Proteolytic Activity Directed Toward Pigment Epithelium-Derived Factor in Vitreous of Bovine Eyes

Implications of Proteolytic Processing

Yan-Q. Wu and S. Patricia Becerra

Purpose. Experiments were designed to identify proteolytic activities that cleave pigment epithelium-derived factor (PEDF), a member of the serpin (serine protease inhibitor) family.

Methods. Proteins in vitreous humor from bovine eyes were analyzed by Western blot with antiserum to human recombinant PEDF protein. Protein fractionation was by ammonium sulfate saturation and by S-Sepharose column chromatography. Proteolytic activities were determined by gelatin zymography and by solution assays against PEDF or chromogenic peptide substrates.

Results. PEDF protein was identified and purified to near homogeneity from vitreous humor of bovine eyes. Limited proteolysis showed that the vitreal protein has a protease-sensitive region at its serpin-exposed peptide loop. Proteolytic activities that cleave the PEDF 49.5 kDa-polypeptide were identified only when proteins from these extracts were separated by 45% to 70% ammonium sulfate fractionation (P70). The degradation product had an apparent molecular weight of 46 kDa. This result is consistent with cleavage at the serpin-exposed loop. The PEDF-cleavage activity in P70 was inhibited specifically by the serine protease inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), but not by aprotinin, EDTA, or pepstatin. The vitreal P70 extracts contained 49- and 53-kDa gelatinolytic activities that also were inhibited by AEBSF and not by EDTA, aprotinin, or pepstatin. The PEDF-cleavage activity did not hydrolyze substrates for thrombin, factor Xa, α-chymotrypsin, trypsin, or plasmin, nor did it immunoreact with antibody to urokinase plasminogen activator.

Conclusions. These data indicated that vitreous has a serine-proteolytic activity associated with a novel 49/53-kDa enzyme that cleaves the PEDF protein in a serpinase fashion. In addition to cleavage in vitro, these proteases might play a role in modulating PEDF in vivo.
demonstrated that cleavage at the serpin-reactive loop does not impair PEDF’s neurotrophic function and that an amino-terminal PEDF region (residue positions 44–121) confers the neurotrophic function to the protein. Thus, cleavage at the vulnerable serpin loop of PEDF would remove unnecessary residues from the neurotrophic active region.

Recently, PEDF protein has been localized to the extracellular matrix next to the retina by biochemical fractionation of the soluble components extracted from the interphotoreceptor matrix (IPM) of bovine eyes. The presence of PEDF in IPM, in addition to its neurotrophic activity in vitro, supports the idea that in vivo PEDF plays a neurotrophic role on the retina. The source of PEDF in IPM is thought to be the retinal pigment epithelium (RPE) because cultured human fetal RPE cells are able to release the protein. Functional PEDF protein derived from other ocular sources has not yet been described. However, reports on PEDF mRNA expression in cells other than RPE, such as fetal lung fibroblasts in culture that also secrete PEDF protein to the media, suggest its presence in extracellular matrices other than IPM.

Serpinas are proteases not inhibited by serpins that cleave at the exposed peptide loop. These proteases have been identified mainly in vitro as inactivators of the inhibitory function of serpins. Here we describe the co-localization in bovine eyes of PEDF and natural proteolytic activities that use PEDF protein as a substrate. We have identified and purified PEDF from bovine vitreous humor. Bovine vitreal extracts were analyzed for proteolysis directed toward the PEDF’s protease-sensitive region. Total bovine vitreal proteins did not exhibit proteolytic activity that cleaves its endogenous PEDF. However, fractionation of vitreal proteins by ammonium sulfate revealed proteolytic activities that cleaved PEDF to a 46 kDa protein product. We show that the vitreal PEDF–cleavage activity behaves as serine proteases of 49/53 X 10$^{-3}$ M$^{-1}$ in gelatin zymogram gels. This activity does not cleave substrates for a-chymotrypsin, trypsin, factor X, thrombin, or plasminogen, nor does it immunoreact against antiserum to urokinase–plasminogen activator. Thus, novel serine proteolytic activities are identified in vitreous that behave like serpinases on PEDF. Their involvement in processing PEDF in an in vivo extracellular space is discussed.

**MATERIALS AND METHODS**

**Reagents and Buffers**

Aprotinin, 4-(2-Aminoethyl)-benzenesulfonyl fluoride (AEBSF) and pepstatin A were all from ICN Biomedica (Aurora, OH). EDTA in solution at 0.5 M and phosphate-buffered saline (PBS; containing 795 mg/1 Na$_2$HPO$_4$, 144 mg/1 KH$_2$PO$_4$, and 9 g/1 NaCl, pH 7.4) was from Quality Biologicals (Gaithersburg, MD). Chromozym X, Chromozym TH, and Chromozym PL came from Boehringer Mannheim Biochemica, (Indianapolis, IN). Buffer S contained 50 mM sodium phosphate, pH 6.3, 1 mM diithiothreitol, and 10% glycerol. Bovine pancreas trypsin (TPCK treated) and bovine pancreas a-chymotrypsin (TLCK treated) were from Worthington Biochemical, Freehold, NJ; human plasma thrombin was from Sigma; bovine plasma factor Xa was from New England BioLabs (Beverly, MA); and rabbit anti-human urokinase-plasminogen activator and human urokinase-plasminogen activator were from American Diagnostica (Greenwich, CT).

**Preparation of Bovine Eye Protein Extracts**

All procedures were performed at 4°C. Fresh adult bovine eyes (purchased from J. W. Treuth & Sons, Baltimore, MD) were dissected by removing the anterior segment and carefully peeling the vitreous body from the neural retina. The vitreous was collected at approximately 11 ml per eye and was homogenized using a Brinkmann Polytron, model PT-MR 3000 (Kinematica AG, Littau, Switzerland) three times at 9500 rpm for 30 seconds. Liquefied homogenates were centrifuged at 1300g for 15 minutes to remove cellular debris. The supernatant was ready to be used or stored at –80°C. Aqueous humor was extracted by keratome for material from every 10 eyes). Alternatively, a S-Sepharose Fast Flow (Pharmacia LKB/Biotechnology AB, Uppsala, Sweden) column (1 ml-bed volume for material from every 10 eyes). Alternatively, a Mono-S HR5/5 column (10 cm × 1 cm; Pharmacia LKB/Biotechnology AB) was used for material from 100 eyes. The PEDF-containing fractions were pooled and ultrafiltered with Centricon-100 (Amicon, Beverly, MA) to remove large proteins. The filtrate was concentrated and desalted by ultrafiltration using...
Centricon-30 (Amicon). The concentrated sample (1.6 mg/ml) was centrifuged at 14,000 g for 10 minutes to remove particulate material, and the supernatant was loaded onto a TSK-SW3000 column (ToSo-Haas, Montgomeryville, PA) attached to a high-performance liquid chromatography system at a flow rate of 1 ml/minute with PBS. Fractions of 1 ml were collected, and the PEDF-containing fractions were stored at −80°C.

Preparation of Vitreal Samples for Proteolytic Activity

Bovine vitreal extract (10 ml) was fractionated by 45% ammonium sulfate saturation. The P₅₀ precipitate was resuspended in 1.5 ml of PBS, desalted, and concentrated 1.5 times, using Centricon-30 (Amicon) as described above. To separate proteases from PEDF, the P₅₀ was applied to an S-Sepharose column, as described above. The different fractions from ammonium sulfate precipitation and from the S-Sepharose column chromatography were used for proteolytic assays.

Solution Assays for Determination of Serine Proteases

Assays with several substrates for serine proteases were used in solution: Chromozym-X (N-Methoxy-carbonyl-D-norleucyl-glycyl-L-arginine-4-nitranilide-acetate), Chromozym TH (Tosyl-glycyl-prolyl-arginine-4-nitranilide-acetate), Chromozym PL (Tosyl-glycyl-prolyl-lysine-4-nitranilide-acetate), and suc-AAPFpNA (succinyl-alanine-alanine-proline-phenylalanine-pnitroaniline). A total of 14 µl of the flow through (FT) from the S-Sepharose column was mixed with either 1 ml of buffer X (Tris 50 mM, pH 8.3, 150 mM NaCl, 16 mM CaCl₂, 5 µg/ml polybrene, 20 µg/ml aprotinin) and 0.05 ml of 20 mM Chromozym X; 0.1 ml of 1.9 mM Chromozym TH, and 0.94 ml of buffer TH (50 mM Tris pH 8.3, 227 mM NaCl); 0.8 ml of buffer PL (50 mM Tris pH 8.2), 0.1 ml 0.9% NaCl and 0.2 ml of 3 mM Chromozym PL; or 50 µl of buffer A (20 mM Tris–HCl pH 7.4, 1 mM EDTA, 150 mM NaCl), and 50 µl of 0.916 mM suc-AAPFpNA. The proteolytic activity was determined spectrophotometrically by measuring the increase in absorbance of 405 nM (DU-30 Spectrophotometer; Beckman Instruments, Irvine, CA). Each substrate was used in a reaction with a known protease to confirm the validity of the assay and was used as a control.

Other Methods

Neurite-outgrowth assays on human retinoblastoma Y-79 cells in culture and SDS–PAGE analysis were performed as described. Molecular weight standards were from Bio-Rad Laboratories (Hercules, CA). Protein concentration determinations were as described by Bradford using Protein Assay by Bio-Rad Laboratories (Hercules, CA) and bovine serum albumin as a standard.

RESULTS

Identification of Pigment Epithelium-Derived Factor in Extracellular Spaces in Bovine Eyes

Figure 1A shows Western transfers of bovine aqueous, vitreous, and a subchoroid wash immunostained with the polyclonal antiserum to PEDF, Abr-PEDF. A 50,000-M, protein is recognized in vitreous and in aqueous. Densitometric scanning indicates that the vitreal immunoreactive signal is 10 times stronger than...
one for aqueous. Based on these results, the vitreal PEDF protein was selected for our studies. First, PEDF was highly purified from bovine vitreous by ammonium sulfate saturation (45% to 80%), Mono-S column chromatography, and gel-filtration column chromatography, as summarized in Figure 1B. The amount of purified PEDF protein obtained through this procedure was approximately 7.2 μg per vitreous of one bovine eye. Vitreal PEDF shared characteristics with the protein from IPM isolated as previously described. Analyses by SDS–PAGE and size-exclusion column chromatography indicated, respectively, an apparent molecular weight (49,500–M₂) and a retention time (18.5 minutes) for the vitreal protein that were identical to those obtained with the IPM-derived PEDF. The neurite-outgrowth activity of the PEDF purified from vitreous was confirmed by treating human Y-79 retinoblastoma cells with 100 ng of pure protein per milliliter of culture for neurite induction (Fig. 2). Induction with protein extracts from aqueous also showed morphologic differentiation of Y-79 cells, revealing its PEDF-mediated neurotrophic activity. Note that control cells treated with bovine serum albumin did not induce differentiation.

Figure 1. Pigment epithelium-derived factor (PEDF) in vitreous of bovine eyes. (A) PEDF immunoreactivity in bovine eye extracts. Protein extracts from aqueous, vitreous, and subchoroid of a bovine eye were resolved by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a membrane by Western blotting for immunoreaction with antisera to human rPEDF, Ab-rPEDF, at a dilution of 1:10,000. Photograph of an immunostained membrane with 4-chloro-l-naphthol is shown. Samples and volumes applied to the polyacrylamide gel were as follows: lane 1, 20 μl of aqueous extract (3.8 μg protein extract); lane 2, 15 μl of vitreal extract (6 μg protein extract); and lane 3, 20 μl of subchoroid wash (8 μg protein extract). (B) Purification of PEDF from vitreous of bovine eyes. PEDF in vitreous was purified by 45% to 80% ammonium sulfate fractionation, S-Sepharose column chromatography, and TSK-3000 column chromatography. The volume of the vitreous humor from one bovine eye had an average of 11 ml and contained a total of 4.4 mg of protein, of which PEDF was less than 1%. The final PEDF protein sample was highly pure and amounted to a total of 7.2 μg PEDF protein purified from each eye. Proteins from each fraction were resolved by 12.5% SDS–PAGE and were stained with Coomassie blue. A fraction representative of each purification step was applied to each lane with the following total protein amount: lane 1, bovine vitreal extract, 8 μg; lane 2, 45% to 40% ammonium sulfate fraction P₃₀, 14.6 μg; lane 3, the 250 mM NaCl eluate from the S-Sepharose column chromatography, 3 μg; and lane 4, fraction from the TSK-3000 column chromatography with a retention time of 18.5 minutes, 4 μg. The numbers to the left correspond to the migration of SDS–PAGE standards.

Figure 2. Biologic activity. Human retinoblastoma Y-79 cells exponentially growing in serum-containing medium were washed twice with phosphate-buffered saline and were plated (1.25 × 10⁶ cell/ml) in serum-free minimal essential medium supplemented with a mix of insulin, transferrin, and selenium. Effectors were then added to the cultures. After 7 days at 37°C in 5% CO₂, the cells were attached to poly-D-lysine-coated plates with fresh serum-free medium. The differentiation state of the cultures was monitored at various intervals after attachment. Morphologic characteristics of 9-day postattachment cultures are shown. The addition of effectors was at the following final protein concentrations: (A) 125 μg/ml bovine serum albumin; (B) 100 ng/ml purified bovine vitreal pigment epithelium-derived factor.
PEDF
Cleaved PEDF

min 120 30 10 120 30 10 120
α-chymotrypsin Trypsin

FIGURE 3. Limited proteolysis of vitreal pigment epithelium-derived factor (PEDF). PEDF protein purified from bovine vitreous was incubated with trypsin or chymotrypsin at a protease:substrate ratio of 1:100 (wt/wt) for increasing periods of time. Each reaction mixture (15 μg) contained a total amount of 4 μg PEDF protein in 20 mM Tris–Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA. Incubations were at 25°C and for the period of time indicated at the bottom of the figure. The proteolytic reactions were stopped by the addition of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer and heating at 100°C for 3 minutes. Samples were applied to a 10% to 20% gradient SDS–PAGE gel; after electrophoresis, proteins were stained with Coomassie blue. A photograph of a stained gel is shown.

Analysis of Purified Vitreal Pigment Epithelium-Derived Factor Protein

Amino-terminal sequence analysis of the purified vitreal PEDF protein in solution was performed. Vitreal PEDF was not sensitive to Edman degradation, unlike the IPM-derived protein. Generally, this is caused by a posttranslational modification at the amino-terminal residue (e.g., acetylation) that blocks the automated Edman degradation. To obtain internal sequence information, the purified vitreal PEDF was treated with either trypsin or chymotrypsin. Under controlled conditions, the 49.5 kDa protein was proteolyzed to generate fragments of 46 kDa (cleaved PEDF) and <5 kDa (Fig. 3). The low-molecular-weight fragment is less sensitive to Coomassie blue staining than the larger one; however, Edman degradation of the reaction mixture confirms the presence of the low-molecular-weight product. Sequence analysis of the proteolytic products yielded an internal PEDF primary structure identical to an internal IPM–PEDF region. The sequences obtained from the tryptic and chymotryptic treatments started at residue positions 382 and 383 in the human PEDF sequence, respectively. The tryptic and chymotryptic cleavage sites correspond to the exposed loop at the carboxy end of the molecule. The results confirmed a folded protein structure for PEDF derived from vitreous that is shared with the one previously demonstrated for the IPM-derived PEDF and serpins.

Stability of Pigment Epithelium-Derived Factor to Cleavage in Total Vitreous

The data presented above demonstrate that the carboxy end of vitreal PEDF is highly vulnerable to proteolytic cleavage. To determine whether PEDF protein undergoes proteolytic cleavage in vitreous, aliquots of total vitreous were incubated at 37°C and then were analyzed by Western blot against Ab-rPEDF. Figure 4 shows that endogenous PEDF did not change its migration pattern on incubation up to 16 hours of total vitreous extract. Coomassie blue staining of the SDS–polyacrylamide gel demonstrated that after incubation, some bovine vitreous proteins undergo degradation and/or formation of protein–protein com-
Activity by Fractionated Vitreal Proteins

Pigment Epithelium-Derived Factor (PEDF) Cleavage Activity in Vitreal Fraction P70

Western blot analysis of vitreal preparations of ammonium sulfate fraction P70 with polyclonal antibody Ab-rPEDF. Immunoblots are shown. In A, two different preparations of P70, P70', and P70", containing PEDF protein (49.5 kDa) were analyzed. Aliquots (20 μl) were incubated with or without 14 mM CaCl2 at 37°C for 16 hours. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed under reducing conditions. Samples were applied as follows: lane 1, P70 without Ca2+; lane 2, P70' with Ca2+; lane 3, P70' without Ca2+; and lane 4, P70" with Ca2+. In B, the effect of inhibitors on the activity of fraction P70" was analyzed. Reaction mixtures of P70" (20 μl) containing the 49.5 kDa PEDF polypeptide were incubated at 37°C for 16 hours in the presence of inhibitors. Final concentration of inhibitors in each reaction was as follows: lane 1, no incubation; lane 2, no inhibitor; lane 3, 5 μg/ml aprotinin; lane 4, 10 mM AEBSF; lane 5, 5 μg/ml pepstatin A; and lane 6, 40 mM EDTA. Arrows indicate the migration positions for the 49.5 kDa and the 46 kDa PEDF proteins.

FIGURE 5. Pigment epithelium-derived factor (PEDF) cleavage activity in vitreal fraction P70. Western blot analysis of vitreal preparations of ammonium sulfate fraction P70 with polyclonal antibody Ab-rPEDF. Immunoblots are shown. In A, two different preparations of P70, P70' and P70", containing PEDF protein (49.5 kDa) were analyzed. Aliquots (20 μl) were incubated with or without 14 mM CaCl2 at 37°C for 16 hours. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed under reducing conditions. Samples were applied as follows: lane 1, P70 without Ca2+; lane 2, P70' with Ca2+; lane 3, P70' without Ca2+; and lane 4, P70" with Ca2+. In B, the effect of inhibitors on the activity of fraction P70" was analyzed. Reaction mixtures of P70" (20 μl) containing the 49.5 kDa PEDF polypeptide were incubated at 37°C for 16 hours in the presence of inhibitors. Final concentration of inhibitors in each reaction was as follows: lane 1, no incubation; lane 2, no inhibitor; lane 3, 5 μg/ml aprotinin; lane 4, 10 mM AEBSF; lane 5, 5 μg/ml pepstatin A; and lane 6, 40 mM EDTA. Arrows indicate the migration positions for the 49.5 kDa and the 46 kDa PEDF proteins.

Novel AEBSF-Sensitive Proteolytic Activity in Vitreous

To separate the endogenous PEDF from the PEDF-cleavage activity in P70', fraction P70' was subjected to S-Sepharose column chromatography as described before.4 Proteins that did not bind were termed flow through (FT70') and were free of PEDF protein. The PEDF-cleavage activity and the gelatinolytic activities in FT70 were analyzed as above. Results showed that FT70' exhibits the PEDF-cleavage activity associated with 49/53 kDa gelatinolytic activities (Fig. 7).

To identify the proteolytic activities present in FT70', serine protease assays were performed. The selection of reagents was based on information reported for known serine proteases (Table 1). Serine proteases not sensitive to aprotinin and/or of similar molecular weight as the ones in FT were considered, such as thrombin, factor X, urokinase–plasminogen activator. Chromozym X, Chromozym TH, Chromozym PL, and Suc-AAPF-pNA are sensitive substrates for determining the activity of factor X, thrombin, plasmin, and α-chymotrypsin, respectively. Table 2 shows that the activities in FT70' did not use the selected chromogenic compounds as substrates. Western transfers of FT70' did not immunoreact with anti-urokinase–plasminogen—
FIGURE 6. Zymography analysis of vitreal P70. (A) A volume of 10 μl of each fraction, P70', (lane 1) and P70'' (lane 2), was applied to a zymogram gel and analyzed for gelatinolytic activities in situ. A Coomassie blue-stained gel is shown. (B) Zymography of P70' in the presence of inhibitors. Aliquots of P70' (10 μl) were applied to each lane, and, after electrophoresis, each lane was excised. Proteolytic reaction in each gel strip was performed in the presence of inhibitors, with final concentrations as follows: lane 1, no inhibitor; lane 2, 5 μg/ml aprotinin; lane 3, 10 mM AEBSF; lane 4, 5 μg/ml pepstatin A; and 40 mM EDTA. Numbers to the left of each panel correspond to the migration position of sodium dodecyl sulfate–polyacrylamide gel electrophoresis standards.

PEG activator. These results indicated that the enzyme that cleaved PEDF in FT70 was not any of these five proteases. Thus, a novel AEBSF-sensitive activity(ies) that cleave PEDF in a serpin fashion is present in vitreous.

DISCUSSION

We have found that the bovine vitreous contains proteolytic activities that use PEDF protein as a substrate. PEDF protein is present in extracellular spaces of the bovine eye, in vitreous, and in aqueous humor (Fig. 1A). As we have reported earlier, PEDF also is localized to the bovine IPM, an ocular extracellular matrix next to the neural retina. These observations, in addition to the account that PEDF is released from cultured human fetal retinal pigment epithelial cells, indicate that under physiological conditions PEDF protein is extracellular. Like other extracellular matrices, vitreous has been reported to contain proteases that can regulate the levels and functions of its extracellular proteins. To investigate how PEDF is modulated in an extracellular matrix, our approach was to identify an in vivo extracellular system for PEDF and for proteolytic activities that specifically cleave PEDF.

PEDF protein accumulates more abundantly in vitreous than in other extracellular spaces of a bovine eye. The concentration of PEDF in vitreous is more than 10 times higher than in aqueous (Fig. 1A). Given the large vitreal volume (approximately six times larger than in aqueous), the levels of PEDF protein in vitreous are more than 60 times higher than in aqueous. With respect to IPM, the PEDF concentrations are estimated to be eight times lower in vitreous (1.6 μg/ml) than in IPM (13.2 μg/ml), where PEDF accumulates next to its putative target cells. However, the total amount of PEDF in vitreous is five times higher than in IPM from an average bovine eye. Although the relative amount of PEDF protein with respect to the total vitreal proteins is less than 1% (Fig. 1B), we have been able to separate PEDF from other vitreal proteins. Following our purification protocol, the yields of highly purified PEDF protein are approximately 7.2 μg per vitreous (Fig. 1B), i.e., they are five times higher than the yields from IPM. Characterization of the PEDF indicates that the vitreal-derived protein shares linear and folded protein characteristics (Fig. 5) of the IPM-derived PEDF protein. One difference is found at the amino terminus of the vitreal PEDF, with an apparent posttranslational modification that does not impair its biologic activity (Fig. 2). This modification suggests that the presence of PEDF in vitreous is not a result of protein diffusion from the IPM.
FIGURE 7. Separation of pigment epithelium-derived factor (PEDF) from cleavage activity. Fraction P701 was applied to an S-Sepharose column to separate PEDF from other proteins. Flow through FT70 corresponded to the fraction of proteins that did not bind to the column. (A) Western blot analysis of reaction mixtures of FT70 (12 μl) with 0.15 μg of pure PEDF added as a substrate and incubated at 37°C for 16 hours. Immunoreaction was with antiseraum Ab-rPEDF at 1:10,000 dilution, and staining was with 4-chloro-l-naphthol. Additions and incubations for each lane were as follows: lane 1, without FT70 and without incubation; lane 2, with FT70 and without incubation; lane 3, with FT70 and with incubation. (B) Zymography analysis of P701 and FT70. A total of 12 μl of each sample was applied to each lane of a zymogram-gelatin gel. After electrophoresis, the gel was treated for a reaction in situ as described in Materials and Methods. A photograph of a Coomassie blue-stained gel is shown. The numbers to the left of each panel correspond to the migration positions of sodium dodecyl sulfate-polyacrylamide gel electrophoresis standards.

The vitreal proteolytic activity identified in this study is the PEDF-cleavage activity that cleaves PEDF in a discrete serpinase fashion (Fig. 5). This activity is associated with 49/53 kDa gelatinolytic activities classified as metalloproteases by their inactivation with EDTA and their insensitivity to other protease inhibitors (see Fig. 6B). Brown et al.20 have reported that vitreous contains an endogenous matrix metalloproteinase-2, MMP-2, known to digest collagen and glycoproteins. Thus, downregulation of PEDF levels in vitreous can be achieved by these endogenous metalloproteases.

One problem with the vitreal preparations is that vitreal samples prepared in a similar fashion show variability in enzyme activities (Figs. 5A, 6A). Demonstration of the existence of the serpinase activity was obtained from 3 of 5 preparations. Furthermore, during purification of PEDF from vitreous, it is not uncommon that several batches result in the preparation of cleaved PEDF (unpublished observations, 1994), indicating the presence of serpinase activities in vitreous. The variability of bovine vitreal protein activities is known, although not well studied. One point to consider is that the activity of the vitreal enzymes depends not only on their dissociation from endogenous inhibitors but on their release from the insoluble framework provided by the collagens in the vitreous.24,25 Proteinases can associate with proteoglycans, as observed in corneal extracts.26 In addition, a lack of knowledge of endogenous mechanisms for enzyme activation, stability, and activity levels can provide another variability factor. In the current study, treatments with exogenously added calcium ions, temperature, and freeze-and-thaw cycles of vitreal samples did not eradicate endogenous serine protease activities.

### Table 1. Aprotinin-insensitive and AEBSF-sensitive Serine Proteases

<table>
<thead>
<tr>
<th>Serine Protease</th>
<th>Protein Size (M, × 10⁻³)</th>
<th>Aprotinin Sensitivity</th>
<th>AEBSF Sensitivity</th>
<th>Reference</th>
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<tr>
<td>Factor Xa (bovine)</td>
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<td>−</td>
<td>+</td>
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<tr>
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<td>37/28</td>
<td>−</td>
<td>+</td>
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not activate the serpinase enzyme in a controlled fashion (Becerra SP, unpublished observations, 1995). Our data indicate that, although activation remains unresolved, demonstration of the existence of the 49/53 kDa PEDF-cleaving serpinase requires release from the insoluble matrix and separation from endogenous inhibitor(s).

Proteases are involved in modulating proteins by either site-specific proteolysis or complete degradation. Site-specific proteolysis is one of the most common forms of posttranslational modifications of proteins. Cleavage by serpinases is of the site-specific type. Serpinases have been studied mainly in vitro, and most of them have been metalloproteases acting on inhibitory serpins as downregulators of the inhibitory function.7-9 In noninhibitory serpins, cleavage at the carboxyl terminal-exposed loop does not have a functional implication, unless this region confers a function to the serpin protein. To this effect, the neurotrophic activity of PEDF is conferred by the amino terminal region of the protein.5 Thus, serpinase-cleaved PEDF retains the biologic activity exhibited by the uncleaved protein. These observations, together with the fact that PEDF is vulnerable to serpinase activities (Figs. 3, 5), support the idea that our PEDF-cleavage activity is involved in processing PEDF. In higher eukaryotes, there is a family of precursor processing endoproteases termed mammalian subtilisin-related proprotein convertases,27 which cleave neuropeptide precursors, prohormones, and various other precursor proteins; all have catalytic domains related to subtilisin, a prokaryotic family of serine proteases. The PEDF-cleavage activity also shares an inhibitory pattern with subtilisin. Altogether, the results presented here reveal a serpinase activity that is identified in a eukaryotic system and that shares particular characteristics with the subtilisin-related proprotein family of convertases. The sequence of the protease protein and physiological target of the vitreal serpinase activity are unknown. However, in addition to being co-expressed with PEDF protein in the vitreous humor and cleaving the protein in vitro, these proteases might be involved in modulating PEDF protein in vivo.

**Key Words**

bovine eyes, pigment epithelium-derived factor, serine proteases, serpins, vitreous humor

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**References**