterizes all TLC methods, this example was no longer followed up in subsequent years.

2.6. Isotachophoresis

Isotachophoresis (ITP) is a versatile application of electrophoretic techniques that can be used for sample preconcentration, separation, purification, and mixing, and to control and accelerate chemical reactions. It consists in the separation of charged constituents in an electric field due to their differences in their electrophoretic mobilities. ITP is usually carried out in capillaries. This method has the advantages of no sample preparation, speed, applicability to a wide sample range [32]. Despite these undoubted advantages and notable versatility, until now ITP has found only one practical application for the determination of prolidase activity. Thus, in 1984 Mikasa and coworkers quantified this parameter by ITP in erythrocytes from a patient with severe imidopeptiduria, measuring in parallel both dipeptides and single amino acids resulting from the enzymatic catalysis [33]. The separations were carried out in a capillary tube which was maintained at a constant temperature of 20 °C. The leading electrolyte consisted of 10 mM HCl and 2-amino-2-methyl-l-propranol buffered at pH 7.5. The terminal electrolyte was 10 mM γ-aminobutyric acid and $Ba(OH)_2$ buffered at pH 10.9.The migration current was set at 75 μA. Under these experimental conditions, the Authors achieved a very good separation of Gly-Pro and glycine allowing to follow the concentrations of both to further validate the analytic method. This technique was compared to the Chinard's reaction as varied by Myara and collaborators (the only technique that in 1984 could be considered as a comparison) revealing considerably greater sensitivity and significance. The report by Mikasa and coworkers clearly revealed how isotachophoresis was a versatile and easy-to-handle analytical methodology able to record prolidase activity with good reliability and reproducibility. So it is quite surprising that no other research team adopted the same and/or modified experimental scheme for the same purpose. Thus, it is hopeful that for the next future also isotachophoresis can be the subject of experimental studies on the same enzyme and, even more importantly, using numerous other biological matrices.

2.7. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF)

Matrix-assisted laser desorption/ionization (MALDI) is an ionization

analytical method that employs a laser energy-absorbing matrix to create ions from large molecules (e.g. biopolymers such as DNA, proteins, peptides and carbohydrates or purely synthetic polymers, dendrimers and other macromolecules) inducing a minimal fragmentation and cannot be fragmented using more conventional ionization techniques due to their chemical liability. The type of a mass spectrometer most times coupled to MALDI is the time-of-flight mass spectrometer (TOF), having a large mass range [34]. The first and until now only application of MALDI-TOF for the determination of prolidase activity has been reported in 2004 by Kurien and coworkers [35]. These Authors used human serum for their investigation and incubated the biological sample with a mixture containing the dipeptide Gly-Pro in Tris–HCl supplemented with Mn^{+2} at 37 °C for 24 h. The samples were finally precipitated with CF_3COOH, centrifuged, and the supernatant mixed with an equal volume of ferulic acid solution prior MALDI-TOF analysis. The activity of the enzyme was determined following the disappearance of the Gly-Pro peak ($m/z = 173$) with the concomitant appearance of the proline peak ($m/z = 116$) and expressed in terms of the ratio of the area beneath the proline to the area beneath the Gly-Pro peaks. The process described by Kurien and coworkers compares favorably to the other most sensitive methodologies mentioned above, like CE, electrochemiluminescence, and ITP, especially in terms of sample processing, reproducibility of results from different biological matrices, and short experimental times. Together with these same techniques the application of MALDI-TOF represents one of the most powerful and effective diagnostic tools in the field of prevention and therapy of syndromes associated with prolidase malfunction.

3. Conclusions and future perspectives

Prolidase is a metallo-exopeptidase hydrolyzing X-Pro and X-Hyp dipeptides. Its reduced level is the basis of prolidase deficiency and malfunctions in general of this enzyme were reported in various diseases. All of the literature reviews concerning prolidase up to now are focused on its role in the clinical practice, while, despite its importance, no surveys about the analytical methodologies to determine its activity have been cited so far. In this manuscript we tried to fill this gap, providing the readers a detailed description of all the analytical process

Table 1
A survey of the methodologies so far reported in the literature aimed at quantification of prolidase assay.

Method	Substrate(s)/Reagent(s)	Sensitivity	Advantages	Disadvantages	Applicability
UV/Vis (Chinard's method)	Proline dipeptides, l-proline-*p*-nitroanilide proline, *p*-nitroaniline/Ninhydrin	150–4500 U/L	Fast, cheap, and easy-to-handle procedure; enhanced sensitivity by addition of GSH and Triton X	Low specifity; interferences from the matrix; poor reproducibility	Cell cultures (e.g. plasma, skin fibroblast, liver collagen, and erythrocytes); rapid screening for prolidase-based syndromes (e.g. prolidase deficiency)
Capillary electrophoresis	Proline dipeptides, L-Pro, α-cyclodextrins	0.40–0.60 μmol Gly-Pro hydrolyzed/mg protein/min	Very good separation efficencies; high accuracy, speed, specifity, and sensitivity; enhanced sensitivity by the addition of α-cyclodextrins and by coupling plasma mass spectrometry	Need of specific equipment (often expensive); poor follow-up	Cell cultures (e.g. skin fibroblast, erythrocytes); screening for prolidase-based syndromes
Electrochemoluminescence	Proline dipeptides, L-Pro/tris (2,2′-bipyridyl)ruthenium (II)	fmol scale (equivalent to 10^{-8} U/L order of magnitude)	Very good separation efficencies; high accuracy, speed, specifity, and sensitivity provision of insights into the enzymatic mechanism of action and binding studies; adaptability for the use of biosensors	Need of specific equipment (often expensive); poor follow-up	Human serum and plasma samples; screening for prolidase-based syndromes
Fluorimetry	Phe-Pro, Gly-Pro, Phe/l-aminoacid oxidase, 3,4-dihydroxyphenylacetic acid, borate and periodate anions	10–60 μM	Cheap, and easy-to-handle procedure	In some cases only Phe-Pro can be used as the substrate and detection limited to Phe after enzymatic reaction promoted by l-aminoacid oxidase; poor follow-up	Cell cultures (human fibroblasts, HeLa cells); screening for prolidase-based syndromes
HPLC	Phe-Pro, Gly-Pro, Phe/UV/Vis and MS detection	Hundreds of pmol scale	Cheap, and easy-to-handle procedure; high accuracy, speed, specifity, and sensitivity; enhanced sensitivity by coupling to MS	Reported studies are narrowly focused on the modification of columns and mobile phases, No other parameters (e.g. sample processing) not taken into proper account so far	Cultured cells (erythtocytes, leukocytes, skin fibroblasts); biological fluids (e.g. urine) screening for prolidase-based syndromes
TLC	Proline dipeptides, L-Pro/Ninhydrin	Low mM scale	Cheap, and easy-to-handle procedure; valid alternative to the Chianrd's reaction	Very low sensitivity; poor follow-up	Cultured cells (skin fibroblasts), useful for a very preliminary screening for prolidase-based syndromes (e.g. prolidase deficiency)
Isotachophoresis	Proline dipeptides, Gly	Low μM scale	No sample preparation; high accuracy, speed, specifity, and sensitivity; good reliability and reproducibility	Need of specific equipment (often expensive); poor follow-up	Cultured cells (erythrocytes); screening for prolidase-based syndromes
MALDI-TOF	Gly-Pro, L-Pro/ferulic acid	Low nmol scale	High accuracy, speed, specifity, and sensitivity; good reliability and reproducibility, short experimental times	Need of specific equipment (often expensive)	Cultured cells and biological fluids (e.g. serum); screening for prolidase-based syndromes

used for this purpose. A survey of the in so far literature reported methodology has been reported in Table 1, in which key elements like substrates, sensitivities, advantages, disadvantages, and applicability are compared to let the reader to have an easier overview of all techniques.

It is evident how the interest towards determination of prolidase activity has continued in parallel with the investigation of the role of this enzyme in the various diseases mentioned above. Over years in fact, the awareness of clinicians and researchers in general increased about the fact that the accuracy in determining the activity of this enzyme could represent a unique way for the diagnosis of severe syndromes like prolidase deficiency and related disorders. The survey reported herein clearly indicated how the Chinard's reaction, although representing the widest employed assay, must be replaced by largely more sensitive analytical methodology. In this sense CE coupled to electroluminescence, ITP, and MALDI-TOF are nowadays the most powerful tool to this concern. On the other hand it has to be considered that these techniques are not easily available to a large audience, requiring expensive equipment and facilities, as well as considerable experience in the field. So it would be desirable to overcome this problem, that more accessible and cheap methodologies like HPLC-, UHPLC-, LC-MS-based ones could subject of a renovated interest. Indeed, from the few examples reported so far in the literature to this regard, it is evident that no detailed investigations on how to increase the performance were practically not accomplished: several parameters influencing the overall chromatographic steps (e.g. sample handling and preparation, derivatization, detection, etc.) were not properly considered. As a consequence, in the field of liquid chromatography techniques there is much space for improvements and in-depth studies. In this way, assays for determining prolidase activity will be more accessible to a wider audience of researchers with positive results also in the clinical practice. The diagnoses of anomalies linked to the abnormal functioning of this enzyme will reveal more easily and consequently the related diagnoses will be easier to identify.

CRediT authorship contribution statement

Serena Fiorito: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Salvatore Genovese:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Francesco Epifano:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Chiara Collevecchio:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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