Purification and Characterization of the Human Stromelysin Catalytic Domain Expressed in *Escherichia coli*

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ABSTRACT: Human stromelysin is a member of the matrix metalloproteinase family involved in connective tissue degradation. The stromelysin catalytic domain (SCD) lacking both propeptide and C-terminal fragment was expressed in *Escherichia coli* in soluble and insoluble forms. The insoluble SCD was refolded to the active form in high yield. The protein showed remarkable thermal stability and was able to cleave a thiopeptolide substrate and its natural substrate proteoglycan. The stable and active 20-kDa protein provides an opportunity to elucidate the structure as well as the mechanism of catalysis and inhibition for matrix metalloproteinases.

Matrix metalloproteinases, such as stromelysins, collagenases, and gelatinases, are believed to be involved in connective tissue degradation (Woessner, 1991) in several physiological and pathological processes including cartilage degradation in arthritis and tumor progression and metastasis (McDonnell & Matrisian, 1990). Therefore, there is great interest in understanding the catalytic mechanism of these matrix metalloproteinases and designing specific inhibitors to control their activity.

Similar to other matrix metalloproteinases, the human fibroblast stromelysin (Whitham et al., 1986; Saus et al., 1988) has a signal peptide for secretion, a propeptide with a cysteine residue for maintaining latency (Van Wart & Birkedal-Hansen, 1990; Park et al., 1991), a catalytic domain with a conserved sequence highly homologous with the zinc binding site in the bacterial zinc proteinase thermolysin (Vallee & Auld, 1990), and a C-terminal fragment which may be involved in substrate and inhibitor binding (Allan et al., 1991; Murphy et al., 1992). The matrix metalloproteinases are all secreted as proenzymes and are activated in vivo by a mechanism not yet determined. However, these enzymes can be activated in vitro with organomercurials, proteolytic enzymes, chaotropic agents, or heat (Okada et al., 1988; Nagase et al., 1990; Koklitis et al., 1991). Removal of the propeptide from prostromelysin by proteinases and organomercurial compounds is a stepwise process (Okada et al., 1988; Nagase et al., 1990) which generates intermediate forms before the propeptide is removed completely by activated stromelysin. The activated enzyme undergoes autolytic cleavage at sites close to the C-terminus, producing a 28-kDa fragment as well as smaller species (Okada et al., 1988; Koklitis et al., 1991). The instability of matrix metalloproteinases due to autodegradation may partially account for the difficulty in structural determination by X-ray crystallography. Matrilysin (formerly called PUMP) is a unique member of this enzyme family in that it lacks the C-terminal portion found in stromelysins, collagenases, and gelatinases (Muller et al., 1988). C-Terminal-deleted stromelysin and collagenase have been made, and they have shown activity similar to the full-length enzymes (Marcy et al., 1991; Lowry et al., 1992; Murphy et al., 1992). Therefore, the N-terminal catalytic domain of stromelysin is responsible and sufficient for the proteinase activity, and the C-terminal portion can be removed without major modification to the active site of the catalytic domain.

We report here the expression, purification, and characterization of a 20-kDa stromelysin catalytic domain (SCD) lacking both the propeptide and the C-terminal fragment (Figure 1). The removal of the propeptide eliminates the need for proteolytic or chemical activation, and the removal of the C-terminal fragment removes autolytic sites, thereby making the protein resistant to autodegradation. We intended to make an active and stable protein with a mass of 20 kDa, suitable for structure determination by nuclear magnetic resonance spectroscopy and X-ray crystallography, as well as mechanistic studies of catalysis and inhibition.

MATERIALS AND METHODS

Materials. Oligonucleotide primers for PCR were synthesized on a Model 392 DNA synthesizer (Applied Biosystems, Foster City, CA) and were purified with an OPC cartridge (Applied Biosystems). The sequence for the forward primer F was GGATCACCA*GCTAGC* TATCCATTGGATGGAGCTGCA, and that for the reverse primer R was GCACTC*GAATTC*TGCAGTCA GGGGGTCTCAGGGGAGTCAG, where the sequences from the stromelysin gene are underlined and the restriction sites *Nhe*I and *Eco*RI are in italics. The cDNA fragment containing the stromelysin gene (Whitham et al., 1986; Saus et al., 1988) was provided by Dr. C. E. Brinkerhoff. The *Escherichia coli* strain DH5αF'IQ was purchased from BRL (Gaitherburg, MD). The plasmid vector pGEMEX-1 was obtained from Promega (Madison, WI), and the M13 phage carrying T7 RNA polymerase (M13/T7) was from Invitrogen (San Diego, CA). Restriction and ligation enzymes, as well as Vent DNA polymerase, were from New England Biolabs (Beverly, MA). The thiopeptolide Ac-Pro-Leu-Gly-thioester–Leu-Leu-Gly-OEt was obtained from Bachem Bioscience (Philadelphia, PA). Inhibitor U24522 (Caputo et al., 1987) was synthesized at Parke-Davis. The protease inhibitors leupeptin, aprotinin, and pepstatin were from Boehringer Mannheim (Indianapolis, IN). Phenyl-Sepharose and Q-Sepharose were purchased from Pharmacia LKB (Piscataway, NJ).

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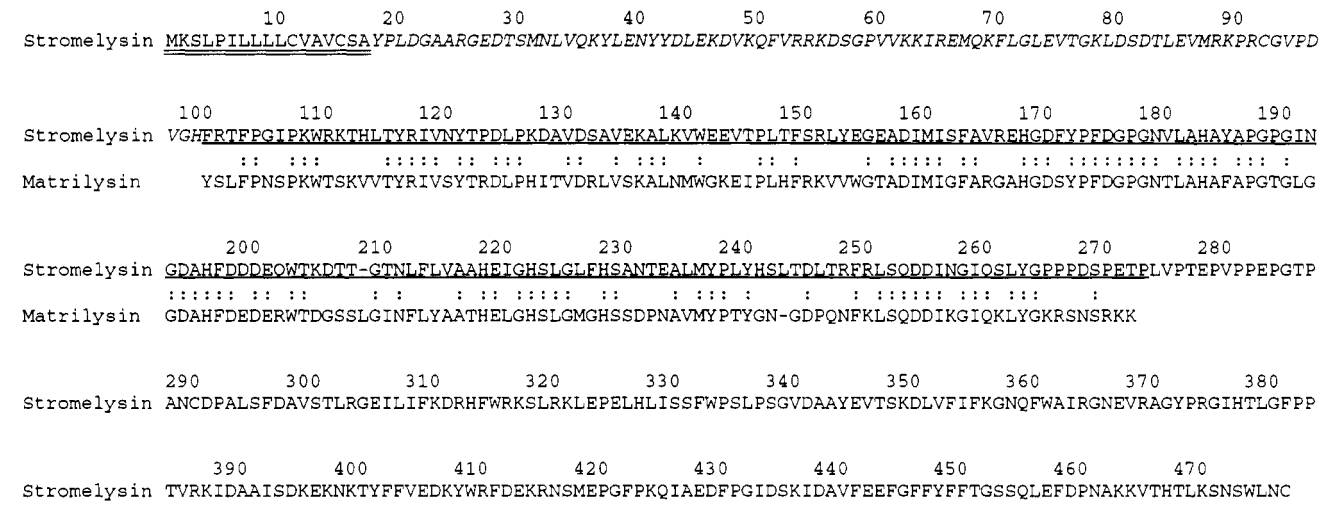

FIGURE 1: Comparison of the amino acid sequences of prostromelysin and the mature matrilysin. The identical amino acids in both prostromelysin and matrilysin are marked by colons. The signal peptide is doubly underlined, and the propeptide is in italics. The sequence for SCD is underlined.

Construction of the Expression Vector pGEMEX-D. Human cDNA containing the stromelysin gene (Whitham et al., 1986; Saus et al., 1988) was amplified by PCR with the primers F and R to obtain the gene fragment coding for amino acid residues 100–273 (Figure 1). The primers were designed to introduce the *Nhe*I restriction site at the beginning of the gene and the *Eco*RI site at the end. The PCR fragment was digested with *Nhe*I and *Eco*RI and ligated with plasmid vector pGEMEX-1, which was previously digested with *Nhe*I and *Eco*RI and dephosphorylated with calf intestine alkaline phosphatase. The recombinant plasmid pGEMEX-D (Figure 2) was transformed into *E. coli* strain DH5αF'IQ. The plasmid was analyzed by restriction digestion, and the DNA sequence for the SCD gene was confirmed by the dideoxy sequencing method.

Expression of the SCD. Two liters of 2×TY medium (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter) containing ampicillin (50 μg/mL) and kanamycin (30 μg/mL) was inoculated with 20 mL of overnight culture of DH5αF'IQ/pGEMEX-D in the same medium. Cells were cultured at 37 °C to an OD_{600} of about 0.8 before induction by adding the phage M13/T7 (plaque forming unit per milliliter = 1×10^{11}, multiplicity of infection = 10) and IPTG (1 mM final concentration). In order to maximize the production of soluble SCD, the temperature was lowered to 27 °C after induction, and the incubation was continued at 27 °C for 4 h before harvesting by centrifugation. In order to maximize the production of insoluble SCD, the incubation at 37 °C was continued for 6 h after induction. The cells were frozen immediately and kept at −20 °C until use.

Purification of Soluble SCD. Cells (6.34 g wet weight) grown at 27 °C were resuspended in 25 mL of 100 mM Tris·HCl (pH 7.6) buffer containing 5 mM $CaCl_2$, 0.5 mM $ZnCl_2$, and the protease inhibitors leupeptin, aprotinin, and pepstatin (1 μg/mL each). The suspension was passed through a French press twice at 14 000 psi, and the lysate was centrifuged at 20000*g* for 30 min at 4 °C. Ammonium sulfate was added to the supernatant to 20% saturation, and the mixture (30 mL) was centrifuged, loaded onto a phenyl-Sepharose column (180 mL) previously equilibrated with 50 mM Tris·HCl (pH 7.6), 5 mM $CaCl_2$, and 1 M $(NH_4)_2SO_4$, and eluted with a linear gradient of decreasing $(NH_4)_2SO_4$ concentration (from 1 to 0 M) and increasing $CaCl_2$ concentration (from 5 to 20 mM) in 50 mM Tris·HCl (pH 7.6). The active fractions were combined and concentrated to a 10-mL volume through a YM10 membrane in an Amicon stirred cell (Amicon, Beverly, MA). The conductivity was adjusted during the concentration with 50 mM Tris·HCl (pH 7.6) and 10 mM $CaCl_2$ to about 8 mS ($m\Omega^{-1}/cm$). The protein solution (10 mL) was loaded onto a Q-Sepharose column (180 mL) previously equilibrated with 50 mM Tris·HCl (pH 7.6) and 10 mM $CaCl_2$, and the protein was eluted from the column with the same buffer containing 200 mM NaCl with a linear gradient (0–100%). The purified SCD was concentrated and stored in 50 mM Tris·HCl (pH 7.6) and 10 mM $CaCl_2$ at 4 °C.

Refolding and Purification of Insoluble SCD. The cells (5.97 g wet weight) grown at 37 °C were resuspended in 25 mL of 50 mM Tris·HCl buffer (pH 7.6) and lysed by two passages through a French press at 14 000 psi. The pellet obtained after centrifugation (20000*g*, 30 min) was washed twice with 50 mM Tris·HCl and solubilized with 10 mL of 8 M guanidine hydrochloride. The mixture was centrifuged at 20000*g* for 20 min. The supernatant was added dropwise to a 100-mL refolding buffer (stirred at 4 °C) containing 50 mM Tris·HCl (pH 7.6), 10 mM $CaCl_2$, 0.1 mM $ZnCl_2$, and the protease inhibitors leupeptin, aprotinin, and pepstatin (1 μg/mL each). The refolding mixture was centrifuged at 20000*g* for 20 min. The pellets were dissolved in another 10 mL of 8 M guanidine hydrochloride, and the refolding was repeated twice. The supernatants from the three refoldings were combined, and ammonium sulfate was added to 20% saturation. The solution containing the SCD was purified on phenyl-Sepharose and Q-Sepharose columns as described for soluble SCD.

Thiopeptolide Assay. The hydrolysis of the thiopeptolide Ac-Pro-Leu-Gly-thioester–Leu-Leu-Gly-OEt, originally developed for vertebrate collagenase (Weingarten & Feder, 1985), was used to follow the SCD activity. A 100-μL assay mixture contained 50 mM MES (pH 6.0), 10 mM $CaCl_2$, 100 μM thiopeptolide substrate, and 1 mM DTNB. The substrate concentration was varied from 10 to 800 μM to obtain K_m and k_{cat}. The change of absorbance at 405 nm was monitored on a ThermoMax microplate reader (Molecular Devices, Menlo Park, CA) at room temperature (22 °C). The calculation was based on ϵ_{412} = 13 600 M^{-1} cm^{-1} for the DTNB-derived product 3-carboxy-4-nitrothiophenoxide (Ellman, 1959).

Proteoglycan Degradation Assay. The proteinase activity was measured using the proteoglycan–polyacrylamide particle assay (Nagase & Woessner, 1980) that was modified for

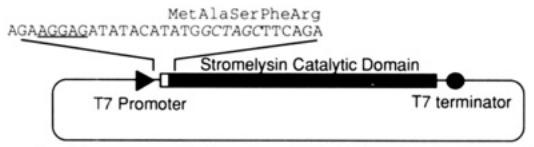

FIGURE 2: Structure of the plasmid pGEMEX-D (3.7 kb) for expressing SCD. Shown in detail is the sequence surrounding the N-terminus of the gene, where the ribosome binding site is underlined and the *Nhe*I site is in italics. Three amino acid residues (Met, Ala, and Ser) were added to the N-terminus of SCD as shown.

screening enzyme inhibitors (Baragi et al., 1992). The assays were conducted in a final volume of 200 μL containing 50 mM Tris·HCl (pH 7.4), 5 mM $CaCl_2$, 200 mM NaCl, 0.02% NaN_3, 7 μg of SCD, and 4 mg of proteoglycan–polyacrylamide particles (150 ± 20 μg of chondroitin sulfate/mg of particles). The incubations were carried out in the presence or absence of inhibitors for 16 h at 37 °C. Enzyme activity was expressed as micrograms of chondroitin sulfate released per hour.

RESULTS

Expression of the SCD. An efficient expression vector was constructed using T7 RNA polymerase (Tabor & Richardson, 1985; Studier et al., 1990) for producing large quantities of SCD (Figure 2). The SCD gene was fused in-frame to the 5′ region of T7 phage gene 10 and replaced most of the gene 10 sequence originally on pGEMEX-1. The recombinant plasmid pGEMEX-D was introduced into DH5αF′IQ, and the SCD expression was initiated by introduction of a M13 phage carrying the T7 RNA polymerase gene. A particularly valuable feature for this expression system is that there is no basal expression of the SCD due to the absence of T7 RNA polymerase prior to the M13 phage infection. This is important for expressing potentially toxic proteins in *E. coli*.

The expression of the SCD under the control of T7 RNA polymerase was efficient. Most SCD was generated as insoluble protein when the expression was carried out at 37 °C. By lowering the incubation temperature to 27 °C, a considerable amount of soluble SCD was obtained. Further lowering of the temperature resulted in a much slower growth rate without increasing the SCD yield. Although the SCD was expressed as an active proteinase without propeptide, no significant effect on cell growth was observed during expression of the SCD either as a soluble or as an insoluble protein.

Purification of the SCD. The cells grown at 27 °C were used for purifying soluble SCD. The SCD bound tightly to phenyl-Sepharose in the presence of 1 M ammonium sulfate and was eluted from the column with a decreasing gradient of ammonium sulfate. The binding of the SCD to Q-Sepharose was weak so that it is necessary to adjust the conductivity of the SCD solution to <10 mS. The protein solution was loaded onto the Q-Sepharose column, and the protein was eluted with a shallow gradient of NaCl (0–200 mM). The protein purified through the two columns appeared to be homogeneous on an SDS–polyacrylamide gel (Figure 3, Table I).

In order to maximize the yield of SCD, the cells were grown at 37 °C for 6 h after induction. Under those conditions, almost all of the SCD was present in the insoluble portion after centrifugation of the lysed cells. This step enriched the SCD in the pellet as the major protein component. The pellet was solubilized in 8 M guanidine hydrochloride. Upon dilution, the SCD refolded quickly to the active form in the presence of Ca^{2+} and Zn^{2+} ions. The high recovery of protein and high specific activity after refolding (Table I) indicated that the refolding was efficient. The refolded SCD was purified to apparent homogeneity the same way as for soluble SCD (Table I). Similar specific activities were obtained for the

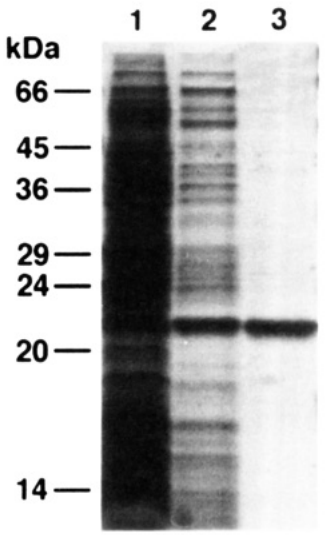

FIGURE 3: Purification of the soluble SCD as analyzed by SDS–polyacrylamide gel electrophoresis and Coomassie blue staining. Lane 1, whole cell extract. Lane 2, after phenyl-Sepharose column. Lane 3, after Q-Sepharose column.

SCD purified from either soluble or insoluble protein, indicating that the SCD was correctly refolded.

Sequence of the SCD. The DNA sequence for the SCD gene in pGEMEX-D was confirmed by DNA sequencing, and it predicted that the expressed SCD would contain three extra amino acids (Met, Ala, and Ser) at the N-terminus. However, the amino acid sequencing for the purified SCD showed the N-terminal sequence as FRTFPGIPKWRKTH-LTYRIVNYTPDLPKDAVDSAVEK. The purified SCD had the same N-terminus as that found in authentic stromelysin with the extra three amino acid residues (Met, Ala, and Ser) removed. This result is consistent with reports that stromelysin has the ability to process intermediates during activation (Okada et al., 1988; Nagase et al., 1990). The molecular weight of the purified SCD was determined by electrospray mass spectrometry. The determined molecular weight of 19 494.1 (±0.1%) was consistent with a SCD starting with Phe-100 and ending with Pro-273 (predicted molecular weight 19 494.1).

Hydrolysis of Thiopeptolide by the SCD. The SCD showed high activity in hydrolyzing the thiopeptolide substrate developed for vertebrate collagenase (Weingarten & Feder, 1985). Under our assay conditions, the thiopeptolide showed no detectable decomposition without enzyme unless the pH was increased to above 8.0. The SCD showed activity at neutral pH but had the highest activity at pH 6.0, more than twice the activity of pH 7.0 (Figure 4). This pH–activity curve is similar to those found for full-length stromelysin (Galloway et al., 1983; Gunja-Smith et al., 1989). At pH 6.0, the SCD has a K_m of 270 μM and a k_{cat} of 127 min^{-1}. Marcy et al. reported a K_m of 1.4 mM and a k_{cat} of 81 min^{-1} for their recombinant stromelysin catalytic domain using Nor-Leu[11]-substance P as the substrate (Marcy et al., 1991).

Hydrolysis of Proteoglycan by the SCD. The SCD cleaves the natural substrate proteoglycan as assayed by the proteoglycan–polyacrylamide particle assay (Nagase & Woessner, 1980; Baragi et al., 1992). The cleavage was inhibited by chelators such as EDTA and 1,10-phenanthroline (Table II). However, inhibitors of serine (PMSF), cysteine (*N*-ethylmaleimide, leupeptin), or aspartyl (pepstatin) proteinases did not significantly inhibit the enzyme. Also, phosphoramidon, a thermolysin inhibitor with no inhibitory effect on matrix metalloproteinases, did not inhibit the enzyme. Fur-

Table I: Purification of SCD

	vol (mL)	protein concn (mg/mL)	protein (mg)	act.[a] (units)	recovery (%)	sp act. (units/mg)	purification (x-fold)
soluble protein[b]							
cell extract	34.7	9.7	337	6.33	100	0.019	1
phenyl-Sepharose	31.0	1.4	43.4	12.02	190	0.277	15
Q-Sepharose	1.25	0.88	1.1	5.19	82	4.72	248
insoluble protein[c]							
8 M guanidine hydrochloride	11.5	13.6	156				
refolding	300	0.16	48	110	100	2.29	1
phenyl-Sepharose	5.2	4.9	25	93.6	85	3.74	1.6
Q-Sepharose	5.0	3.8	19	76.7	70	4.04	1.8

[a] The activity was followed by a thiopeptolide assay as described under Materials and Methods. A unit of activity is defined as micromoles of product per minute at room temperature (22 °C). [b] From a 2-L culture, 6.34 g of cell paste. [c] From a 1-L culture, 5.97 g of cell paste.

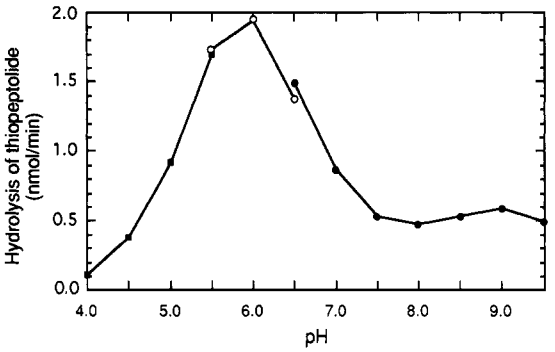

FIGURE 4: Hydrolysis of the thiopeptolide substrate at different pHs. The activity of the purified SCD was determined as described under Materials and Methods in 100 mM acetate (closed squares), MES (open circles), and Bis-Tris propane (closed circles) at the pHs indicated.

Table II: Inhibition of Proteoglycan-Degrading SCD

inhibitor	concn	proteoglycan degradation (μg of chondroitin sulfate/h)	% inhibn[a]
no inhibitor		8.0	0
EDTA	5 mM	0.7	91
1,10-phenanthroline	4 mM	0.0	100
PMSF	500 μM	7.4	7
N-ethylmaleimide	10 mM	6.9	30
leupeptin	10 μg/mL	5.6	14
pepstatin	1 μg/mL	7.2	10
phosphoramidon	25 μM	8.2	0
U24522	5 μM	0.8	90

[a] Ratio of proteoglycan degradation in the presence and absence of inhibitors.

thermore, U24522, a synthetic inhibitor known to inhibit proteoglycan-degrading metalloproteinases (Caputo et al., 1987), effectively inhibited the SCD. On the basis of these data, the activity measured using the proteoglycan substrate is consistent with the activity of proteoglycan-degrading matrix metalloproteinases.

Stability of the SCD. The purified SCD is stable in 50 mM Tris·HCl (pH 7.6) and 10 mM $CaCl_2$ at 4 °C. At 37 °C, it was stable at pH 6–9 in the presence of 10 mM $CaCl_2$, and it showed a significant decrease in activity when the pH was dropped to 5.0 in acetate buffer (Figure 5A). However, at the lower $CaCl_2$ concentration (0.5 mM), the activity decreased over a 6-h period, most quickly at pH 5.0 (Figure 5B). In another experiment, SCD retained considerable amounts of activity at pH 6.0, 7.0, or 8.0 with 10 mM $CaCl_2$ (43%, 69%, and 83%, respectively) after incubation at 37 °C for 24 days. SDS–PAGE analysis showed that the activity correlated with the protein band at 20 kDa and that the loss of activity accompanied the appearance of lower molecular

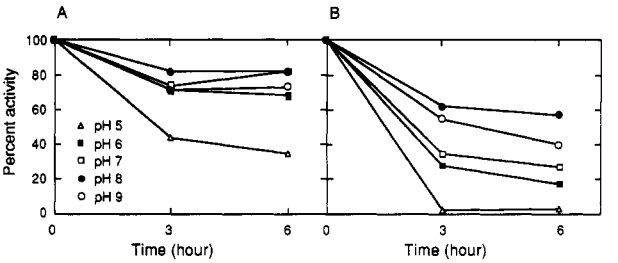

FIGURE 5: Thermal stability of the SCD in the presence of $CaCl_2$. The SCD was incubated at 37 °C at the pHs indicated in the presence of 10 (A) or 0.5 mM (B) $CaCl_2$, and aliquots were taken after the time intervals. Activity was assayed as the thiopeptolide hydrolysis at pH 6.0.

weight proteins. The Ca^{2+} ion appeared to stabilize the SCD as found by Lowry et al. (1992) from a similar collagenase fragment.

DISCUSSION

Structural information is vital for computer-assisted inhibitor design, and this study was carried out to provide a catalytically active segment of stromelysin suitable for structural studies. The deletion of the stromelysin C-terminal portion and propeptide generates the SCD (Figure 1) with a mass of 20 kDa, which is the challenging but manageable size for structure determination by nuclear magnetic resonance spectroscopy. Attempts to make truncated matrix metalloproteinases have been reported. Marcy et al. (1991) expressed a truncated stromelysin containing the catalytic domain and the propeptide. The propeptide was removed in vitro to generate the catalytic domain (Marcy et al., 1991; Salowe et al., 1992). Lowry et al. (1992) described a stability study using a recombinant 19-kDa collagenase catalytic domain. However, the expression and purification of the collagenase catalytic domain have not been described. More recently, Murphy et al. (1992) expressed the C-terminal-deleted procollagenase and prostromelysin in mouse cells.

The unique feature of our expression system is that the active proteinase was expressed without propeptide attached so that there was no need for in vitro activation. Also, the *E. coli* expression system with the deletion of the propeptide made it more efficient to incorporate stable isotopes (^{15}N, ^{13}C, etc.) into the protein for NMR studies. Although it lacks the C-terminal portion of stromelysin, the SCD is capable of cleaving the thiopeptolide substrate and the natural substrate proteoglycan. It showed remarkable stability in the presence of $CaCl_2$, a property required for the extended time involved in NMR data acquisition.

In summary, we have constructed an efficient system for the SCD expression and have devised a simple scheme for its purification. The SCD is stable and fully active, and therefore

it provides a useful target for structure determination, as well as for mechanistic studies on catalysis and inhibition.

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Registry No. Stromelysin, 79955-99-0.