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Purification of Active Matrix Metalloproteinase Catalytic Domains and Its Use for Screening of Specific Stromelysin-3 Inhibitors

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Abstract

The matrix metalloproteinase (MMP) stromelysin-3 (ST3) has been shown to be involved in malignant tumor progression and therefore represents an attractive therapeutical target. In order to screen for ST3 synthetic inhibitors, we have produced and purified the catalytic domain of ST3, matrilysin, stromelysin-2, and membrane type-1 MMP from inclusion bodies in a bacterial system. Our strategy allowed the purification of MMPs directly in the active form, thereby avoiding *in vitro* activation. A total of 140,000 synthetic compounds from the Bristol-Myers Pharmaceutical Research Institute chemical deck were tested, using a substrate-based colorimetric enzymatic assay, in which ST3 activity was evaluated through its ability to cleave and inactivate α-1 proteinase inhibitor. One ST3 inhibitor belonging to the cephalosporin family of antibiotics was thereby identified.

KeyWords : α-1 proteinase inhibitor ; cephalosporin ; matrix metalloproteinase inhibitors ; stromelysin-3.

The growing family of matrix metalloproteinases (MMPs) represents a group of zinc endopeptidases which share sequence and structural similarities and which are active extracellularly. The MMPs, which are also known as matrixins (1), are believed to be mediators of both normal and pathological tissue remodeling processes (2, 3). In malignant processes, there is accumulating evidence by *in situ* hybridization and immunohistochemical studies that MMP expression in cancer tissue is increased compared to noncancerous tissue. The MMP activity is believed to promote the expansion and invasion of primary tumors, the movement of cancer cells across the vascular membrane, the local growth and invasion of secondary tumors, and the formation of new blood vessels, which is an important requisite for tumor growth.

MMPs are produced as proforms which are activated by proteolytic cleavage of the prodomain, and the activity of mature enzymes is controlled by physiological inhibitors. Native inhibitors of MMP activity are the general serum proteinase inhibitor, α-2 macroglobulin and, more specifically, the family of tissue inhibitors of metalloproteinases (TIMPs). To date, four members of the family have been characterized, and TIMP-1, TIMP-2, TIMP-3, and TIMP-4 can inhibit the activity of all known matrix metalloproteinases (4). TIMP-2 has been shown to inhibit tumor growth, angiogenesis, invasion, and metastasis in experimental models, an observation consistent with a role of MMP activity in these processes (5). However, the therapeutic use of these proteins is likely to be limited because of their low oral bioavailability and limited tissue penetration.

As there is growing experimental evidence that malignant tumors use MMPs for tumor growth and spreading, inhibitors of MMPs represent a new class of anticancer agents (6, 7). Synthetic inhibitors ideally have the desirable properties of high water solubility, oral bioavailability, low toxicity, and ease of production. So far, the most extensively used inhibitor in clinical trials is marimastat, a hydroxamic acid analogue which exhibits broad spectrum inhibitory activity against MMPs (8).

Several laboratories have initiated the development of new MMP inhibitors. In our laboratory, for the purpose of screening inhibitors, we have used a bacterial system to purify the catalytic domain of stromelysin-3 (ST3), an MMP which is known to be expressed in most human carcinomas (9, 10) and to promote tumor growth in mice

(11). In order to check for inhibitor specificity, we have also purified matrilysin, stromelysin-2 (ST2), and membrane type-1 MMP (MT1-MMP). ST3 and MT1-MMP belong to a subgroup of MMPs which are activated in intracellular compartments by furin or furin-like convertases, unlike other MMPs including ST2 and matrilysin that are processed extracellularly (12). An enzymatic assay based on the cleavage of α-1 proteinase inhibitor (α1-PI), a common substrate to MMPs (13, 14), has been designed and utilized for screening of several tens of thousands of small synthetic molecules in order to evaluate their ability to inhibit ST3 activity. The screening has led to the identification of one inhibitor which appears to be of potential interest for further characterization.

MATERIALS AND METHODS

Materials

Polymerase chain reaction (PCR) amplification of cDNAs was carried out with synthetic oligonucleotides using a Perkin-Elmer Cetus thermal cycler (Foster City, CA). *Taq*1 polymerase and Lennox Broth medium were obtained from GIBCO BRL (Gaithersburg, MD). Tris-HCl, sodium chloride, calcium chloride, and zinc chloride were purchased from Euromedex (Strasbourg, France); pepstatin, antipain, aprotinin, leupeptin, chymostatin, and Chaps were obtained from Boehringer Mannheim (Mannheim, Germany). Brij-35 was purchased from Sigma Chemical Company (St. Louis, MO). Q-Sepharose and Superdex 200 chromatographic resins were obtained from Pharmacia (Uppsala, Sweden). Centriprep YM10 units were purchased from Amicon (Millipore, Bedford, MA).

Expression Plasmid Constructs

The nucleotide fragments encoding the catalytic domains of mouse ST3 (15) and human matrilysin, ST2, and MT1-MMP (16, 17) were obtained by DNA amplification corresponding to residues Phe102-Ser276 for ST3, Tyr78-Lys247 for matrilysin, Phe82-Lys260 for ST2, and Tyr111-Arg298 for MT1-MMP, respectively, by PCR. Primer 1 (5'-AACCGACAGCATATGTTCGTC-CTG-3') and primer 2 (5'-GCCCCAACTTTGAGCT-GATAGCTCGAGACAGATACC-3') for ST3, primer 3 (5'-GAATTCCATATGTACTCACTATTTCCAAATA GCC-3') and primer 4 (5 '-CGCGGATCCTCACTATT-TCTTTCTTGAATTACTTCT-3') for matrilysin, primer 5 (5'-GGAATTCCATATGGGGTTCAGCTCCTTTC-CTGGCATGCCG-3') and primer 6 (5'-CCGCTCGAG-TCACTATTTTGTGGGCACCAGGGGT-3') for ST2, and primer 7 (5'-GGAATTCCATATGTACGCCATC-CAGGGTCTCA-3') and primer 8 (5'-GTCAGTA-GATCTTCACTACCTGGGTTGAGGGGGCAT-3') for MT1-MMP were used respectively for the reaction. Oligonucleotide primers were designed with an *Nde*I restriction site at the 5' end in order to introduce the translational initiating methionine. The 3' primer provides the stop codon and a unique *Xho*I site in the case of ST3 and ST2, a *Bam*HI site in the case of matrilysin, and a *Bgl*II site in the case of MT1-MMP. PCR was carried out for 25 cycles (94°C, 30 s; 55°C, 1 min; 72°C, 1 min 30 s) with *Taq*1 polymerase to generate the cDNA fragments corresponding to the catalytic domains of ST3, matrilysin, ST2, and MT1-MMP, respectively. These fragments were then digested with *Nde*I and *Xho*I (or *Bam*HI or *Bgl*II as specified above) and ligated into the unique *Nde*I and *Xho*I (or *Bam*HI or *Bgl*II) sites of a modified T7 expression vector pET3b (15). Clones containing cDNAs of ST3, matrilysin, ST2, and MT1-MMP respectively were obtained by transformation in the nonexpressing *Escherichia coli* strain XL1-Blue. After bacterial selection for the MMP-containing clones, the different plasmids were transformed into the expressing *E. coli* strain BL21(DE3)pLysS, which contains a copy of the T7 RNA polymerase under the control of an inducible lac promoter. Complete sequencing of the PCR fragments of the catalytic domain of the different MMPs ensured a 100% identity with the original cDNA sequences.

Purification of MMP Catalytic Domains

Inclusion Body Isolation

BL21(DE3)pLysS cells containing the plasmid pET3b-ST3, pET3b-matrilysin, pET3b-ST2, or pET3b-MT1-MMP were grown in 2 liters of Lennox Broth medium in the presence of ampicillin (100 μg/ml) with shaking at 37°C up to a cell density corresponding to an OD_{600} of 0.6. The expression of the recombinant protein was induced by adding 0.4 mM isopropyl-1-β-D-galactopyranoside and incubation was continued at 37°C for 3 h. Bacterial cells were collected by centrifugation for 10 min at 5000*g* at 4°C and were resus-pended in 800 ml of 50 mM Tris, pH 7.5, 100 mM KC1, 0.1% Brij-35, 1 mM EDTA, pH 7.5, 5 mM β-mercapto-ethanol, 0.1 mM diisopropyl fluorophosphate (DFP) at 4°C, in the presence of a protease inhibitor cocktail mixture containing pepstatin, leupeptin, aprotinin, an-tipain, and chymostatin, each at a final concentration of 2.5 μg/ml. The cells were lysed using a microtip sonicator and the cell debris was cleared by centrifu-gation for 30 min at 8000*g* at

4°C. The resulting pellets were washed twice in the same buffer but in the absence of DFP and the protease inhibitor cocktail and in the presence of 2 M NaCl to release nonspecifically bound bacterial proteins. The bacterial inclusion bodies thus obtained were harvested by two final washing steps in 20 mM Tris, pH 7.5, and 5 mM β-mercapto-ethanol and collected by centrifugation for 30 min at 8000*g* at 4°C.

Recovery, Refolding, and Purification from Inclusion Bodies

Partially purified enzyme preparation. The catalytic domains of the MMPs were solubilized from inclusion bodies at 22°C for 2 h in 8 M urea, 20 mM Tris, pH 8.5, 100 mM DTT and purified by anion-exchange chromatography. A 15-ml Q-Sepharose column was equilibrated with 6 M urea, 20 mM Tris, pH 8.5, 10 mM DTT and the solubilized inclusion bodies, isolated from 2 liters of bacterial culture as described above, were loaded (1 ml/min) using a liquid chromatographic separation apparatus BioLogic (Bio-Rad, Hercules, CA). The concentration of denaturing agent in the solubilized inclusion bodies was lowered from 8 to 6 M urea prior to loading. The resin-bound MMP was eluted (1 ml/min) by an increasing step gradient from 0 to 500 mM NaCl in the equilibrating buffer. The protein concentration in the fraction pool eluted with 250 mM NaCl was determined, and the refolding was performed at a protein concentration between 50 and 250 µg/ml. The concentration of urea in the enzyme was gradually diluted out over a period of 16 h in 50 mM Tris, pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$, 10 µM $ZnCl_2$ at 22°C. Typically, 400 ml of pooled enzyme was placed in a dialysis tubing in 2 liters of dialysis buffer described above containing 6 M urea and diluted with 4 liters of dialysis buffer without urea. Further elimination of urea in the sample was carried out in a stepwise manner by dialysis against 4 liters of 50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $CaCl_2$, 1 µM $ZnCl_2$, containing 1 M urea, for 2 h at 4°C, followed by a final dialysis step at 4°C against the above buffer in the absence of urea. The resulting partially purified preparation of enzyme was centrifuged at 12,000*g* for 30 min at 4°C to eliminate precipitates and either frozen in aliquots and stored at -80°C in the case of partially purified enzyme or stored overnight at 4°C after addition of 0.1% Chaps (3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate) when highly purified enzyme is needed.

Purification by gel filtration chromatography. The refolded enzyme preparation containing 0.1% Chaps stored at 4°C was concentrated to about 10 ml using an Amicon-stirred cell fitted with a YM10 membrane in a helium atmosphere, at 4°C. Further concentration of the protein sample to 1 ml was carried out using Centriprep concentrators fitted with YM10 membranes at 700*g* at 4°C. The concentrated protein sample (gel filtration pool) was applied to a Superdex 200 gel filtration column at a flow rate of 2 ml/min. Eluate fractions were collected in 50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $CaCl_2$, 1 µM $ZnCl_2$ containing 0.1% Chaps. The protein aggregates and most of the degradation products were eliminated in this size-exclusion chromatography. In order to ensure protein stability, pure enzymes were stored at —80°C.

Protein Analyses

Protein concentrations were determined by the Bradford assay, using a kit from Bio-Rad. Protein samples were analyzed by SDS-PAGE. The protein species were detected by staining with Coomassie blue.

Determination of Enzymatic Activities

Determination of k_{cat}/K_m Constants

The specificity constants k_{cat}/K_m were determined for matrilysin, ST2, and MT1-MMP using the matrixin substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2, and for ST3 the substrate, Mca-Pro-Leu-Ala-Cys(OMe-Bzl)-Trp-Ala-Arg-Dpa-NH_2, as previously described (18).

Quantitative Colorimetric Substrate Assay with α1-PI

MMPs (100 ng to 1 µg) were incubated at 22°C with α1-PI at a final concentration of 30 µg/ml in 33 µl of 50 mM Tris, pH 7.5, 200 mM NaCl, 10 mM $CaCl_2$, and 0.1% Triton X-100. The cleavage of α1-PI was stopped after 1 h with 2 µl of 100 mM 1,10-orthophenanthroline (1,10-OP). α-Chymotrypsin (α-CT) diluted in the same buffer as above was then added at a final concentration of 2.4 µg/ml (total volume 95 µl). Incubation of the mixture containing the MMP, α1-PI, and α-CT was carried out for 20 min. At the end of this incubation period, 7 µl of a synthetic substrate for α-CT (*N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide), which carries a chromogenic moiety, was added at a final concentration of 140 µg/ml and incubated for 2 min. The reaction was stopped by the addition of 7 µl of 15 mM phenyl-methylsulfonyl fluoride and the optical density at 405 nm was determined using a Beckman DU640 spectrophotometer (Fullerton, CA). A color change of the substrate from colorless to

yellow indicates the presence of MMP activity.

Direct Evaluation of α 1-PI Cleavage

MMP preparations were incubated with α1-PI as described above for 2 h at 22°C. After addition of 1,10-OP, the incubation mixture was solubilized in sample buffer. The samples were analyzed by 7.5% SDS-PAGE and visualized by Coomassie blue staining.

Inhibitor Screening

From the deck of synthetic chemical compounds made available by the Bristol-Myers Pharmaceutical Research Institute (BMSPRI, Wallingford, CT), 140,000 compounds were screened using the quantitative α1-PI assay and preparations of partially purified ST3. The α1-PI-based assay was adapted to the screening conditions by performing the test in 96-well polystyrene plates (Corning, Acton, MA). Each reaction contained 500 ng of ST3. Test compounds were solubilized using dimethyl sulfoxide (DMSO) and preincubated for 20 min before addition of α1-PI. DMSO did not interfere with the screening reaction up to 1% final concentration. After addition of α-CT substrate, the optical density at 405 nm was determined in a THERMOmax microplate reader (Molecular Devices, Sunnyvale, CA).

Preparation of Recombinant TIMP-2

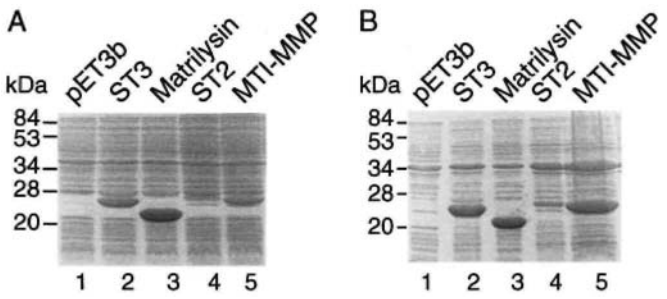
TIMP-2 was produced from transformed Chinese hamster ovary (CHO) cells, using the dihydrofolate reductase-methotrexate selection and amplification system, and purified from CHO-conditioned medium on Hitrap Cu^{2+}-chelating affinity column and a Hitrap Q-column (Pharmacia) according to the procedure described (19).

RESULTS

Expression of ST3, Matrilysin, ST2, and MT1-MMP Catalytic Domains in E. coli

BL21(DE3)pLysS cells were used for the production of the catalytic domain of ST3, matrilysin, ST2, and MT1-MMP. No obvious toxicity was observed in bacteria expressing the MMPs. Gel electrophoretic analysis of total bacterial cultures showed overexpression of ST3, matrilysin, and MT1-MMP, followed by a lower level of expression for ST2 (Fig. 1A). All four MMPs were found to be preferentially partitioned in the insoluble fraction (Fig. 1B).

FIG. 1. *Electrophoretic analysis of ST3, matrilysin, ST2, and MT1-MMP catalytic domains expressed in BL21(DE3)pLysS cells. (A) Analysis of control expression vector pET3b (lane 1), pET3b-ST3 (lane 2), pET3b-matrilysin (lane 3), pET3b-ST2 (lane 4), and pET3b-MT1-MMP (lane 5) by SDS-PAGE under reducing conditions. Pellets from 1 ml of bacterial culture were solubilized in 100 µl of sample buffer, and 10 µl of each sample was separated on 15% gels and stained with Coomassie blue. (B) Insoluble fractions were obtained by lysing pellets from 1 ml of bacterial culture with 250 µl of PBS containing 0.1% Triton X-100. The samples were frozen and thawed in liquid nitrogen (three times) and sonicated prior to centrifugation at 8000g for 30 min. The pellet was solubilized in 250 µl sample buffer, and 25 µl of the samples was separated by SDS-PAGE on 15% gels under reducing conditions and stained with Coomassie blue. The mobilities of standard molecular weight markers (kDa) are indicated.*



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Refolding and Purification of ST3, Matrilysin, ST2, and MT1-MMP Catalytic Domains

After solubilization of inclusion bodies, ST3, matrilysin, ST2, or MT1-MMP were applied to a Q-Sepharose column and refolded by dialysis to remove urea as described under Materials and Methods. During these steps, most of the bacterial proteins were eliminated. On an average, this experimental procedure allowed us to obtain 10 to 20 mg of ST3, matrilysin, and MT1-MMP starting from 2 liters of bacterial culture. These refolded catalytic domains of MMPs (partially purified enzyme) have been further purified on a gel filtration column (Fig. 2 and data not shown). This step, carried out using a Superdex 200 resin, allows the separation of the inactive aggregates from the active monomeric species and the elimination of most of the low molecular weight degradation products that are often formed during MMP purification. One to 5 mg of purified enzymes were then obtained and they were at least 90% homogenous as shown in Fig. 3. The estimated molecular weights of the catalytic domains of MMPs are 23 kDa for ST3, ST2, and MT1-MMP and 20 kDa for matrilysin. Production of ST2 was about five times lower than that of the other three MMPs. The yields of the MMPs from the different purification steps are indicated in Table 1. Sequencing of the N-terminus of ST3, matrilysin, ST2, and MT1-MMP confirmed complete homogeneity of the purified catalytic domains (data not shown).

TABLE 1 : *Purification Table*

Sample protein	MMP	Volume (ml)	Concentration (mg/ml)	Total protein (mg)	% Recovery
Solubilized inclusion bodies	ST3	30	2.5	76	
	Matrilysin	30	1.8	55	
	ST2	30	0.6	18	
	MT1-MMP	30	2.1	64	
Partially purified	ST3	400	0.055	22	28
	Matrilysin	400	0.037	15	27
	ST2	400	0.012	5	27
	MT1-MMP	400	0.045	18	28
Gel filtration pool	ST3	1	9.6	9.6	13
	Matrilysin	1	8	8	15
	ST2	1	2.3	2.3	13
	MT1-MMP	1	8.1	8.1	13
Final product	ST3	30	0.166	5	7
	Matrilysin	28	0.107	3	5
	ST2	12	0.083	1	6
	MT1-MMP	36	0.111	4	6

Note. The yields of the different MMPs are representative of at least two independent preparations for each MMP from 2 liters of bacterial cultures. Protein fractions from different steps of the purification scheme were analyzed by the Bradford assay.

Determination of k_{cat}/K_m Constants

The specificity constant k_{cat}/K_m for the hydrolysis of the matrixin substrate Mca-Pro-Leu-Ala-Leu-Dpa-Ala-Arg-NH_2 has been determined for gel filtration-purified matrilysin, ST2, and MT1-MMP at pH 7.5 and 25°C. The values were 107,000 M^{-1} s^{-1} for matrilysin, 113,000 M^{-1} s^{-1} for ST2, and 240,000 M^{-1} s^{-1} for MT1-MMP, which are in accordance with previously reported values in the literature (20, 21). As ST3 does not cleave efficiently the above matrixin substrate, the k_{cat}/K_m value (36,000 M^{-1} s^{-1}) was derived from the hydrolysis of the Mca-Pro-Leu-Ala-Cys(OMeBzl)-Trp-Ala-Arg-Dpa-NH_2 substrate (18). All together, these findings indicate that the MMP catalytic domains produced and purified were properly refolded and corresponded mostly to homogenous active enzyme.

FIG. 2. *Chromatogram of gel filtration purification of ST3 on a Superdex 200 column. 400 ml of partially purified ST3 was concentrated to a volume of 1 ml (9.6 mg) and separated on a Superdex 200 column as described under Materials and Methods. The major peak (fractions 45-55) represents the monomeric protein which was separated from the aggregates (fractions 22-44).*

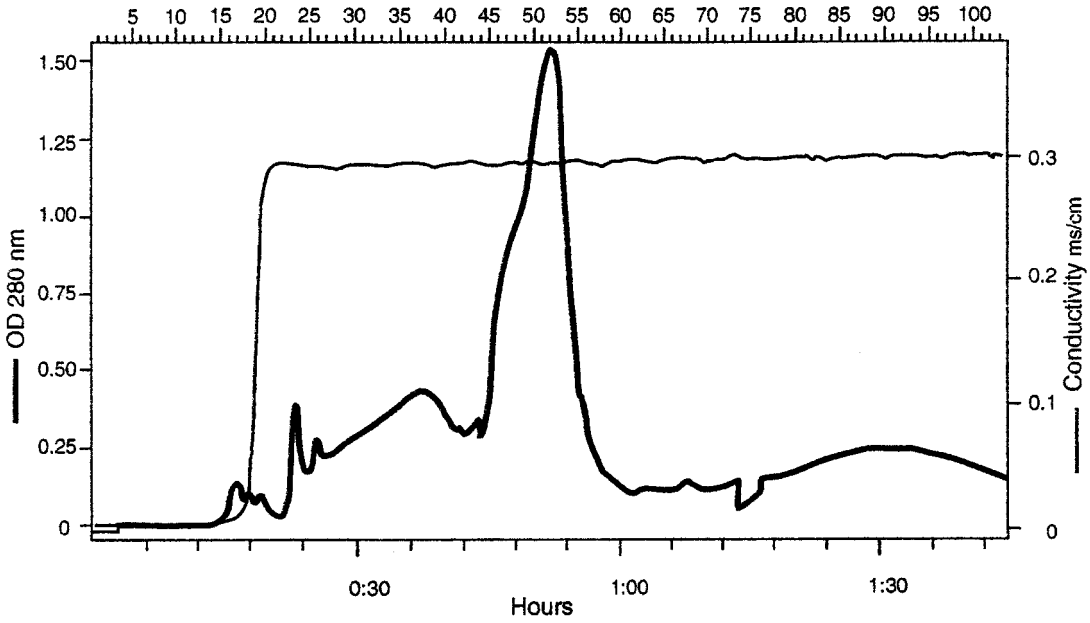
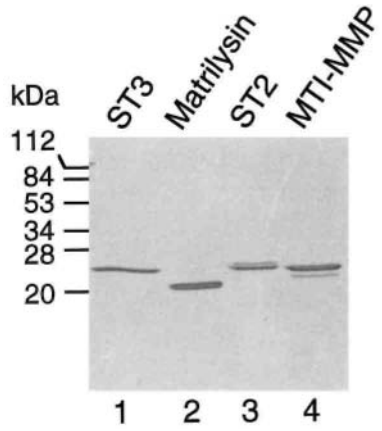


FIG. 3. *Electrophoretic analysis of purified ST3, matrilysin, ST2, and MT1-MMP catalytic domains after refolding and gel filtration chromatography. One microgram of gel filtration-purified ST3 (lane 1), matrilysin (lane 2), ST2 (lane 3), and MT1-MMP (lane 4) were separated by SDS-PAGE on 15% gels under reducing conditions and stained with Coomassie blue. The mobilities of standard molecular weight markers (kDa) are indicated.*



Random Screening of ST3 Inhibitors

The quantitative assay based on α1-PI cleavage (Fig. 4) was used for screening of synthetic compounds that could inhibit ST3. A total of 140,000 compounds from the BMSPRI deck were tested on partially purified ST3 preparations. Since it has been previously shown that TIMP-2 can inhibit ST3 and other MMPs as well, each preparation was tested for inhibition by TIMP-2, in order to verify that the enzymatic activity evaluated in the α1-PI quantitative assay was due to ST3 alone and not due to contaminating proteases. This screening led to the identification of some 100 specific inhibitors of α-CT. Among compounds that did not directly inhibit α-CT, one of them, compound EK 2900, was found to inhibit ST3 with an IC_{50} value of 15 μM. This compound also

inhibits MTl-MMP (IC_{50} = 45 μM) and ST2 (IC_{50} = 70 μM). However, there is no inhibition of matrilysin observed at the highest possible concentration of the inhibitor (500 μM) that can be utilized in the α1-PI quantitative assay. These results were double checked by directly evaluating α1-PI cleavage by SDS-PAGE analysis. In the presence of EK 2900, α1-PI cleavage was prevented by ST3, ST2, and MT1-MMP (Figs. 5A, 5C, and 5D) but not by matrilysin (Fig. 5B), while TIMP-2 inhibited the enzymatic activity of all four MMPs (Figs. 5A-5D).

FIG. 4. *Principle of indirect colorimetric substrate assay used for ST3 inhibitor screening. The synthetic substrate for α-CT, N-succi-nyl-Ala-Ala-Pro-Phe-p-nitroanilide (A) is colorless and yields a yellow product (B) when cleaved by α-CT. When α1-PI inhibits α-CT, no change in color is observed with the substrate. The catalytic domain of ST3 cleaves and inactivates α1-PI, thereby preventing inhibition of α-CT and leading to a color change. However, in the presence of an inhibitor of ST3, the ST3 catalytic domain is inhibited leading in turn to the inactivation of α-CT and no resulting color change of the substrate is observed. (+) The presence and (—) absence of reagents.*

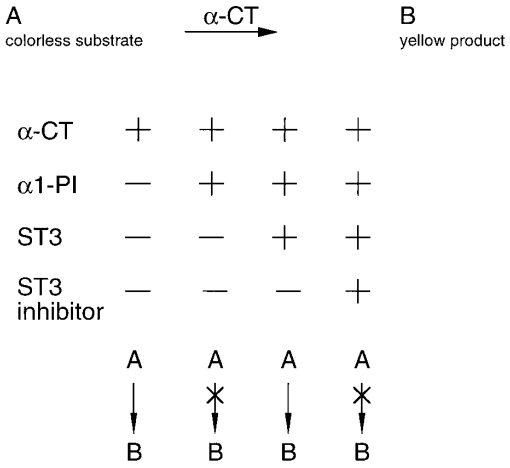
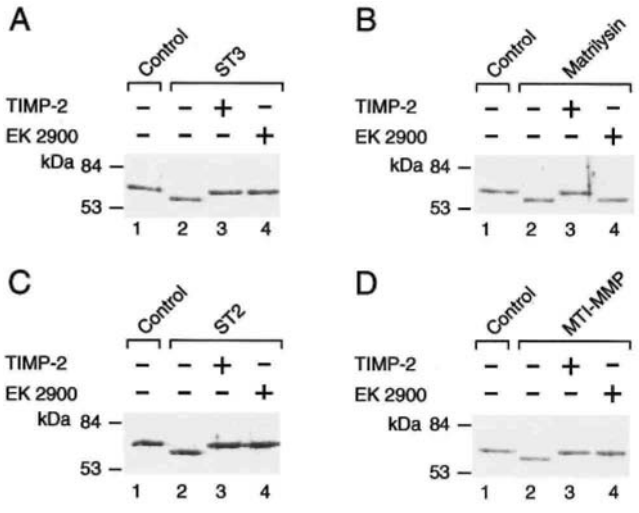


FIG. 5*. Electrophoretic analysis of α1-PI cleavage by ST3, matrilysin, ST2, and MT1-MMP. 100 ng of gel filtration-purified ST3 (A), 850 ng of matrilysin (B), 250 ng of ST2, and MT1-MMP (C and D, respectively) were incubated with α1-PI (1 μg) for 2 h and separated by SDS-PAGE on 7.5% gels under reducing conditions, before being stained with Coomassie blue. In all four panels, lane 1 represents α1-PI alone incubated under the experimental conditions as a control, lane 2 contains MMP incubated with α1-PI, lane 3 contains MMP incubated with α1-PI and TIMP-2 (1 μg), and lane 4 contains MMP incubated with α1-PI and EK 2900 (2 μg = 130 μM).*



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DISCUSSION

The expression of some MMPs, including ST3, matrilysin, ST2, and MT1-MMP during the progression of human cancer, has been well documented (2, 3, 22). In this context, obtaining inhibitors that would neutralize their enzymatic activity could allow the development of new therapeutic agents (6, 7). ST3 was chosen as a target for a first round of screening for synthetic inhibitors, as it is an attractive candidate being present in a vast majority of invasive carcinomas and exhibiting unusual functional properties for an MMP (23). In order to screen the BMSPRI chemical deck, we have expressed in bacteria the catalytic domain of ST3. Matrilysin, ST2, and MT1-MMP were similarly expressed and used to evaluate inhibitor specificity. The MMPs were predominantly found in bacterial inclusion bodies, from which they could be purified after separation on a Q-Sepharose anion-exchange column and a refolding step. Progressive removal of urea resulted in the gradual restoration of the activity of the MMPs and the resulting enzymes were obtained in high yields.

While proST3 has recently been expressed in baculovirus-infected insect cells (24), there are several significant advantages in our method for purifying the MMP catalytic domains directly. First, the MMPs can be expressed in high amounts in the inclusion bodies of bacteria, and this provides a readily available source of recombinant protein. Second, the refolding of the protein is rapid and results in 10 to 20 milligrams of partially purified enzyme starting from 2 liters of bacterial culture. The possibility of producing and using enzymes directly in the active form possesses a distinct advantage, as the activation steps often lead to a loss of enzyme quantitatively. Third, in our procedure, even the partially purified MMP preparations can be used for inhibitor screening. Fourth, highly purified enzyme is obtained after further chromatographic steps in order to eliminate the inactive aggregates of the MMPs which are inevitably formed during refolding, from the active monomeric protein. The resulting MMP preparations can be used for extensive characterization of inhibitors obtained from the primary screening, which was carried out with partially purified enzyme.

α1-PI was used as a common MMP substrate for inhibitor screening. In this assay, we could not detect the activity of any contaminating bacterial proteinases, as demonstrated by the absence of α1-PI cleavage in the presence of TIMP-2. The screening of the BMSPRI composite chemical deck with ST3 as target has led to the identification of EK 2900, a compound with inhibitory activity toward ST3, ST2, and MT1-MMP but not matrilysin. EK 2900 is a cephalosporin, which belongs to the general class of antibiotics. Several antibiotics, including tetracyclines, cephalosporins, anthraquinones, and aranciamycin, have been reported to be MMP inhibitors. Cephalosporins themselves have been found to inhibit MMP activity in reactive periprosthetic tissue, suggesting that they could be used to reduce complications associated with loose hip prostheses (25). Cephalosporins have also been implicated in MMP inhibition in abdominal aortic aneurysms (26, 27). Thus, EK 2900 provides a potential lead compound that merits further exploration.

In cancer, the use of synthetic MMP inhibitors may also open novel therapeutical perspectives. Several MMP inhibitors have demonstrated antitumor activity in various experimental models of cancer and phase III clinical trials have been recently initiated with mari-mastat, a hydroxamate-type inhibitor (8). In this context, the observation that ST3 (9) and some other MMPs (16, 17, 22) implicated in carcinoma progression originate from the tumor stroma and not from the cancer cells is interesting. Indeed, targeting stromal cells (or stromal cell products) rather than cancer cells, as is the case in conventional chemotherapy, should reduce the development of drug resistance and to generate antitumor agents capable of acting synergistically with the conventional cytotoxic agents directed against cancer cells. However, compounds such as ma-rimastat are broad spectrum inhibitors and have been associated with undesirable side effects in cancer patients, which prevent their use in optimal doses. It is therefore reasonable to postulate that inhibitors with specificity limited to only a few MMPs should be used in the future (28) and that compounds such as EK 2900 could serve as starting material to obtain such inhibitors.

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