Three Activity-Based Assays for Serp-1

A Viral-Derived Serine Proteinase Inhibitor

Xing Li, Elaine King, Colin Macaulay, Alexandra Lucas, Grant McFadden

The production of recombinant proteins as therapeutics requires careful assessment of protein quality, including measurements of identity, quantity, purity, and activity. Because each protein has its unique set of activities, assays must be customized for each product — and activity assays are often the most difficult to develop.

Serp-1 represents the first of a new class of therapeutic antiinflammatory proteins derived from viruses. Viruses have evolved many mechanisms to prevent, disable, or subvert a host's innate immune and inflammatory defense systems. These mechanisms include producing viral proteins that affect chemokine, cytokine, and complement signaling cascades and the signals that induce apoptosis and major histocompatibility complex (MHC) class-1 surface expression (**1**, **2**). Serp-1 was originally isolated from myxoma virus and shown to be a member of the serpin superfamily of serine proteinase inhibitors (**3**). Biochemical studies have demonstrated Serp-1 to be an efficient inhibitor of human uPA (urokinase type plasminogen activator), tPA (tissue type plasminogen activator), and plasmin (**4**). Studies in animal model systems have demonstrated that Serp-1 is a highly effective inhibitor of restenosis and of the vasculopathy that leads to organ failure during chronic rejection of organ transplants (**5–7**).

Recombinant Serp-1 has been expressed in engineered CHO (Chinese hamster ovary) cells and purified using standard column chromatography methods (**4**). To ensure the quality of the purified product, a number of Serp-1 activity indicating assays have been developed. We describe the development of three assays, two of which measure the interaction or inhibition of the serine proteinase uPA. The third assay is a capture ELISA (enzyme-linked immunosorbent assay) that uses a conformation-specific monoclonal antibody. This antibody is specific for the active form of Serp-1, which enables the ELISA to function as a surrogate Serp-1 activity-indicating assay. All three activity-indicating assays produced similar profiles in a series of Serp-1 stability studies.

PHOTODISC (WWW.PHOTODISC.COM)

PRODUCT FOCUS: ANTI-INFLAMMATORY VIRAL PROTEIN

PROCESS FOCUS: ASSAY DEVELOPMENT

INTENDED AUDIENCE: LABORATORY RESEARCHERS, QA/QC

KEY WORDS: ACTIVITY ASSAY DEVELOPMENT; CONFORMATION-SPECIFIC MONOCLONAL ANTIBODY; VIRAL SERPIN, ELISA

LEVEL OF COVERAGE: INTERMEDIATE AUDIENCE IN ASSAY DEVELOPMENT

METHODS AND MATERIALS

Band-shift assay. The stock uPA (Sigma-Aldrich, U1131) was diluted to 2.7 unit/mL (equal to 10 µg/mL) in reaction buffer (100 mM NaCl, 2 mM $CaCl_2$, 0.005% Triton X-100, 100 mM Tris-HCl, pH 7.5). Serp-1 was diluted in the same reaction buffer to three concentrations: 40 µg/mL, 20 µg/mL, and 10 µg/mL. A 5-µL sample of uPA was mixed with 5 µL of the three different Serp-1 concentrations and incubated at room temperature for two hours. A 10-µL aliquot of 2× SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) loading buffer (0.1 M Tris, 4% SDS, and 20% glycerol, pH 6.8) was added to stop the reaction. The mixture was separated by electrophoresis on a 12% SDS-polyacrylamide gel. The separated proteins were electrophoretically transferred from the gel to a Hybond-C membrane (Amersham

Biosciences, RPN303E). The membrane was blocked by mixing in blotto using 5% nonfat milk in 1× phosphate-buffered saline (PBS) and 0.1% Tween for one hour at room temperature.

The Serp-1 protein was detected by first incubating the membrane with an anti-Serp-1 monoclonal antibody (VT-mAbA) diluted in blotto, washing the membrane in PBS, and then incubating with a horseradish peroxidase (HRP)-conjugated goat antimouse IgG (Pierce Biotechnology, P21124) diluted 1:20,000 in blotto. After a one-hour incubation, the blot was rinsed in PBS, the antibodies detected using a chemiluminescence kit (Amersham, 1059243), and the images recorded on Kodak scientific film (NEN Life Science Products, NNK-8646770). Densitometry and image analysis were performed with Quantity One software (Bio-Rad Laboratories).

Chromogenic uPA inhibition assay. The activity of each lot of uPA was determined using the substrate Chromozym U (Roche Applied Science, 836583). The amount of uPA that gave an absorbance reading (OD405 nm) of 0.90 ± 0.10 after one hour at 37 °C was selected for use in this assay. A 25 µL aliquot of uPA at this concentration was mixed with a 25 µL aliquot of Serp-1 and incubated at room temperature for two hours in a 96-well microplate. Typically, six serial dilutions of Serp-1 (0.2 µg/mL, 0.4 µg/mL, 0.8 µg/mL, 1.2 µg/mL, 1.6 µg/mL, and 2.0 µg/mL) were first prepared in reaction buffer (100 mM NaCl, 2 mM $CaCl_2$, 0.005% Triton X-100, 100 mM Tris-HCl, pH 7.5). After the two-hour incubation, a 100-µL aliquot of 0.5 mM Chromozym U was added to the uPA mixture. The plate was placed in a 37 °C microplate reader (Thermo LabSystems), and the absorbance at 405 nm was determined for one hour.

Capture ELISA. A 50-µL aliquot of anti-Serp-1 monoclonal antibody diluted to 1.6 µg/mL in coating buffer (Sigma, C-3041) was adsorbed to the surface of a 96-well microplate (Corning, 3590) by an overnight incubation at 4 °C. Each well was then coated with blocking buffer (1 × PBS containing 2% BSA and 0.05% Tween-20) to prevent nonspecific binding. The Serp-1 test solution was diluted to a proper range and added to the antibody-coated plate. After a one-hour incubation at room temperature, unbound proteins were washed away and each well incubated with a second biotin-labeled anti-Serp-1 antibody for one hour at room temperature. The wells were washed and each well incubated with HRP-conjugated streptavidin (Pierce, P21124) for one hour at room temperature. The unbound streptavidin was washed away, and the bound HRP-streptavidin was detected with the chromogenic substrate TMB (3, 3′, 5, 5′ tetramethy benzidine chromgen, Pierce P34021). Color development was stopped with 2N H_2SO_4, and the absorbance (450nm) values were recorded using a microplate reader (Bio-Tek Instruments).

The Serp-1 concentration in a sample was calculated by comparing to a standard curve created using purified Serp-1. All samples and standards were run in duplicate. The assay linear range was between 1 and 20 ng/mL.

Results and Discussion

Band-shift assay. Serpins share a conserved core structure and a common mechanism of serine proteinase inhibition (**8**). A target proteinase binds to the serpin's reactive center loop (RCL) and tries to cleave the peptide bond between the P1 and P1′ residues. The P1 residue is therefore critical for determining proteinase specificity. As soon as the P1-P1′ peptide bond is cleaved, the serpin snaps into a more stable conformation. This change in conformation traps the serpin/proteinase complex at an intermediate step in the cleavage process in which a covalent linkage is formed between the P1 residue of the serpin and the active site serine of the proteinase. This serpin/proteinase complex is stable to the detergent SDS and can be separated from both the free serpin and proteinase by SDS-PAGE.

When Serp-1 is mixed with uPA, the Serp-1/proteinase complex is formed. This complex has a slower mobility on a polyacrylamide gel compared to that of the free Serp-1, resulting in an apparent band-shift (Figure 1). In this particular example, a monoclonal antibody against Serp-1 (VT-mAbA) is used to detect both the native and shifted forms on an immunoblot. This makes the band-shift assay very sensitive in that very little Serp-1 (50 ng) is required for detection. When the molar ratio of Serp-1 and uPA is 1:1 (Figure 1, lanes 3, 6, and 9), nearly 100% of the Serp-1 is shifted. This indicates that nearly all of the Serp-1 is active, because any

Figure 1: Results of a band-shift assay. The activity of three different lots of Serp-1 (A, B, and C) was compared. Serp-1 at various amounts (200 ng: lanes 1, 4, 7; 100 ng: lanes 2, 5, 8; 50 ng: lanes 3, 6, 9) was mixed with uPA (50 ng) and analyzed in immunoblot. Serp-1 bands were probed using an anti-Serp-1 monoclonal antibody (VT-mAbA) and an HRP-conjugated goat anti-mouse secondary antibody. The Serp-1/uPA complex is seen in the shifted position, and the native Serp-1 is seen in the unshifted position.

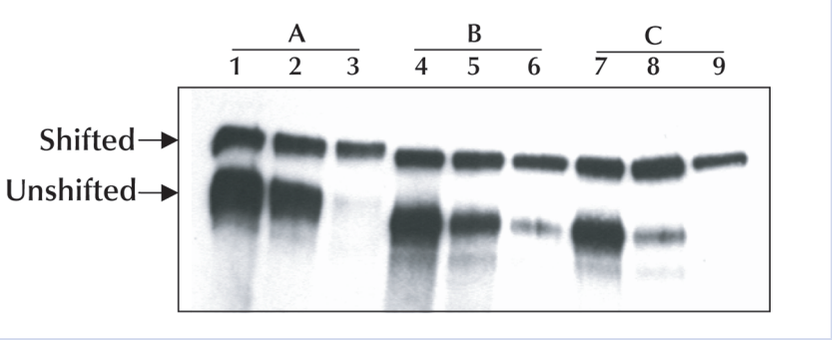
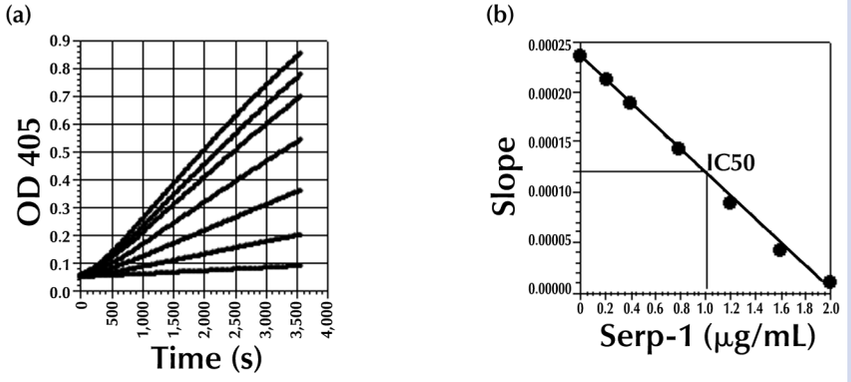


Figure 2: Results of a chromogenic uPA inhibition assay. A given concentration of uPA was mixed with Serp-1 at 0.0 µg/mL, 0.2 µg/mL, 0.4 µg/mL, 0.8 µg/mL, 1.2 µg/mL, 1.6 µg/mL, and 2.0 µg/mL (a, top to bottom). After two hours, 0.5 mM Chromozym U was added to each sample, and the absorbance (405 nm) monitored at 37 ºC for one hour. The reaction rates of seven samples are presented (a). A replot of the slopes of each sample in (a) versus the concentration of Serp-1 is presented in (b). The IC_{50} is extrapolated from the slope value that is one half the maximum slope value in the uninhibited control.



inactive Serp-1 would appear at the unshifted position. When the molar ratio of Serp-1 to uPA is 2:1 (lanes 2, 5, and 8) or 4:1 (lanes 1, 4, and 7), then the portion of Serp-1 that cannot react with uPA remains at the unshifted position. The use of a SDS-polyacrylamide gel allows for the separation of the native Serp-1 from the Serp-1/proteinase complex and from any proteolytically digested Serp-1. Using such a band-shift assay, numerous proteinases have been screened for their ability to form SDS-stable complexes (**4**, **9**). In addition to uPA, both tPA and plasmin can also efficiently form SDS-stable complexes with Serp-1.

One advantage of the band-shift assay is that active Serp-1 is physically separated from inactive Serp-1. Any treatment that affects Serp-1 activity or the proportion of shifted to unshifted Serp-1 is easily monitored. One disadvantage is that the assay is not precisely quantitative. Although immunoblots can be scanned and the ratio of shifted to unshifted Serp-1 calculated, this has a limited linear range. In addition, the antibody used to detect the Serp-1 may not have the same reactivity toward the native Serp-1 and the Serp-1/proteinase complex.

Chromogenic uPA inhibition assay. The amount of proteinase in solution is commonly quantitated using a proteinase-specific chromogenic substrate. As the substrate is cleaved, the concentration of the chromophore released into solution is quantitated spectrophotometrically. Chromozym U has been used as a chromogenic substrate for uPA. A quantitative assay of Serp-1 activity was developed by mixing serial dilutions of Serp-1 with a single concentration of uPA. After a two-hour incubation, excess chromogenic substrate was added and the mixture incubated at 37 °C. The amount of residual uPA activity was determined by measuring the amount of chromophore released into solution over time (Figure 2a). The amount of uPA activity is represented by the increase of chromophore (OD405) with time, or the slope of the curves shown in Figure 2a. As the concentration of Serp-1 increases, the amount of residual uPA activity (slope) decreases. The slopes of all curves were linear, indicating that the amount of substrate was not limiting during the time course of this reaction.

The amount of Serp-1 activity can be expressed in a number of ways. The simplest way is to determine the concentration of Serp-1 that inhibits half of the uPA activity (IC_{50}). Figure 2b shows a plot of the slopes of the curves in Figure 2a versus the concentration of Serp-1 in the reaction. Uninhibited uPA activity is indicated by the slope value at a Serp-1 concentration of zero. The IC_{50} can be determined by extrapolating the Serp-1 concentration needed to give a slope that is 50% or half the value of the uninhibited uPA. This typically gives an IC_{50} of ~1 µg/mL for pure Serp-1 with an interassay coefficient of variation of less than 10% (data not shown).

For this assay to work consistently, it is important to control the amount of uPA that is used in the reaction. The amount of uPA is always determined by the control reaction without Serp-1. Alternatively, one can use a standard preparation of uPA with a defined number of international units (IU), which can be obtained from the NIBSC (National Institute for Biological Standards and Controls). Assuming that one unit of Serp-1 inhibits one unit of protease, then an IC_{50} of 1 µg/mL corresponds to a Serp-1 specific activity of ~60,000 Units/mg.

The chromogenic assay has a number of advantages over the band-shift assay: It can be adapted to a high throughput plate-based format; it provides a quantitative determination of Serp-1 activity; and it is highly reproducible.

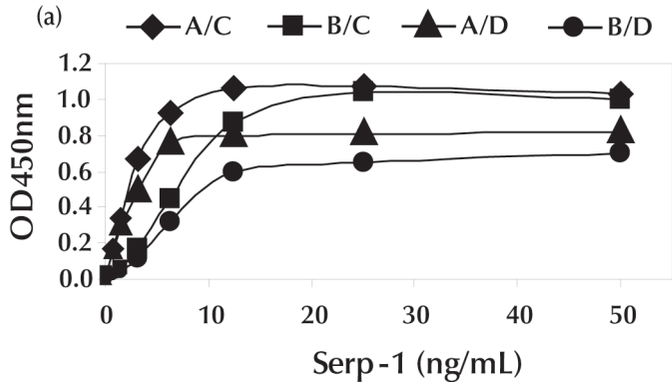
Conformation-specific capture ELISA. A capture ELISA often is used to quantitate the level of a specific antigen in solution. A pair of antigen-specific antibodies is required for this type of assay (**10**). The first antibody is immobilized on the surface of a microtiter plate, which is then exposed to antigen-containing solution during the capture phase of the assay. The first antibody binds the antigen, and unbound material is washed from the plate. A second antibody is then added to detect the presence of the immobilized antigen. The second antibody is usually labeled with an enzyme or marker that can then be quantitated by absorbance,

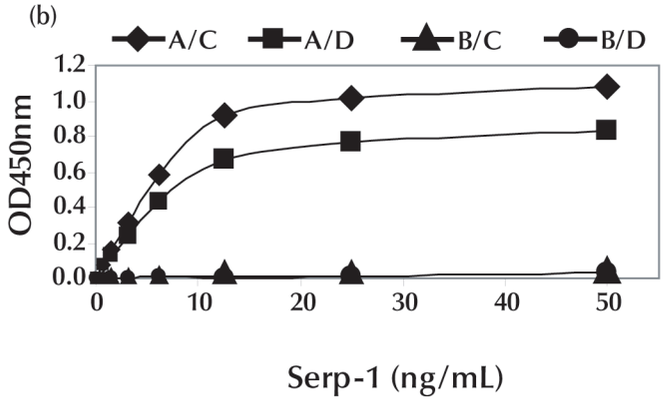
fluorescence, or chemiluminescence. The level of antigen in a solution is determined by comparing the ELISA signal to a standard curve created using pure antigen of known concentration.

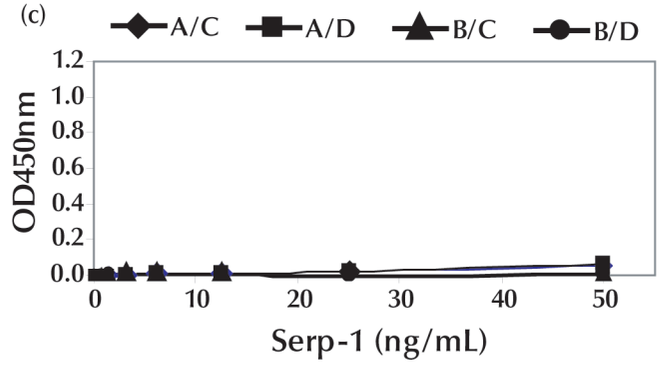
Twelve anti-Serp-1 monoclonal antibodies were available for conformation-specific ELISA development. Two antibodies (VT-mAbC and VT-mAbD) were tested as capture antibodies; the two other antibodies (VT-mAbA and VT-mAbB) were labeled with biotin and tested as detection antibodies. These four antibody pairs (A/C, A/D, B/C, and B/D) were tested for their ability to detect different conformational forms of Serp-1. Three different conformational forms of Serp-1 were prepared: native Serp-1 as typically purified; Serp-1 in an inhibitory complex with uPA; and heat-denatured Serp-1 (75 °C, 5 minutes). The three conformational forms of Serp-1 were adjusted in concentration from 50 ng/mL to 0.75 ng/mL and tested in the capture ELISA. Figure 3 presents the results.

All four antibody pairs were able to detect native Serp-1 (Figure 3a). The A/C pair was the most sensitive, the B/D pair the least sensitive, and the B/C pair had the widest linear range (0.75–24 ng/mL). None of the antibody pairs were able to detect heat-denatured Serp-1 (Figure 3c). It is known that VT-mAbA can detect Serp-1 on an immunoblot (Figures 1 and 4a), suggesting it may be able to detect heat-denatured Serp-1, but that neither of the capture antibodies (VT-mAbC and VT-mAbD) appeared to bind to heat denatured Serp-1. Two of the antibody pairs (A/C and A/D) were able to detect Serp-1 while it was in a complex with uPA (Figure 3b). The other two pairs (B/C and B/D) could not detect Serp-1 while it was in a complex with uPA, suggesting that the epitope for the detection antibody VT-mAbB was masked or altered by the presence of the bound uPA. Table 1 summarizes the conformational specificity of each antibody. The B/C pair of antibodies apparently can be used in a capture ELISA that is specific for native Serp-1 and would not detect Serp-1 that was denatured or in a complex with a target proteinase. The detection limit for this assay is ~2 ng/mL with a coefficient of variation less than 20% (data not shown). Thus, the capture ELISA with this pair of MAbs will be not only an assay to measure Serp-1 concentrations, but a surrogate assay to indicate Serp-1's activity.

Figure 3: Results of a capture ELISA detection of three conformational forms of Serp-1. The four combinations of capture and detection antibodies (A/C, A/D, B/C, and B/D) were used to detect different conformational forms of Serp-1: (a) Serp-1 in its native conformation. (b) Serp-1 that was incubated with uPA for 2 hours. (c) Serp-1 that was heated to 75 °C for 5 minutes. All three Serp-1 solutions were adjusted to the same concentrations (X axis) and detected in ELISA assays. The OD (450 nm) values were measured (Y axis).







Serp-1 Stability Studies

Serp-1 stability has been examined by subjecting the purified protein to

Figure 4: Thermal stability of Serp-1. Serp-1 was incubated at various temperatures for five minutes and analyzed using either: the band shift assay; the chromogenic uPA inhibition assay; the conformation-specific capture ELISA with the (B/C) MAb pair; or absorbance at 280nm. (a) An image of the X-ray film showing the shifted and unshifted positions of Serp-1 in the band shift assay. (b) A summary of the Serp-1 activity data from the capture ELISA assay, the band shift uPA banding assay, and the chromogenic uPA inhibition assay. Serp-1 activity was expressed as percentages (left Y axis) of control samples, which were kept at 4 °C. A second axis indicates the values (OD280nm) for each sample.

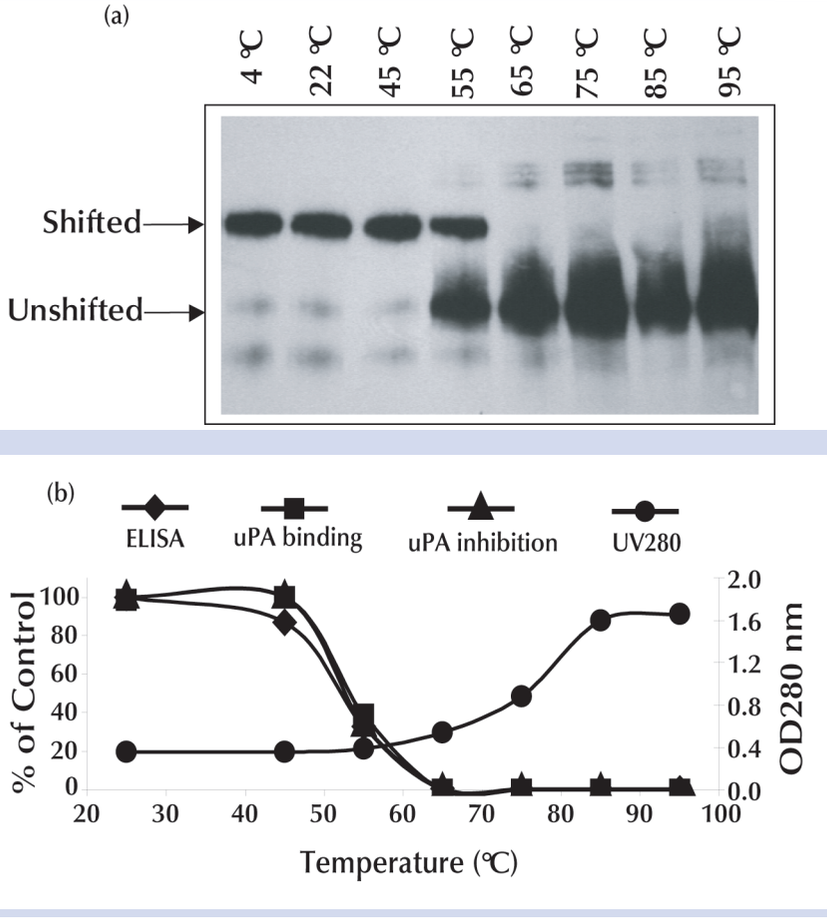


Table 1: Interaction of four anti-Serp-1 monoclonal antibodies (VT-mAbA, VT-mAbB, VT-mAbC, and VT-mAbD) with Serp-1 of three different conformational forms in capture ELISAs; (+) indicates that the MAb could recognize Serp-1 molecules.

	Detecting MAb		Coating MAb	
	A	B	C	D
Native Serp-1	+	+	+	+
Serp-1/uPA complex	+	–	+	+
Heated Serp-1	*+	–	–	–

* See Figure 4a for the detection of Serp-1 by immunoblot using VT-mAbA.

a variety of stress conditions including heating, cycles of freezing and thawing, and exposure to low pH conditions. Absorbance at 280 nm, the band shift assay, the chromogenic uPA inhibition assay, and the capture ELISA using the B/C pair of antibodies were all used to monitor Serp-1 during the various treatments.

For measuring the stability of Serp-1 to heat, samples were incubated at different temperatures for five minutes and then returned to 4 °C. The effect of the heat treatments on the ability of Serp-1 to form an SDS-stable complex with uPA was measured using the band-shift assay (Figure 4a). No difference in Serp-1 activity was seen between 4 °C and 45 °C, but inactivation started to occur at 55 °C. At 65 °C and higher, no Serp-1 activity was detected using the band-shift assay. The level of Serp-1 activity can be quantified using a densitometer by measuring the ratio of shifted to unshifted Serp-1. When this ratio is compared to that of the control sample that was stored at 4 °C, the remaining Serp-1 activity can be expressed as a percent of the control sample, and this is plotted in Figure 4b.

In addition to the band-shift assay, a simple measurement of absorbance at 280 nm was performed on each heated sample. The absorbance increased with temperature. This could be due to an increase in light scattering due to denaturation and aggregation of the heated protein. The amount of Serp-1 activity in each sample was also measured using the chromogenic uPA inhibition assay and expressed in terms of a percent of the control (4 °C) sample (Figure 4b). Similarly, the level of Serp-1 protein in each sample was determined using the capture ELISA with the B/C antibody pair. This ELISA used unheated Serp-1 for the standard curve, and the level of Serp-1 detected in each heated sample was expressed as a percent of the untreated (4 °C) control sample (Figure 4b). There is a clear correlation between the loss of Serp-1 activity as measured by both the band-shift assay and the chromogenic uPA inhibition assay, and the loss of Serp-1 detected by the capture ELISA. This suggests that the capture ELISA could be used as a sensitive surrogate activity-indicating assay for Serp-1.

To determine the stability of Serp-1 to repeat freezing and thawing, test samples were treated to five cycles of freeze/thaw. This consisted of freezing to less than –60 °C and thawing to 22 °C. After each freeze-thaw cycle, the amount of Serp-1 activity was measured using the chromogenic uPA inhibition assay, and the level of active Serp-1 protein was determined using the conformation-specific capture ELISA with the B/C antibody pair (Table 2). No significant change was seen in either Serp-1 activity or protein level,

Figure 5: The pH stability of Serp-1. Six separate aliquotes of Serp-1 (pH 7.2 in PBS) were adjusted by the addition of acetic acid to a final pH of 4.00; 3.75; 3.70; 3.60; 3.40; or 3.30; and held for one hour before being neutralized to ~pH 7.2 with Tris-HCl. Each sample was tested using either the band-shift assay or the capture ELISA. Serp-1 activity in samples was expressed as percentages of the activity of the untreated control sample.

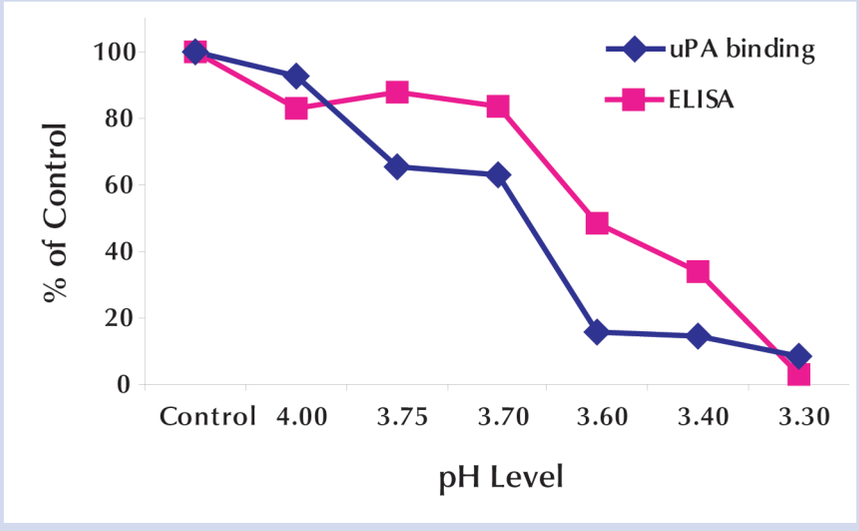


Table 2: Freeze–thaw stability of Serp-1 over five cycles, measuring the Serp-1 activity using the capture ELISA with the MAb pair of B/C and chromogenic uPA inhibition assay (measured as specific activity)

Freeze–thaw cycle	Adsorbance (OD280nm)	ELISA (mg/mL)	Activity (unit/mg)
1	0.895	0.968	59406
2	0.913	—*	58252
3	0.924	0.900	57692
4	0.942	0.971	—*
5	0.978	0.911	63830

*Not tested

indicating that the purified protein is stable to at least five cycles of freeze thaw treatment.

Exposure to low pH, either during elution from an affinity matrix or as a means of virus inactivation, is a relatively common occurrence during the production of a protein therapeutic. To examine the stability of Serp-1 in an acidic environment, purified Serp-1 was exposed to a number of acidic conditions for 60 minutes and then neutralized (to ~pH 7.0). The amount of Serp-1 activity remaining in each sample was measured using the band-shift assay, and the level of active Serp-1 protein was determined using the conformation-specific ELISA (Figure 5). A significant drop in Serp-1 activity was seen when the pH was less than 3.7, and by pH 3.3, virtually all Serp-1 activity was lost. A similar activity profile was seen using the conformation-specific ELISA.

Conclusions

Each protein therapeutic requires a customized set of assays to monitor its biological activity. For Serp-1, we have developed three assays, two of which are based on the ability of Serp-1 to inhibit the serine proteinase uPA. The third assay is a capture ELISA that uses a conformation-specific monoclonal antibody. In a series of stability studies, any loss in Serp-1 activity that was measured using the uPA-based assays was also reflected by a loss in Serp-1 protein using the conformation-specific ELISA. These results indicate that this ELISA is specific for active Serp-1 and that it can be used as a surrogate activity-indicating assay. The high sensitivity and high throughput abilities of the ELISA will facilitate the development of Serp-1 as a protein therapeutic.

Acknowledgments

We are grateful to Heather Schneider and Ling Chen for their excellent technical assistance. The monoclonal antibodies used in this study were provided by Biogen.

References

1 Alcami, A; Koszinowski, UH. Viral Mechanism of Immune Evasion. *Immunol. Today* 2000, 9, 447–455.

2 McFadden, G; Murphy, PM. Host-Related Immunomodulators Encoded By Poxviruses and Herpesviruses. *Curr. Opin. Microbiol.* 2000, 3 (4), 371–378

3 Upton, C; et al. Myxoma Virus and Malignant Rabbit Fibroma Virus Encode a Serpin-Like Protein Important for Virus Virulence. *Virology* 1990, 179, 618–631.

4 Nash, P; et al. Inhibitory Specificity of the Anti-Inflammatory Myxoma Virus Serpin, SERP-1. *J. Biol. Chem.* 1998, 273 (33), 20982–20991.

5 Lucas, A; et al. Transplant Vasculopathy: Viral Anti-Inflammatory Serpin Regulation of Atherogenesis. *J. Heart Lung Transplant.* 2000, 19 (11), 1029–38

6 Miller, LW; et al. Inhibition of Transplant Vasculopathy in a Rat Aortic Allograft Model After Infusion of Anti-Inflammatory Viral Serpin. *Circulation* 2000, 101, 1598–1605.

7 Hausen, B; et al. Viral Serine Proteinase Inhibitor (Serp-1) Effectively Decreases the Incidence of Graft Vasculopathy in Heterotopic Heart Allografts. *Transplantation* 2001, 72, 364–368.

8 Silverman, GA; et al. The Serpins Are an Expanding Superfamily of Structurally Similar but Functionally Diverse Proteins. *J. Biol. Chem.* 2001, 276 (36), 33293–33296.

9 Lomas, DA; et al. Inhibition of Plasmin, Urokinase, Tissue Plasminogen Activator, and C1s By a Myxoma Virus Serine Proteinase Inhibitor. *J. Biol. Chem.* 1993, 268, 516–521.

10 Baker, K; et al. Rapid Monitoring of Recombinant Protein Products: A Comparison of Current Technologies. *Trends in Biotechnology* 2002, 20 (4), 149–156.

*Corresponding author **Dr. Xing Li** Senior Scientist, **Elaine King** is Laboratory and Manufacturing Manager, **Dr. Colin Macaulay** is Research and Development Manager, **Dr. Alexandra Lucas** is Chief Medical Officer, and **Dr. Grant McFadden** is Chief Scientific Officer, at Viron Therapeutics, Inc., UWO Research Park, 100 Collip Circle, Suite 103, London, ON, Canada N6G 4X8, xli@vironinc.com. Additionally, Drs. Lucas and McFadden are associated with the Vascular Biology Research Group of the John P. Robarts' Research Institute, London, ON, Canada; and the Department of Microbiology and Immunology, University of Western Ontario (UWO), London, ON, Canada.*