Endocrine, vol. 7, no. 1, 33–36, August 1997 0969-711X/97/7:33-36/$9.00

Insulin-Like Growth Factor Binding Protein (IGFBP) Substrate Zymography

A New Tool to Identify and Characterize IGFBP-Degrading Proteinases

John L. Fowlkes,[1] Kathryn M. Thrailkill,[1] Delila M. Serra,[2] and Hideaki Nagase[3]

[1]*Department of Pediatrics, University of Kentucky College of Medicine, Lexington, KY;*
[2]*Department of Pediatrics, Duke University Medical Center, Durham, NC;*
and [3]*Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, KS*

Insulin-like growth factor binding protein (IGFBP) degrading proteinase activities have been described in biological fluids and conditioned media from numerous cell lines. To identify and characterize IGFBP-degrading proteinases, our laboratory has developed IGFBP substrate zymography. Herein, we illustrate how IGFBP substrate zymography can be used both to identify candidate IGFBP-degrading proteinases and characterize their degradative capabilities. For this purpose, human matrix metalloproteinase-3 (MMP-3), a proteinase that degrades IGFBP-3 in human fibroblast cultures, was first electrophoresed through a polyacrylamide gel containing IGFBP-3 as substrate and then analyzed for its ability to degrade the substrate into immunoreactive fragments that were absorbed onto a polyvinylidene difluoride membrane. IGFBP-3 substrate zymography was capable of detecting as little as 20 ng of human MMP-3, demonstrating a sensitivity similar to casein substrate zymography. Using the zymogram as a template, MMP-3 was identified in a standard SDS-polyacrylamide gel run in parallel with the zymogram, and the corresponding area of the gel was excised. Electroelution of the gel slice yielded active MMP-3 when examined by casein substrate zymography. Furthermore, digestion of IGFBP-3 in solution by the electroeluted MMP-3 revealed the same fragmentation pattern of the binding protein as that produced by MMP-3, which had not been electroeluted. Together, these studies demonstrate that IGFBP substrate zymography can be a useful tool for both the identification and the characterization of IGFBP-degrading proteinases.

Key Words: Insulin-like growth factor binding proteins; zymography; proteinase; matrix; metalloproteinase.

Received February 21, 1997; Revised April 3, 1997; Accepted May 7, 1997.
Author to whom all correspondence and reprint requests should be addressed: John L. Fowlkes, MD, Department of Pediatrics, Division of Pediatric Endocrinology and Metabolism, J464 Kentucky Clinic, 740 S. Limestone, Lexington, KY 40536-0284. E-mail: jlfowlk@pop.uky.edu

Introduction

Proteinases that degrade insulin-like growth factor (IGF) binding proteins have been described for at least four (IGFBP-2, 3, 4, and 5) of the six known IGFBPs (reviewed in refs. *1–4*). It is believed that under a variety of physiologic and pathologic conditions, proteolysis of IGFBPs occurs, resulting in the production of IGFBP fragments that have little or no affinity for IGFs. Through this mechanism, it is possible that IGFs are released from IGFBP/IGF complexes to interact with cell-surface IGF receptors. Despite their potential impact on IGF action, the identity of these IGFBP-degrading proteinases remains largely unknown. In order to clarify the identity of these IGFBP-degrading proteinases and to determine if the various IGFBP-degrading proteinases represent one or more distinct proteinases, appropriate analytical methods must be developed. To this end, a number of investigators have utilized a variety of chromatographic methods, including ion-exchange and size-exclusion chromatography, to purify partially IGFBP-degrading proteinase activity from a number of biological fluids. Alternatively, we have recently developed a zymographic method using recombinant human (rh)IGFBPs as substrates to analyze fluids for IGFBP-degrading proteinase *(5,6)*. Zymography studies have demonstrated that matrix metalloproteinases (MMPs) are involved in the degradation of IGFBP-5 in osteoblast cultures *(5)*, and in the degradation of IGFBP-3 in rat pregnancy serum *(7)* and human fibroblast cultures *(6)*. Herein, we have used human MMP-3 (stromelysin 1) as a prototypical IGFBP-degrading proteinase to illustrate the utility of IGFBP substrate zymography in identifying IGFBP-degrading proteinases, and as an initial step in isolating the proteinase and characterizing its IGFBP-degrading action.

Results

Because MMP-3 readily degrades IGFBP-3 under physiologic conditions *(6)*, we chose this enzyme to illustrate the overall utility of IGFBP substrate zymography. Figure 1A demonstrates that MMP-3 readily degraded casein, pro-

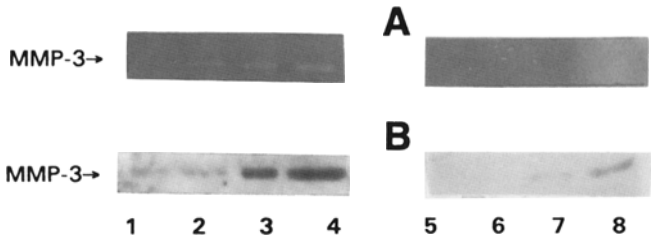

Fig. 1. Analysis of MMP-3 proteinase activity using casein substrate zymography **(A)** and IGFBP-3 substrate zymography **(B)** as described in Materials and Methods. Lanes 1 and 5: 20 ng MMP-3; lanes 2 and 6: 75 ng MMP-3; lanes 3 and 7: 125 ng MMP-3; lanes 4 and 8: 250 ng MMP-3. Lanes 1–4 were incubated with 50 m*M* Tris-HCl, 5 m*M* $CaCl_2$, pH 7.5, whereas lanes 5–8 were incubated with the same buffer containing 10 m*M* EDTA.

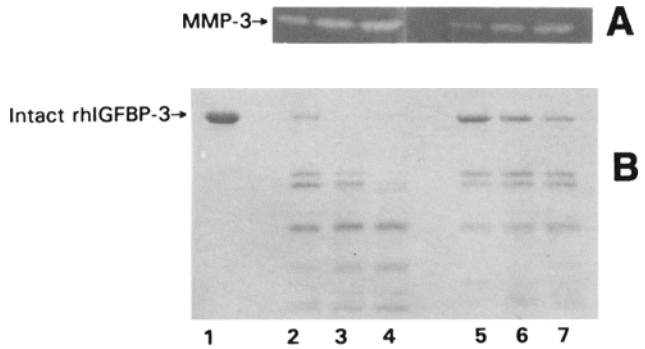

Fig. 2. Degradation of casein and rhIGFBP-3 by MMP-3 and electroeluted MMP-3. 5 µL (lanes 2 and 5), 10 µL (lanes 3 and 6), or 20 µL (lanes 4 and 7) of either MMP-3 (10 µg/mL; lanes 2–4) or electroeluted MMP-3 (lanes 5–7) were analyzed by casein substrate zymography **(A)** or were incubated with rhIGFBP-3 (2 µg) in solution **(B)** as described in Materials and Methods. Lane 1 is rhIGFBP-3 with no added proteinase.

ducing "lytic" areas within the casein substrate gel in a dose-dependent manner (lanes 1–4). Similarly, MMP-3 degraded the immobilized IGFBP-3 substrate into immunoreactive (dark) bands in a dose-dependent fashion (Fig. 1B, lanes 1–4). Comparing the sensitivity of both methods demonstrates that under these conditions, IGFBP-3 substrate zymography is as sensitive as casein substrate zymography in detecting as little as 20 ng of the metalloproteinase (lane 1). Inclusion of EDTA, a chelating agent, in the incubation buffer resulted in almost complete inhibition of MMP-3 activity when analyzed by either method (lanes 5–8). Together, these studies demonstrate that IGFBP-3-degrading proteinase activity is detectable at low proteinase concentrations, and they suggest that the observed proteinase may be classified by including various proteinase inhibitors in the incubation buffer.

Figure 2A demonstrates that electroeluted MMP-3, identified by the IGFBP-3 substrate zymogram, retained its ability to digest casein (lanes 5–7) similar to that seen using MMP-3, which had not been subjected to SDS-PAGE and electroelution (lanes 2–4). When the electroeluted and dialyzed MMP-3 was incubated with rhIGFBP-3 in solution (Fig. 2B, lanes 5–7), it degraded the binding protein into the same-sized fragments as did naive MMP-3 (Fig. 2B, lanes 2–4). No degradation of rhIGFBP-3 occurred when incubated in buffer alone (Fig. 2B, lane 1). There did appear to be some loss of activity of the electroeluted proteinase, and this may be explained by incomplete electroelution, loss of protein during dialysis, and/or partial denaturation of the enzyme owing to residual urea and SDS in the sample. Overall, recovery was estimated at 20–50% in this instance. Nonetheless, these data demonstrate that MMP-3 can be recovered from substrate gels by electroelution, and that the enzyme retains sufficient activity in order to characterize and analyze its IGFBP-3-degrading properties.

Discussion

Extracellular proteinases that degrade intact IGFBPs may function as significant regulators of IGF bioavailability and bioactivity at the cellular surface. It has been speculated that for each IGFBP, a unique proteinase exists, or that a "family" of IGFBP proteinases may exist, which are capable of degrading one or more of the IGFBPs. IGFBP-degrading proteinases have been reported by several groups: Cohen et al. have reported that prostate-specific antigen (PSA) degrades IGFBP-3 in seminal plasma *(8)*; Campbell et al. have shown that plasmin can degrade IGFBPs in solution *(9)*, and Conover and DeLeon have demonstrated that cathepsin-D degrades IGFBP-3 in acidified fibroblast conditioned media *(10)*. Recently, using a variety of techniques, including IGFBP substrate zymography, our laboratories have identified the MMPs as IGFBP-3- and IGFBP-5-degrading proteinases *(5–7)*. Thus, several enzyme systems may be involved in IGFBP degradation in different cell lines and under various conditions.

To help clarify the identity of IGFBP-degrading proteinases, we have developed a zymographic method that can help rapidly analyze IGFBP-degrading proteinases, compare IGFBP-degrading proteinase profiles from different sources, identify the molecular weights of IGFBP-degrading proteinases, determine the individual proteinase inhibitor profiles of each proteinase, characterize the proteolytic capacities of each proteinase, and potentially serve as an initial step in their purification. To illustrate these points, MMP-3 was used in the present study. IGFBP-3 substrate zymography proved to be a sensitive method for detecting MMP-3 degradation of IGFBP-3, similar to the sensitivity achieved with casein substrate zymography, but using 1000-fold less substrate. We have previously demonstrated that the antisera produced against *Escherichia coli*-derived IGFBP-3 readily detects several IGFBP-3 fragments *(6,11)*, and obviously, this is a requirement for IGFBP-substrate zymography to be successful. Although we used only EDTA as a proteinase inhibitor to demonstrate its ability to

block MMP-3 activity, the addition of different proteinase inhibitors to the incubation buffer may be useful in characterizing an IGFBP-degrading proteinase in regard to its proteinase classification (i.e., serine, cysteine, metallo-, or aspartic proteinase). This has important applications when studying a complex fluid that may contain more than one IGFBP-degrading proteinase, each of which may represent one or more class of proteinase. Unlike more traditional methods of enzyme purification, such as size-exclusion or ion-exchange chromatography, IGFBP substrate zymography may have several advantages in identifying IGFBP-degrading proteinases because:

1. A number of samples can be analyzed on the same substrate gel, allowing for comparison of samples from various sources, making it more efficient than chromatographic methods;
2. In SDS-PAGE, proteinase/proteinase inhibitor complexes should be disrupted, whereas in conventional chromatographic methods, IGFBP-degrading proteinases might not be detected if complexed with endogenous inhibitor(s); and
3. Latent or zymogen forms of proteinases may be detected by zymography, whereas they may remain inert during chromatographic purification schemes.

Along with the capability of identifying IGFBP-degrading proteinases by IGFBP substrate zymography comes the possibility of recovering the active enzyme. We have demonstrated in the current study that MMP-3 could be localized within a standard SDS-polyacrylamide gel using the IGFBP-3 substrate zymogram as a template. Localization and excision of the identified proteinase, and electroelution of the gel slice were successful in yielding active MMP-3 capable of degrading casein and rhIGFBP-3. Since some enzymes may be denatured irreversibly by SDS, some enzyme preparations may need to be analyzed using nondenaturing polyacrylamide gel electrophoresis. Furthermore, if complex fluids are analyzed, more than one protein may migrate at the same molecular mass as the identified IGFBP-degrading proteinase. In this instance, other methods, such as HPLC, may need to be employed to separate and purify the electroeluted proteinase further. Nevertheless, using this step, several goals in characterizing IGFBP-degrading proteinases may be achieved. First, electroeluted proteinases can be used to digest IGFBPs, and the pattern of IGFBP fragments produced can be compared to those observed using other proteinases and/or crude starting material; second, detailed proteinase inhibitor profiles can be obtained on electroeluted proteinases; third, the electroeluted proteinase may serve as an antigen for use in developing antisera; and/or finally, the electroeluted proteinase might be used for N-terminal, amino acid analysis.

In conclusion, although we have previously characterized IGFBP substrate zymography as a useful tool in identifying potential IGFBP-degrading proteinases, we have expanded on these studies to show that when used in combination with other methods, such as electroelution, it can be useful as a first step in enzyme purification and characterization. Thus, these studies suggest that IGFBP substrate zymography may be a powerful tool in the identification, characterization, and purification of a variety of IGFBP-degrading proteinases present in numerous biological fluids.

Materials and Methods

Nonglycosylated rhIGFBP-3 produced in *E. coli (12)* and antiserum produced against it were kindly provided by Christopher Maack, Celtrix Pharmaceuticals, Santa Clara, CA. Reagents used for SDS-PAGE were purchased from Bio-Rad Laboratories, Richmond, CA. Immobilon-P polyvinylidene difluoride (PVDF) membranes were purchased from Millipore Corp., Bedford, MA. ECL-Western blot kits, Rainbow low-mol-wt markers, and Hyperfilm-ECL were obtained from Amersham Corp., Arlington Heights, IL. Active recombinant human MMP-3 (~45 kDa) was produced as described elsewhere *(13)*.

IGFBP-3 Substrate Zymography

IGFBP-3 substrate zymography was performed similarly to that described elsewhere *(6)*. In brief, rhIGFBP-3 (1 µg/mL) was added to a 12.5% SDS-polyacrylamide gel solution before gel casting (Mini-PROTEAN II electrophoresis system, Bio-Rad). Various amounts of MMP-3 (20–250 ng) were diluted 1:1 in nonreducing 2X sample buffer *(14)*, and unheated samples were electrophoresed through the substrate gel (Fig. 3A). Substrate gels were washed in 100 mL of 2.5% Triton X-100 at 4°C for 1 h to remove SDS and then thoroughly rinsed in dH_2O. Using the capillary transfer apparatus demonstrated schematically in Fig. 3B, IGFBP-3-degrading proteinases present in the substrate gel *[5]* were allowed to degrade IGFBP-3 present in the substrate gel by constant exposure of the gel to a wick *[6]* made of double-thickness 3MM chromatography paper (Whatman International Inc., Maidstone, UK) saturated in a calcium-containing buffer (50 m*M* Tris-HCl, pH 8.0/5 m*M* $CaCl_2$) *[7]*. The incubation was for 6 h at 37°C. IGFBP-3 fragments produced by the proteinases were then eluted from the substrate gel and transferred by capillary action onto PVDF membranes (Millipore, Bedford, MA) *[4]*. A constant flow of buffer from the chamber through both the gel and PVDF membrane was achieved by backing the PVDF membrane with two sheets of 3MM chromatography paper *[3]*, 4–6 in of paper toweling *[2]*, topped by a 0.5-lb. weight *[1]*. After the incubation was completed, PVDF membranes were air-dried, rewetted in 10 m*M* Tris, pH 7.4, 150 m*M* NaCl, 0.1% Tween-20 (TBST buffer), and then blocked for 1 h at room temperature in TBST buffer containing 5% (w/w) powdered milk. Membranes were incubated with a polyclonal antiserum generated against rhIGFBP-3 (1:2500) in TBST buffer containing 1.0% BSA for 1 h at room temperature. Membranes were washed in

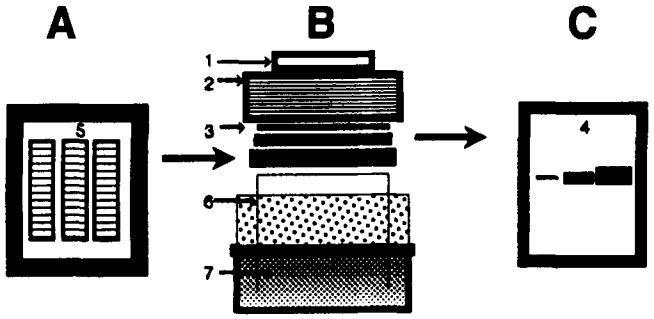

Fig. 3. Schematic of IGFBP substrate zymography. For details *see* Materials and Methods. Legend: 1—0.5 lb weight; 2—4–6 in of paper toweling; 3—two sheets of 3MM chromatography paper; 4—capillary transfer setup; 5—substrate gel; 6—wick made of double-thickness 3MM chromatography paper; 7—calcium-containing buffer.

TBST buffer, incubated with an antirabbit IgG horseradish peroxidase conjugate (1:1000) in TBST containing 1.0% BSA for 30 min at room temperature, washed again in TBST buffer, then reacted with the chemiluminescent reagent provided in the ECL-Western blot kit for 1 min, and finally exposed to Hyperfilm-ECL for 10–30 s (Fig. 3C). Inhibition of proteinase activity was achieved by adding EDTA (10 m*M*) to the calcium-containing buffer. Casein zymography was performed by standard zymography methods using casein at a concentration of 1 mg/mL *(6)*.

Isolation and Recovery of MMP-3 Using IGFBP-3 Substrate Zymography

To determine if IGFBP-substrate zymography can be used as an initial step in the isolation and characterization of IGFBP-degrading proteinases, we used the IGFBP-3 zymogram as a template to localize an IGFBP-3 proteinase (i.e., MMP-3 [10 μg]), which had been electrophoresed through a standard 12.5% SDS-polyacrylamide gel under identical conditions to those used for the IGFBP-3 zymogram. The area of the gel corresponding to the proteolytic band on the zymogram was excised and cut into ~2 × 3 mm slices. MMP-3 present in the slices was then electroeluted overnight at 200 V using Centricon-3 concentrators and a Centrilutor apparatus (Amicon, Danvers, MA) according to the manufacturer's instructions. Concentrated electroeluted samples were exchanged into 50 μL of TNC buffer (50 m*M* Tris-HCl, 5 m*M* $CaCl_2$, 0.05% Brij-35, 0.02% NaN_3, pH 7.5) and then analyzed by casein-substrate zymography to estimate recovery. To remove SDS, samples were first dialyzed against 6 *M* urea and then into TNC buffer. To characterize the IGFBP-3 fragments produced by the electroeluted proteinase, rhIGFBP-3 (2 μg) was incubated for 6 h at 37°C with various amounts of electroeluted MMP-3, and the digestion pattern was compared with that produced by MMP-3, which had not been subjected to SDS-PAGE. Digestion products were analyzed by separation on 15% SDS-polyacrylamide gels under reducing conditions, and the gels were stained with Coomassie blue.

Acknowledgments

We thank Christopher Maack (Celtrix Pharmaceuticals) for providing the rhIGFBP-3 and IGFBP-3 antiserum used in these studies. This work was supported by National Institutes of Health Grants AR39189 (H. N.), DK02776, a March of Dimes Basil O'Connor Starter Scholar Research Award (#5-FY93-0953) and a Duke University Medical Center Small Research Grant (J. L. F.), and a Duke Children's Telethon Grant (K. M. T.).

References

1. Rechler, M. M. (1993). *Vitamin. Horm.* **47,** 1–114.
2. Bach, L. A. and Rechler, M. M. (1995). *Diabetes Rev.* **3,** 38–61.
3. Jones, J. I. and Clemmons, D. R. (1995). *Endocr. Rev.* **16,** 3–34.
4. Rechler, M. M. (1995). In: *Molecular Endocrinology: Basic Concepts and Clinical Correlations.* Weintraub, B. D. (ed.) Raven: New York, pp. 155–180.
5. Thrailkill, K. M., Quarles, L. D., Nagase, H., Suzuki, K., Serra, D. M., and Fowlkes, J. L. (1995). *Endocrinology* **136,** 3527–3533.
6. Fowlkes, J. L., Enghild, J. J., Suzuki, K., and Nagase, H. (1994). *J. Biol. Chem.* **269,** 25,742–25,746.
7. Fowlkes, J. L., Suzuki, K., Nagase, H., and Thrailkill, K. (1994). *Endocrinology* **135,** 2810–2813.
8. Cohen, P., Graves, C. B. G., Peehl, D. M., Kamarei, M., Giudice, L. C., and Rosenfeld, R. G. (1992). *J. Clin. Endocrinol. Metab.* **75,** 1046–1053.
9. Campbell, P. G., Novak, J. F., Yanosick, T. B., and McMaster, J. H. (1992). *Endocrinology* **130,** 1401–1412.
10. Conover, C. A. and DeLeon, D. D. (1994). *J. Biol. Chem.* **269,** 7076–7080.
11. Fowlkes, J. L. (1994). *Endocrinology J.* **2,** 63–68.
12. Sommer, A., Maack, C. A., Spratt, S. K., Mascarenhas, D., Tressel, T. J., Rhodes, E. T., Lee, R., Roumas, M., Tatsuro, G. P., Flynn, J. A., Gerber, N., Taylor, J., Cudney, H., Nanney, L., Hunt, T. K., and Spencer, E. M. (1991). In: *Modern Concepts of Insulin-Like Growth Factors.* Spencer, E. M. (ed.) Elsevier: New York, pp. 715–728.
13. Nagase, H., Suzuki, K., Cawston, T. E., and Brew, K. (1997) *Biochem. J.* **325,** 163–167.
14. Laemmli, U. K. (1970). *Nature* **227,** 680–685.