Current Topics

Applications of Fluorescence Detection in Current Pharmaceutical Research

Regular Article

A Facile Method to Determine Prolidase Activity Using a Peptide-Specific Fluorometric Reaction

Tsutomu Kabashima,*[,a] Nana Hamasaki,[a] Keiko Tonooka,[b] and Takayuki Shibata[c]

[a] *Faculty of Pharmaceutical Sciences, Nagasaki International University, 2825–7 Huis Ten Bosch machi, Sasebo, Nagasaki 859–3298, Japan:* [b] *Department of Pathophysiology, Yokohama University of Pharmacy, 601 Matano-cho, Totsuka-ku, Yokohama 245–0066, Japan: and* [c] *Department of Laboratory Sciences, Gunma University Graduate School of Health Sciences, 3–39–22 Showa-machi, Maebashi, Gunma 371–8514, Japan.*

Received June 28, 2022; accepted September 27, 2022

Prolidase is the only enzyme capable of cleaving imidodipeptides containing C-terminal proline (Pro) or hydroxyproline and plays a crucial role in several physiological processes such as wound healing and cell proliferation. Here, we developed a new method to determine prolidase activity. This method is based on a novel fluorescence (FL) reaction selective for N-terminal glycine (Gly)-containing peptides using 3,4-dihydroxyphenylacetic acid (3,4-DHPAA). The 3,4-DHPAA can selectively react with Gly–Pro, the substrate for prolidase, and the prolidase activity is measured by monitoring the decrease in FL intensities. The prolidase activities in fibroblasts and HeLa cells were successfully measured by the proposed method. Compared with classical Chinard's method, our method does not require any caustic acids, pre-incubation to activate the enzyme, and heating for reaction with the detection reagent. The proposed method enables facile and specific measurement for biogenic prolidase activity.

Key words prolidase, proline, fluorescence reaction, iminodipeptide

Introduction

Prolidase is a manganese-dependent metallopeptidase that is widely distributed from animals to bacteria. Prolidase hydrolyzes iminodipeptides containing proline or hydroxyproline at the C-terminus (X-Pro) and releases the proline moiety.[1)] In humans, prolidase is located in different cells[2–7)] and is related to protein catabolism and circulation of proline through collagen metabolism.[8,9)] Therefore, prolidase affects matrix remodeling and cell growth. In addition, physiological and pathological importance of prolidase is suggested in wound healing, inflammation, vascularization, cell proliferation, and protein synthesis.[10,11)]

The human prolidase gene was identified by Endo *et al.*[12)] During prolidase deficiency caused by congenital defects in the prolidase gene, collagen metabolism and proline circulation are inhibited, resulting in a variety of disorders including chronic skin disease, intellectual disability, and respiratory infection.[13,14)] In prolidase-deficient patients, accumulation of proline (Pro) or hydroxyproline-containing dipeptides in urine and cells is observed owing to lack or reduction in prolidase activity caused by mutations.[15)] Significant changes in prolidase activity have been reported in various diseases, including cancer, renal, hepatic, and respiratory disorders. Prolidase serves as ligands for various receptors and modulates p53 during cellular regulation. Therefore, accurate measurement of prolidase activity in biological samples is important not only to diagnose prolidase deficiency but also to elucidate the pathophysiology of other diseases.[16)]

Existing assay methods of prolidase activity include colorimetric determination of proline produced by enzymatic reaction,[17)] capillary electrophoresis,[18)] matrix-assisted laser desorption/ionization time-of-flight mass spectrometry,[19)] and HPLC.[20)] In particular, Chinard's method by colorimetric determination using ninhydrin is the simplest method and currently the most frequently used in determining of prolidase activity.[17,21)] However, this method has low specificity for proline and is complicated; therefore, developing a rapid and facile method to determine prolidase activity is highly desirable.

We developed a peptide-specific fluorescence (FL) reaction to measure the activities of various enzymes.[22–24)] We found that 3,4-dihydoroxyphenylacetic acid (3,4-DHPAA) selectively reacts with short peptides containing N-terminal glycine (Gly), specifically N-terminal Gly–Pro containing peptide and produced FL[25)] (Fig. 1). In this study, we utilized this reaction to develop a novel fluorometric assay to determine prolidase activity using Gly–Pro as a substrate.

Experimental

Reagents Gly–Pro was purchased from BACHEM (Bubendorf, Switzerland); 3,4-DHPAA was purchased from Tokyo Chemical Industry (Tokyo, Japan); and prolidase from porcine kidney (121 U/mg) was purchased from Sigma-Aldrich (Saint Louis, MO, U.S.A.).

Preparation of Cultured Cell Extracts Fibroblasts and HeLa cells were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum in a humidified

* To whom correspondence should be addressed. e-mail: kabashima@niu.ac.jp

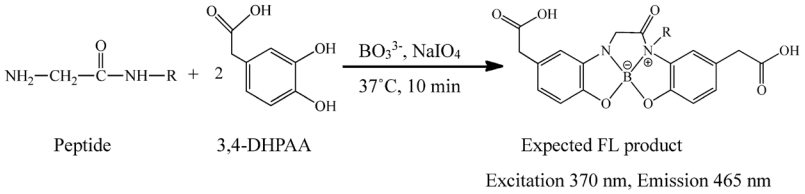

Fig. 1. FL Reaction of an N-Terminal Gly-Containing Peptide with 3,4-DHPAA

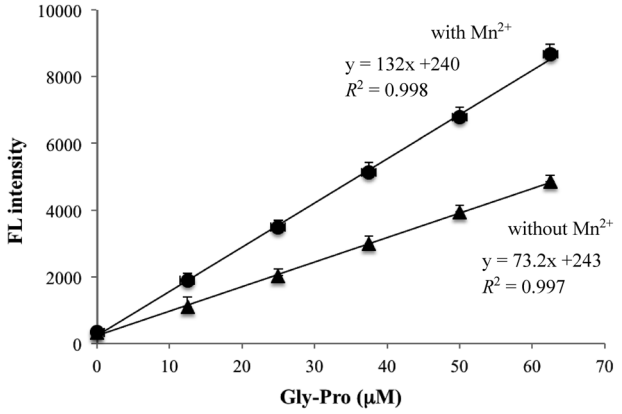

Fig. 2. Calibration Curve of Gly–Pro Using 3,4-DHPAA

Gly–Pro at different concentrations was reacted with 3,4 DHPAA at 37 °C for 10 min either in the presence of 62.5 μM $MnCl_2$ or without $MnCl_2$ ($n = 3$ each).

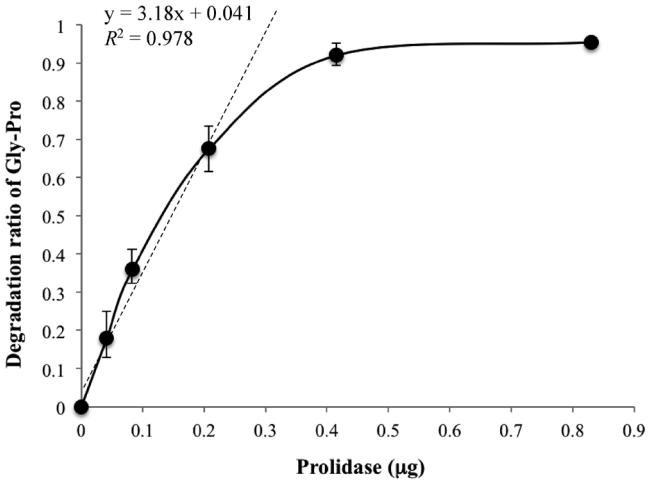

Fig. 3. Calibration Curve of Prolidase

Commercially available purified prolidase was used. The degradation ratio of Gly–Pro was calculated from the decrease in FL intensity with/without (enzyme $n = 3$).

chamber containing 5% CO_2 at 37 °C. The cells were collected and lysed by sonication at 4 °C. The lysates were centrifuged at 12000 × ***g*** for 10 min. The supernatants were collected and stored at −80 °C until further use. Total proteins in the cells were measured with a Quick-Start Bradford Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Measurement of Prolidase Activity Using 3,4-DHPAA The supernatants (25 μL) containing prolidase were mixed with 50 μL of 0.5 mM Gly–Pro, 25 μL of 100 mM borate buffer (pH 8.0) containing 1 mM $MnCl_2$. The mixture was incubated at 37 °C for 20 min. The enzymatic reaction was stopped by heating the reaction mixture at 90 °C for 10 min and cooling in an ice bath for 5 min.

To the above solution (100 μL), 100 μL of 1.25 mM $NaIO_4$, 100 μL of 125 mM borate buffer (pH 8.0), and 100 μL of 0.75 mM 3,4-DHPAA were added and incubated at 37 °C for 10 min. The FL reaction was stopped by cooling in an ice bath for 10 min.

FL intensity was measured at 370 nm excitation and 465 nm emission wavelengths using an FP-8200 Spectrofluorometer (Jasco Co., Tokyo, Japan). The amount of degraded Gly–Pro was calculated from the decrease in FL intensity.

Results and Discussion

Measurement of Prolidase Activity by 3,4-DHPAA Prolidase requires Mn^{2+} for its activity. The FL reaction between 3,4-DHPAA and the peptide takes place in the presence of an oxidant; therefore, the effect of Mn^{2+} on the reaction was investigated. FL intensity increased with the addition of $MnCl_2$ to the reaction mixture, indicating that $MnCl_2$ did not interfere with this assay (Fig. 2). Figure 2 shows the calibration curve of Gly–Pro in the presence of 62.5 μM $MnCl_2$. Since significant linearity was observed between Gly–Pro concentration and FL intensity even in the presence of Mn^{2+}, the amount of degraded Gly–Pro by prolidase could be calculated by the decrease in FL intensity.

In this study, prolidase activity was measured using commercially available purified enzyme, and a calibration curve was prepared (Fig. 3). The FL intensity decreased with increasing the amount of the enzyme, therefore confirming the successful measurement of prolidase activity. The linear regression equation of the calibration curve for prolidase (0–0.2 μg) was $y = 3.18x + 0.041$ (x = amount of prolidase and y = degradation ratio of Gly–Pro) with a correlation coefficient (R^2) of 0.978. The relative standard deviation (RSD) was determined for each assay as an indicator of the inter-assay variation. The RSD values in 3 different runs were 9–13%. However, when Gly–Gly was used as a substrate instead of Gly–Pro, FL intensity did not decrease. This result is consistent with the reports that prolidase specifically cleaves X-Pro as a substrate and not Gly–Gly,[26] indicating that this assay can specifically determine prolidase activity.

Evaluation of Prolidase Assay Using 3,4-DHPAA The present assay was evaluated by measuring prolidase activity in cultured cell extracts. The biochemical properties of prolidase from fibroblast cells are well known.[27] And prolidase activity has been found to be changed in a variety of cancers.[28,29] Therefore, we selected fibroblasts and HeLa cells for experiments.

First, the effect of $MnCl_2$ in the range of 0–0.5 mM in the enzymatic reaction solution was studied (Fig. 4). In the absence of $MnCl_2$, slightly decrease in FL intensity after enzyme reaction was observed, which indicated that Gly–Pro was not efficiently degraded. With an increase in $MnCl_2$, concentration from 0.15 to 0.5 mM, a linear decrease in FL

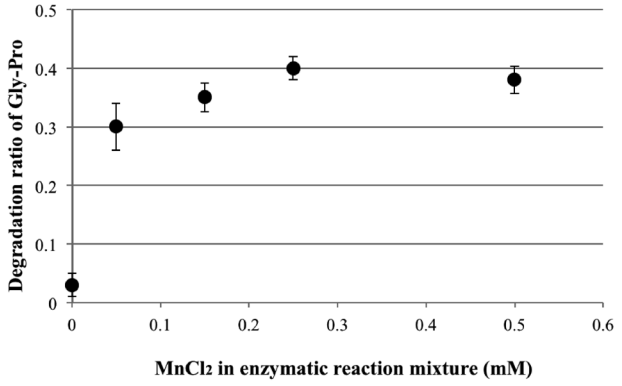

Fig. 4. Effect of Mn^{2+} for Prolidase Activity

Cell extracts (12.5 μg/tube) were used as enzyme sources. The degradation ratio of Gly–Pro was calculated from the decrease in FL intensity with/without enzyme. Different concentrations of Mn Cl_2 were used in the enzymatic reaction mixture (n=3).

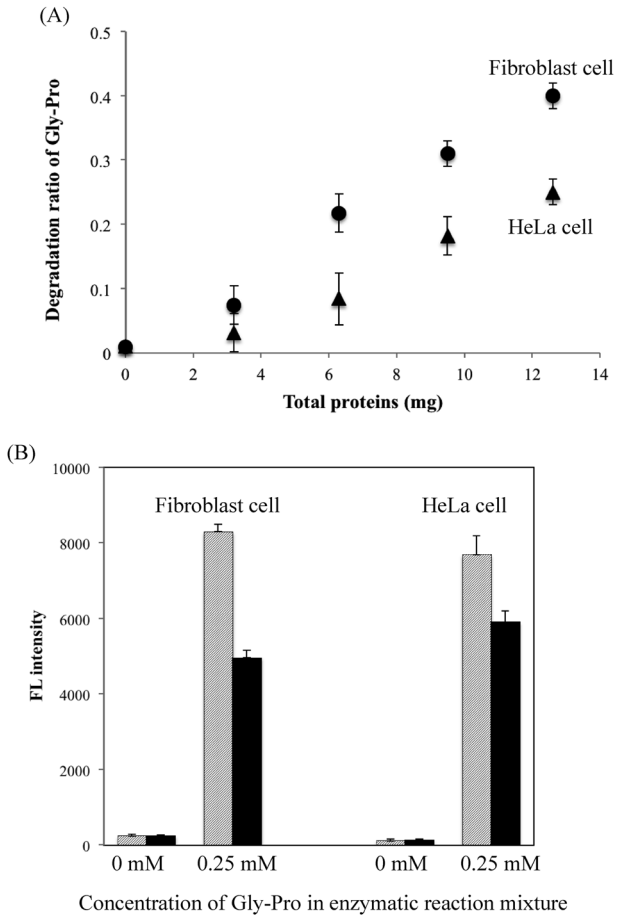

Fig. 5. Prolidase Activity in Fibroblast and HeLa Cells

(A) Prolidase activity in cell extracts measured by the present method. (B) Cell extracts (12.5 μg/tube) were used as enzyme sources. The enzymatic reaction was carried out with or without the substrate (Gly–Pro), and FL intensities at 0 min (gray) and 20 min (black) were monitored. Each measurement was performed in triplicate.

intensity was noticed; therefore, we selected 0.25 mM as the optimal concentration of $MnCl_2$ for this method.

Using this method, prolidase activities were successfully measured in both fibroblasts and HeLa cells, and Gly–Pro degradation increased with an increase in the amount of cell extract in the reaction mixture (Fig. 5). The low FL intensity in the absence of Gly–Pro indicating that 3,4-DHPAA did not react with the biological substances in the cell extracts. This result is consistent with previous reports[25] and indicates that this assay can specifically measure prolidase activity without the interference of any biological substance. From the calibration curve of the purified enzyme, the contents of prolidase were calculated to be approximately 0.06 and 0.04 μg/10 μg protein in fibroblasts and HeLa cells, respectively.

Conclusion

We developed a new method for determining prolidase activity using 3,4-DHPAA. This assay method is very simple because it does not require pre-incubation or deproteinization as observed in ninhydrin-based assay.

Prolidase may act as a potential biomarker as it regulates cell cycle and its activity is altered during diseased conditions. Therefore, this assay method may find its potential role in disease diagnosis.

Acknowledgments This work was supported by a Grant-in-Aid from the Japan Society for the Promotion of Science (KAKENHI: 21K05120).

Conflict of Interest The authors declare no conflict of interest.

References

1) Cunningham D. F., O'Connor B., *Biochim. Biophys. Acta*, **1343**, 160–186 (1997).
2) Endo F., Hata A., Indo Y., Motohara K., Matsuda I., *J. Inherit. Metab. Dis.*, **10**, 305–307 (1987).
3) Myara I., Cosson C., Moatti N., Lemonneir A., *J. Biochem.*, **26**, 207–214 (1994).
4) Cosson C., Myara I., Miech G., Moatti N., Lemonnier A., *Int. J. Biochem.*, **24**, 427–432 (1992).
5) Masuda S., Watanabe H., Morioka M., Fujita Y., Ageta T., Kodama H., *Acta Med. Okayama*, **48**, 173–179 (1994).
6) Ohhashi T., Ohno T., Arata J., Sugahara K., Kodama H., *Clin. Chim. Acta*, **187**, 1–9 (1990).
7) Oono T., Yasutomi H., Ohhashi T., Kodama H., Arata J., *J. Dermatol. Sci.*, **1**, 319–323 (1990).
8) Yaron A., Naider F., Scharpe S., *Crit. Rev. Biochem. Mol. Biol.*, **28**, 31–81 (1993).
9) Emmerson K. S., Phang J. M., *J. Nutr.*, **123**, 909–914 (1993).
10) Surazynski A., Liu Y., Miltyk W., Phang J. M., *J. Cell. Biochem.*, **96**, 1086–1094 (2005).
11) Palka J. A., Phang J. M., *J. Cell. Biochem.*, **67**, 166–175 (1997).
12) Endo F., Tanoue A., Nakai H., Hata A., Indo Y., Titani K., Matsuda I., *J. Biol. Chem.*, **264**, 476–481 (1989).
13) Goodman S. I., Solomons C. C., Muschenheim F., McIntyre C. A., Miles B., O'Brien D., *J. Med.*, **45**, 152–159 (1968).
14) Powell G. F., Rasco M. A., Maniscalco R. M., *Metabolism*, **23**, 505–513 (1974).
15) Lupi A., Tenni R., Rossi A., Cetta G., Forlino A., *Amino Acids*, **35**, 739–752 (2008).
16) Kurien B. T., Patel N. C., Porter A. C., D'Souza A., Miller D., Matsumoto H., Wang H., Scofield R. H., *Anal. Biochem.*, **349**, 165–175 (2006).
17) Myara I., Charpentier C., Lemonnier A., *Clin. Chim. Acta*, **125**, 193–205 (1982).
18) Yuan J., Li T., Yin X. B., Guo L., Jiang X., Jin W., Yang X., Wang E., *Anal. Chem.*, **78**, 2934–2938 (2006).
19) Kurien B. T., Patel N. C., Porter A. C., Kurono S., Matsumoto H., Wang H., Scofield R. H., *Anal. Biochem.*, **331**, 224–229 (2004).

20) Harada M., Fukasawa K. M., Hiraoka B. Y., Fukasawa K., Mogi M., *J. Chromatogr.*, **530**, 116–121 (1990).
21) Chinard F. P., *J. Biol. Chem.*, **199**, 91–95 (1952).
22) Rahman M. S., Kabashima T., Yasmin H., Shibata T., Kai M., *Anal. Biochem.*, **433**, 79–85 (2013).
23) Ejupi V., Dragusha S., Kabashima T., Zhu Q., El-Mahdy A. F. M., Yin S., Shibata T., Kai M., *Adv. Enzyme Res.*, **3**, 19–29 (2015).
24) Kabashima T., Tonooka K., Takada M., Kai M., Shibata T., *Sci. Rep.*, **9**, 9150 (2019).
25) Yasmin H., Shibata T., Rahman M. S., Kabashima T., Kai M., *Anal. Chim. Acta*, **721**, 162–166 (2012).
26) Adams E., Smith E. L., *J. Biol. Chem.*, **198**, 671–682 (1952).
27) Oono T., Yasutomi H., Ohhashi T., Kodama H., Arata J., *J. Dermatol. Sci.*, **1**, 319–323 (1990).
28) Karna E., Surazynski A., Palka J., *Int. J. Exp. Pathol.*, **81**, 341–347 (2000).
29) Gecit I., Aslan M., Gunes M., Pirincci N., Esen R., Demir H., Ceylan K., *J. Cancer Res. Clin. Oncol.*, **138**, 739–743 (2012).