ISSN 1671-4083 Acta Pharmacol Sin 中国药理学报 2002 Feb; 23 (2): 143 - 151
http://www.chinaphar.com

Expression and purification of catalytic domain of human macrophage elastase for high-throughput inhibitor screening

CHENG Dong-Hang, SHEN Qiang, QIAN Jing, QIAN Zhen, YE Qi-Zhuang[1]
(*National Center for Drug Screening, Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 201203, China*)

KEY WORDS matrix metalloproteinases; human macrophage elastase; gene expression; high-throughput screening

ABSTRACT

AIM: To obtain a catalytically active human macrophage elastase catalytic domain (hMECD) and to establish an efficient high-throughput method for screening macrophage elastase inhibitors. **METHODS**: Catalytic domain of human macrophage elastase was expressed in *E coli* and characterized to establish a high-throughput screening assay using a colorimetric method. A set of 8560 pure compounds and mixtures were screened. **RESULTS**: We have constructed an efficient *E coli* system for this human protein expression, and the recombinant hMECD protein was purified to homogeneity using anion-exchange chromatography after *in vitro* refolding from inclusion bodies. The yield of active hMECD protein was 23 mg from one liter of *E coli* culture after purification. Calcium and zinc ions were required both in refolding and enzymatic activity, but high concentration of zinc inhibited the refolding and activity. The hMECD cleaved several synthetic substrates including a chromogenic thiopeptolide and fluorogenic peptides with optimal activity around pH 8.0. Screening of 8560 compounds and mixtures led to identify 27 pure compounds and 14 natural products with inhibitory activity higher than 80 % at 20 mg/L. **CONCLUSION**: An efficient expression and purification method for hMECD protein has been established, and the assay is effective, reliable, and fast in identifying the recombinant protein inhibitors.

[1]Correspondence to Prof YE Qi-Zhuang.
Phn 86-21-5080-1313. Fax 86-21-5080-0721.
Email yeq@mail.shcnc.ac.cn
Received 2001-09-13 Accepted 2001-10-31

INTRODUCTION

The matrix metalloproteinases (MMP) are a family of calcium and zinc-dependent endo-proteinases that can be classified into five subgroups according to their substrate specificity and structure: collagenases, stromelysins, gelatimases, membrane-type MMP and other MMP[1]. The family of proteolytic enzymes together can degrade all components of the extracellular matrix and basement membranes[2]. MMP thus implicated in both normal tissue turnover and pathological matrix remodeling. Human macrophage elastase (MMP-12) is one of the MMP family members with typical MMP features. It is a matrix-degrading enzyme that requires zinc and calcium for activity and shares sequence homology to other MMP. Its activity can be inhibited by tissue inhibitors of metalloproteinases (TIMP). With unique macrophage-specific pattern of expression, MMP-12 is associated with several macrophage-mediated destructive diseases in human, including atherosclerosis, tumor invasion, metastasis, and pulmonary emphysema[3-5]. MMP-12 plays a central role in the pathogenesis of pulmonary emphysema because of its capability of degrading elastin that imparts elastic recoil to arterial vessels and lung parenchyma[6]. The consequent elastin destruction will ultimately result in the distinctive changes of pulmonary emphysema. Study of the progression on smoking-induced emphysema by using mouse knockout has suggested that macrophage elastase is required for both macrophage accumulation and emphysema[5].

The activity of the naturally occurring matrilysin lacking a hemopexin-like domain suggests that the catalytic domain alone can function as an active enzyme independent of other domains. The hemopexin-like domain of MMP such as gelatinase A was involved in binding to TIMP-2. Meanwhile, some of the MMP catalytic domain proteins, when expressed alone, have shown increased stability, probably due to removal of

ISSN 1671-4083 Acta Pharmacol Sin 中国药理学报 2002 Feb; 23 (2)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6471-2629

autolytic sites in the C-terminal domain. During purification, M_r 54 000 proenzyme of MMP-12 readily shed both the prodomain and the C-terminal domain, leading to get an active enzyme of M_r 20 000, suggesting that direct expression of this active domain could be achieved in *E coli*[7]. Moreover, it has been known that the MMP catalytic domains usually have similar catalytic efficiency of the full length MMP and can be used as a tool for screening MMP inhibitors[8-10]. We reported here the construction of an efficient *E coli* expression system, the purification for the hMECD protein, and its application in random high-throughput screening (HTS) of specific MMP-12 inhibitors.

MATERIALS AND METHODS

Materials The EST clone (GenBank number: AI368844, EST clone number: 1989528) containing the gene for human macrophage elastase catalytic domain was purchased from Genome Systems (St Louis, MO, USA). Primers were synthesized by Sangon Company (Shanghai, China) and purified by polyacrylamide gel electrophoresis. The plasmid pGEMEX-1 and restriction enzymes were from Promega (Madison, WI, USA). *E coli* strain BL21 (DE3)/pLysS cells were purchased from Novagen (Madison, WI, USA). Hi-performance Q-Sepharose and HiPrep 26/10 desalting column were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Thiopeptolide Ac-PLG-[2-mercapto-4-methylpentanoyl]-Leu-Gly-OEt (TPL) and fluorogenic peptides Mca-Pro-Leu-Gly-Leu-Dap-Ala-Arg-NH_2 (FP1), Dnp-Pro-β-cyclohexyl-Ala-Gly-Cys (Me)-His-Ala-Lys (N-Me-Abz)-NH_2 (FP2), Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2 (FP3), and DABCYL-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-EDANS (FP4) were purchased from Bachem Bioscience (King of Prussia, PA, USA). 5,5′-Dithiobis (2-nitro) benzoic acid (DTNB) was obtained from Sigma (St Louis, MO, USA). Other regents and solvents used in experiments were of analytic grade or reagent grade as appropriate.

Samples used in the HTS assays were purchased or collected from different sources with widely structural diversity. Polymerase chain reaction (PCR) amplification of human macrophage elastase catalytic domain DNA was carried out with synthetic oligonucleotides using MJ thermal cycler (Watertown, MA, USA). Biomek 2000 pipeting workstation from Beckman (Fullerton, CA, USA) and HYDRA-96 multichannel pipetor from Robbins (Sunnyvale, CA, USA) were used for liquid handling. SPECTRAmax 340 96-well plate reader and *f*max 96-well plate reader from Molecular Devices (Sunnyvale, CA, USA) were used for end point readings and kinetic monitoring.

Construction of the expression vector pGEMEX-hMECD The nucleotide fragments encoding the catalytic domains of human macrophage elastase was obtained by PCR amplification of the EST clone with synthetic primers. Primer 1 (5′-CCTA-TT*GCTAG*CTTCAGGGAAATGCCAGGGGGG-3′) and Primer 2 (5′-CGCGCA*GGATC*CTTATCCATACAGGG-ACTGAATGCC-3′) (the coding sequences are underlined and the restriction sites are in italics) were designed based on the published human macrophage elastase[6] (GenBank Accession, No L203080). The PCR was carried out for 30 cycles (94 ℃, 1 min; 55 ℃, 1 min; 72 ℃, 1 min). The resulting DNA fragments coded for amino acid residues 100 – 263 of human macrophage elastase (Fig 1) were digested with *Nhe* Ⅰ and *Eco*R Ⅰ and subcloned into the plasmid pGEMEX-1 for expression in *E coli* under control of T7 promoter. The nucleotide sequences of the insert were confirmed by DNA sequencing. The sequence contained two silent mutations (T714C, C741T) when it was compared to the sequence reported by Shapiro *et al*[6]. For expression of the recombinant protein, the recombinant plasmid pGEMEX-hMECD containing the hMECD was transformed into *E coli* strain BL21(DE3)pLysS.

Protein expression and purification BL21 (DE3) pLysS cells containing the plasmid pGEMEX-hMECD was cultured in 1 L of TY medium in the presence of ampicillin (100 mg/L) with shaking at 37 ℃ to a cell density at OD_{600} of 0.6. Four hours after induction with isopropyl β-*D*-thiogalactopyranoside (IPTG) 1 mmol/L, cells were harvested by centrifugation for 5 min at 8000 × *g* at 4 ℃. The cells were washed with Tris·HCl buffer 50 mmol/L (pH 7.5) and lysed by sonication at 4 ℃. The pellets were washed three times by suspension in Tris·HCl 50 mmol/L (pH 7.5) followed by centrifugation. Then the pellets containing inclusion bodies were washed with Tris·HCl 50 mmol/L (pH 7.5) buffer containing urea 2 mol/L. Finally the pellets were solubilized in urea 8 mol/L.

The concentration of denaturing agent in the solubilized inclusion bodies was lowered from 8 mol/L to 6 mol/L and clarified by centrifugation (15 000 × *g*, 20

```
         1        10        20        30        40        50        60        70        80
hMECD    FREMPGGPVWRKHYITYRINNYTPDMNREDVDYAIRKAFQVWSNVTPLKFSKINTGMADILVVFARGAHGDFHAFDGKGGIL
              : : :     :::: :: :     :     ::  :   :: : :   : :::   :::::::::   ::: : :
hMat     YSLFPNSPKWTSKVVTYRIVSYTRDLPHITVDRLVSKALNMWGKEIPLHFRKVVWGTADIMIGFARGAHGDSYPFDGPGNTL
                       EEEEEE          HHHHHHHHHHHHHHHHH      EEEEE      EEEEEE
              90        100       110       120       130       140       150       160
hMECD    AHAFGPGSGIGGDAHFDEDEFWTTHS-GGTNLFLTAVHEIGHSLGLGHSSDPKAVMFPTYKYVDINTFRLSADDIRGIQSLYG
         :::: :: : :::::::::: ::  :  : :      :: ::::: :::::: ::: :::   :   : :: ::: ::: :::
hMat     AHAFAPGTGLGGDAHFDEDERWTDGSSLGINFLYAATHELGHSLGMGHSSDPNAVMYPTYGNGDPQNFKLSQDDIKGIQKLYG
         EEE          EEEE              HHHHHHHHHHHHHH                              HHHHHHHHHHH
```

Fig 1. Comparison of the amino acid sequences of hMECD and the catalytic domain of matrilysin (hMat). The identical amino acids in both hMECD and hMat are marked by colons. The secondary structures in hMat are indicated by H (α-helix) and E (β-sheet).

min), and the supernatant fluid was collected for purification.

The urea extract 6 mol/L containing the hMECD protein was loaded on to a Hi-Performance Q-Sepharose column (20 mL) equilibrated previously with Tris·HCl 50 mmol/L-urea 6 mol/L (pH 7.8) and eluted with a linear NaCl gradient (0 – 1 mol/L) in the Tris·HCl 50 mmol/L-urea 6 mol/L (pH 7.8), and fractions containing the hMECD protein were collected. The fractions were added slowly dropwise to a refolding buffer containing 50 mmol/L Tris·HCl (pH 7.8) and zinc and calcium ions. The mixture was centrifuged (15 000 × *g*, 20 min) to remove insoluble protein and then concentrated by using Amicon 8200 stirred cell (Millipore, Bedford, MA, USA) with a 10 000 NMWL Amicon PM 10 membrane for ultrafiltration. The concentrated refolded hMECD protein was desalted by using HiPrep 26/10 desalting column (53 mL; Uppsala, Sweden) and were further purified on Hi-Performance Q-Sepharose column (20 mL; Uppsala, Sweden) as described above under conditions without denaturant at 4 ℃. Protein samples taken through out the purification procedure were analyzed by 12 % reducing SDS-PAGE gel.

Enzyme activity assays The proteolytic activity of hMECD was evaluated at 37 ℃ and 25 ℃ by monitoring the hydrolysis of the thiopeptolide substrate (TPL) and a few fluorogenic peptides, respectively. Cleavage of thiopeptolide generates a free thiol group, which reacts immediately with DTNB to produce a substance with UV absorption (ε412 nm = 13 600 mol·L^{-1}·cm^{-1}). The cleavage was monitored at 412 nm on 96-well plates by using an absorbance microplate reader[11] in a 100 μL mixture containing hMECD proteins, TPL (100 μmol/L), DTNB (1 mmol/L), $CaCl_2$(50 mmol/L), $ZnCl_2$(50 μmol/L), and Tris·HCl (50 mmol/L, pH 7.8). A unit activity is defined as nmoles product per minute at 37 ℃.

The activity of hMECD in cleaving fluorogenic peptides FP1, FP2, FP3, and FP4 were monitored by measuring fluorescence emission at 393 nm with excitation at 328 nm in a 100 μL mixture containing hMECD (60 nmol/L), Tris·HCl (50 mmol/L, pH 7.8), $CaCl_2$(50 mmol/L), $ZnCl_2$(50 μmol/L), and different concentration of fluorogenic peptide, respectively.

The k_{cat} and K_m values for hMECD were estimated by nonlinear regression of the plot of initial velocity of product *vs* substrate concentration[12]. Protein concentrations were determined by Bradford method with bovine serum albumin as the standard[13].

For investigating the effects of zinc and calcium concentrations on hMECD refolding, the purified hMECD about 15 μmol/L in urea 6 mol/L were refolded by directly diluting it 10 folds into refolding buffers containing Tris·HCl buffer 50 mmol/L (pH 7.8), $CaCl_2$10 mmol/L, and $ZnCl_2$ concentration ranging from 0.01 μmol/L to 2 mmol/L or refolding buffers containing Tris·HCl buffer 50 mmol/L (pH 7.8), $ZnCl_2$ 10 μmol/L, $CaCl_2$ concentration ranging from 0.1 mmol/L to 200 mmol/L, respectively. After refolding 3 h, a 10 μL refolded hMECD from different refolding

ISSN 1671-4083 Acta Pharmacol Sin 中国药理学报 2002 Feb; 23 (2)
E-mail aps@mail.shenc.ac.cn Phn/Fax 86-21-6474-2629

buffer mixtures was added into reaction mixtures. The reaction mixtures finally contained Tris·HCl 50 mmol/L pH 7.8, $ZnCl_2$ 200 μmol/L, $CaCl_2$ 100 mmol/L, DTNB 1 mmol/L, and TPL 100 μmol/L for testing the effects of zinc and calcium concentrations on hMECD refolding. The effects of zinc and calcium ion concentrations on hMECD activity were evaluated using 60 nmol/L refolded and dialyzed hMECD in buffer containing 50 mmol/L Tris·HCl pH 7.8. In the presence of $CaCl_2$ 10 mmol/L, the hMECD activity as the initial rate was determined with $ZnCl_2$ concentration ranging from 10 nmol/L to 10 mmol/L. In the presence of $ZnCl_2$ 50 μmol/L, the activity was determined with $CaCl_2$ concentration ranging from 10 μmol/L to 2 mol/L. The determinations were repeated at least three times.

The pH dependence of k_{cat}/K_m for hMECD-catalyzed complete hydrolysis of TPL was studied at 37 ℃ using a buffer system containing $ZnCl_2$ 50 μmol/L and $CaCl_2$ 50 mmol/L. Buffer consisting of NaOAC 50 mmol/L, MES 50 mmol/L, Tris·HCl 50 mmol/L, or ethanolamine 50 mmol/L respectively was used for pH ranging from 5.0 to 10.0. Activities at pH 8.5, 9.0, 9.5, and 10.0 were corrected for noenzymtic degradation of the thiopeptolide. The time course of the product generation was fitted exponentially as a first-order reaction to give k_{app}[10]. The catalytic efficiency, k_{cat}/K_m, was calculated from k_{app} with the equation $k_{cat}/K_m = k_{app}/[E]$, Where [E] is the concentration of hMECD (60 nmol/L) used.

Inhibition of the hMECD by known MMP inhibitors Reference MMP inhibitors were prepared in Me_2SO. Reference MMP inhibitors at various concentrations such as Boc-*L*-Trp-OH (0.1 – 4 mmol/L), Cbz-*L*-Trp-OH (20 – 1000 μmol/L), 4-Abz-Gly-Pro-*D*-Leu-*D*-Ala-NHOH (8 – 100 μmol/L), or Galardin (20 – 200 nmol/L) were tested for calculating IC_{50}. Assays were performed by using 60 nmol/L hMECD to cleave TPL in Tris·HCl 50 mmol/L (pH 7.8) buffer containing $ZnCl_2$ 50 μmol/L, $CaCl_2$ 50 mmol/L.

MMP inhibitor screening From the compounds and mixtures collection of our center, 7036 compounds and 1524 mixtures were screened using purified hMECD in 96-well polystyrene plates (Corning, Action, MA) according to the method described earlier[14]. In brief, test compounds or mixtures were solubilized using Me_2SO at 1 g/L, and then distributed on the daughter plates. The Me_2SO (2 μL) as test vehicle was distributed in eight blank wells. An assay mix (88 μL) containing 10 μL of Tris·HCl (500 mmol/L, pH 7.5), 1 μL of $ZnCl_2$ (5 mmol/L), 1 μL of $CaCl_2$ (5 mol/L), 10 μL of thiopeptolide (1 mmol/L), 1 μL of DTNB (100 mmol/L in ethanol), and 65 μL of water, was added to each well by using 12 succession with 8 channel pipette on Biomek 2000. Then 10 μL of hMECD protein (600 nmol/L in Tris·HCl 50 mmol/L, pH 7.8) was added by HYDRA-96 (syringe size 100 μL). The plate was transferred to the microplate reader for immediate ready at 412 nm for 3 min. The initial rates of product formation were monitored at 37 ℃, and slope of the absorption change was recorded. The percentage of control activity of screening compounds was obtained by dividing the slope from compound wells by the average of the slopes from the 8 blank in each plate. IC_{50} values were determined when the compound showed 80 % inhibition near or below 20 mg/L.

RESULTS

Expression, refolding, and purification of hMECD The hMECD protein, expressed in *E coli* BL21(DE3)pLysS cells, represented a truncated form of the enzyme. By removing the N-terminal propeptide domain and the C-terminal hemopexin-like domain, requirement for *in vitro* activation is thereby eliminated. SDS-PAGE analysis (Fig 2, lane 2) indicateed that the expression of this protein in *E coli* was found almost exclusively in inclusion bodies, which was solubilized in urea 8 mol/L at neutral pH after washing with lysis buffer and urea 2 mol/L. The hMECD protein in the urea extract was purified by anion-exchange chromatography before it was refolded *in vitro* to an active form. The refolded hMECD had been further purified on a Q-Sepharose anion-exchange column. SDS-PAGE analysis showed that the protein purified through the final column migrated as a single protein bind (Fig 2, lane 4). The yield was 23 mg of active and purified enzyme from 1 L of culture (Tab 1).

Effect of calcium and zinc on the hMECD refolding and activity Both calcium and zinc ions were required for refolding, since either metal ion alone was not sufficient for hMECD to refold efficiently and to show activity. When hMECD was rapidly refolded by diluting into refolding mixtures containing different concentration of $ZnCl_2$ in the presence of $CaCl_2$ 10 mmol/L, the optimal refolding was obtained around $ZnCl_2$ 50 μmol/L (Fig 3A; closed circles). In the

Tab 1. Enzyme (hMECD) purification and refolding.

Sample	Volume/ mL	Protein/ $g \cdot L^{-1}$	Total protein/ mg	Activity/ $kU \cdot L^{-1}$	Total activity/U	Specific activity/ $kU \cdot g^{-1}$	Recovery/ %	Fold purification
Inclusion bodys solubilized in urea 8 mol/L	40	3.27	130	3000	120000	923	100	1
Q-Sepharose pool	200	0.29	60	1080	216000	3600	46	1
Dilution refolding	1800	0.02	35	267	480600	13890	27	15
Q-Sepharose purification	70	0.33	23	6657	466000	20000	18	22

Representative preparation from a 1-L preparation of cells. Protein fractions from the purification and refolding scheme were taken at various stages and analyzed for protein using the Bradford assay. Enzymatic activity was measured using the thiolpeptiloide as described in Materials and Methods. A unit activity is defined as nmoles product per minute at 37 ℃.

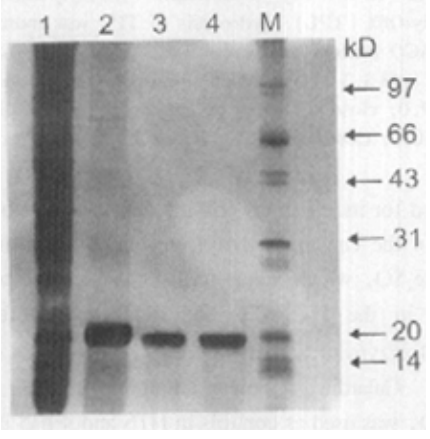

Fig 2. SDS-PAGE analysis of fractions taken at each step of the hMECD expression, purification, and refolding procedures on 12 % polyacrylamide gels. Lane 1: the total induced cell lysate; Lane 2: hMECD solution in Tris/6 mol · L^{-1} urea (pH 7.8) before loading the Q-Sepharose column; Lane 3: combined active fractions after Q-Sepharose column; Lane 4: refolded hMECD after Q-Sepharose column; Lane M: protein markers (low range standards).

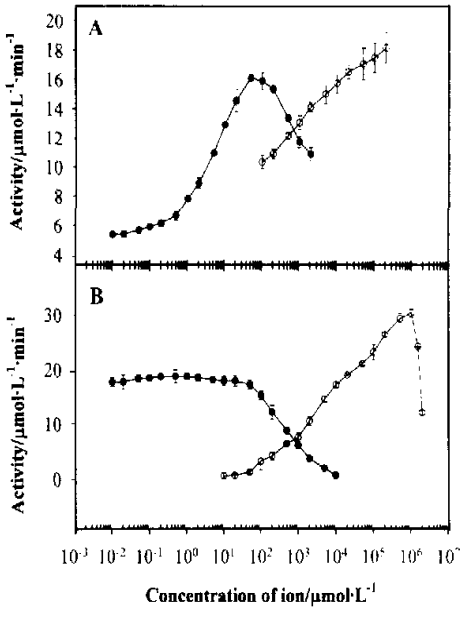

Fig 3. Effects of zinc and calcium ions on hMECD refolding (A) and activity (B). Hydrolysis of the thiopeptolide was monitored in Tris·HCl buffer (pH 7.8) at 37 ℃. Activities at different $ZnCl_2$ concentrations in the presence of $CaCl_2$ 10 mmol/L are shown as closed circles, and activities at different $CaCl_2$ concentrations in the presence of $ZnCl_2$ 10 μmol/L (A) or $ZnCl_2$ 50 μmol/L (B) are shown as open circles. $n=3$. $\bar{x} \pm s$.

presence of $ZnCl_2$ 10 μmol/L, the amount of refolded hMECD was increased with the increasing of $CaCl_2$ concentration up to 200 mmol/L (Fig 3A; open circles). Higher zinc concentration (> 50 μmol/L) significantly inhibited hMECD refolding.

A dialyzed hMECD with trace metals removed was used to evaluate the optimal effects of calcium and zinc ions on enzymatic activity. Varying amount of zinc concentration did not significantly increase the activity of hMECD, probably due to the incorporation of zinc atoms at the catalytic and structural sites already during refolding process. In the presence of $CaCl_2$ 10 mmol/L, hMECD showed higher activity at $ZnCl_2$ 10 nmol/L – 50 μmol/L (Fig 3B; closed circles). In the presence of $ZnCl_2$ 50 μmol/L, the optimal activity of hMECD was achieved around $CaCl_2$ 1 mol/L (Fig 3B; open circles). Higher calcium concentration (> 1 mol/L) inhibited hMECD activity. Zinc concentration higher than 50 μmol/L inhibited hMECD activity.

Determination of k_{cat}/K_m constants The hMECD showed activity against the synthetic thiopeptolide and peptide substrates (Tab 2). The hMECD

ISSN 1671-4083 Acta Pharmacol Sin 中国药理学报 2002 Feb; 23 (2)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

cleaved a substrate that was designed for stromelysin with highest catalytic efficiency (k_{cat}/K_m = 100 700 mol·L^{-1}·s^{-1}). Cleavage by the hMECD of the commonly used MMP substrate FP1 (k_{cat}/K_m = 36 694 mol·L^{-1}·s^{-1}), FP2 (k_{cat}/K_m = 53 660 mol·L^{-1}·s^{-1}), a substrate designed for gelatinase B and collagenase-1, or TPL (k_{cat}/K_m = 58 230 mol·L^{-1}·s^{-1}) was also efficient. It was interesting to note that hMECD could cleave FP4 (k_{cat}/K_m = 32 685 mol·L^{-1}·s^{-1}), a substrate that was designed for evaluation of pro-tumor necrosis factor-α converting enzyme (TACE) activity. This is consistent with the report that hMECD could release TNF from a pro-TNF fusion protein[7].

Tab 2. The constants of steady state kinetics of hMECD with different substrates. n = 3 experiments. $\bar{x} \pm s$.

Substrates	hMECD		
	K_m/μmol·L^{-1}	k_{cat}/s^{-1}	k_{cat}/K_m(mol·L^{-1}·s^{-1})
TPL	396 ± 63	23 ± 3	58230
FP1	51 ± 8	1.9 ± 0.3	36694
FP2	38 ± 14	3.1 ± 1.0	53660
FP3	59 ± 12	6.0 ± 1.5	100710
FP4	65 ± 22	2.1 ± 0.9	32685

TPL, Ac-Pro-Leu-Gly-[2-mercapto-4-methylpentanoyl]-Leu-Gly-OEt; FP1, Mca-Pro-Leu-Gly-Leu-Dap-Ala-Arg-NH_2; FP2, Dnp-Pro-β-cyclohexyl-Ala-Gly-Cys (Me)-His-Ala-Lys (N-Me-Abz)-NH_2; FP3, Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys (Dnp)-NH_2; FP4, DABCYL-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-EDANS.

pH-Dependence of catalytic efficiency for cleavage of TPL by hMECD Previous reports demonstrated that the catalytic domains of stromelysin[11] and gelatinase A[10] had the pH profiles similar to corresponding full-length stromelysin and gelatinase A. Therefore, the ability of hMECD in hydrolyzing the TPL at the pH ranging from 5.0 to 10.0 was investigated. The catalytic efficiency was found to be maximal at pH 8.0 (k_{cat}/K_m = 0.76 μmol·L^{-1}·s^{-1}). The optimal pH is consistent with the previous report of full-length human macrophage elastase[15]. The bell-shaped pH profile (Fig 4) fits a two proton ionization model. These are observable differences in catalytic efficiency in different buffer systems at the same pH value, however these differences did not change the shape of pH profile.

High-throughput screening of hMECD inhibitors Firstly, the inhibitor vehicle, Me_2SO was evaluated for its effect on enzyme activity. Higher than 90 % of the enzyme activity remained with even up to 2 % Me_2SO, which indicates that the presence of 2 % Me_2SO in the HTS did not significantly affect the hMECD enzymatic activity. Me_2SO solvent was used as blanks. Galardin, a potent no nmole inhibitor of the hMECD, was used as controls in HTS and it had IC_{50} and K_i values of 85.9 nmol/L and 68.6 nmol/L, respectively. IC_{50} and K_i values of other reference inhibitors are showed in Tab 3. The hMECD protein at 60 nmol/L showed reasonable signal to noise ratio under the assay condition. A total of 8560 compounds and mixtures from our center collection was tested on the purified hMECD. This screening led to the identification of 171 compounds and mixtures with 40 % – 60 % inhibition, 66 compounds and mixtures with 60 % – 80 % inhibition, and 49 compounds and mixtures with larger than 80 % inhibition and the rest with no or weak inhibition. The 49 compounds and mixtures with inhibition higher than 80 % were further tested to obtain IC_{50} values. Eight compounds and mixtures were eliminated because they showed precipitation and were considered false positives. After taking their molecular weights into account for each active compound, there was 10 of 27 compounds among 41 hits that showed IC_{50} better than 1 μmol/L. There was 1 of 14 mixtures that showed IC_{50} better than 1 mg/L. These compounds and mixtures will be further characterized for developing potent and selective MMP inhibitors.

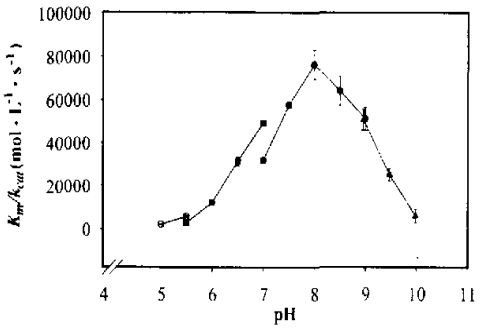

Fig 4. pH effects on the catalytic activity of hMECD with Ac-Pro-Leu-Gly-[2-mercapto-4-methylpentanoyl]-Leu-Gly-OEt (TPL). Hydrolysis of TPL was monitored in NaACO buffer (pH 5.0 – 5.5; open circles), Mes buffer (pH 5.5 – 7.0; closed squares), Tris buffer (pH 7.0 – 9.0; closed circles) or Ethanolamine buffer (pH 9.0 – 10.0; closed triangles). n = 3. $\bar{x} \pm s$.

Tab 3. IC_{50} (95 % confidence interval, CI) and K_i values for inhibition of the hMECD by reference matrix metalloproteinase inhibitors. $n = 3$ experiments.

Inhibitor	hMECD	
	IC_{50}/μmol·L^{-1} (95 % CI)	K_i/μmol·L^{-1}
4-Abz-Gly-Pro-*D*-Leu-*D*-Ala-NHOH	53.8(45.6-62.0)	43.0
Boc-*L*-Trp-OH	1652.4(1431.3-1873.6)	1319.6
Cbz-*L*-Trp-OH	185.4(172.3-198.6)	148.1
Galardin	0.086(0.080-0.092)	0.069

Abz, aminobenzoyl; Boc, tert-butyloxycarbonyl; Cbz, benzyloxycarbonyl. K_i values were derived from IC_{50} values by using the equation $K_i = IC_{50}/(1 + [\text{substrate}]/K_m)$, where substrate concentration was 100 μmol/L, K_m for hMECD was 396.5 μmol/L.

DISCUSSION

A catalytic domain (Phe 100 to Gly 263) of human macrophage elastase has been expressed in *E coli*. This molecule represents a truncated form of the enzyme and is missing prodomain and hemopexin-like domain. A similar construct which encodes from Gly 106 to Asn 268 has recently been expressed and purified in *E coli* by Parkar *et al*[16]. In their report, the catalytic domain MMP-12 was expressed in both an intact "A" and as a 10-amino-acid N-terminally truncated "B" form. Therefore, the methods of purification and refolding are very complex because there are two similar forms of protein in inclusion bodies. They hypothesize that B form may be produced by active MMP-12 or by *E coli* proteases cleaving the intact protein. Since a uniform protein in *E coli* was found almost exclusively in inclusion bodies in our study, we speculated that the different boundaries of two sequences were the reason that resulted in the differences between two expressions. Using our procedures, 23 mg of highly purified hMECD was obtained from per liter of bacterial culture. This result was the same as the report of Parkar *et al*. However, the specific activity of the final purified hMECD against the commonly used fluorescent substrate FP1 using our assay conditions (1.1 mmol·min^{-1}·g^{-1}) is higher than the activity (0.6 mmol·min^{-1}·g^{-1}) reported by them. The formation of the Phe 100 ▸ Asp 253 salt bridge may play a crucial role in hMECD having higher activity. For some MMP, a several folds larger activity has been demonstrated for active enzymes, starting with a highly conserved Phe 100 residue compared with species truncated for several residues[17,18].

Both calcium and zinc ions were required for hMECD refolding by dilution, since hMECD could not refold efficiently and show activity without zinc and calcium ions in refolding buffer. However hMECD could not refold efficiently by using direct dialysis like that of GaCD[10], because hMECD became insoluble and lost activity after it was dialyzed in the presence or absence of zinc and calcium ions. When the hMECD was rapidly refolded by dilution, the optimal refolding required a 30-fold molar excess of zinc ion in the presence of $CaCl_2$ 10 mmol/L. hMECD have incorporated zinc ions in refolding process, therefore refolded hMECD do not show significantly increased activity by increasing zinc concentration. Conversely, higher zinc concentration (> 50 μmol/L) was significantly inhibited refolded hMECD activities. The zinc and calcium ions play important roles in hMECD structure and catalytic activity. Zinc and calcium may change structure and activity of protein conformation by arranging the active site of enzyme. Therefore, the excess zinc and calcium ions might lead to some improperly folded protein or inhibit activity.

From pH-dependent profiles for the hMECD catalytic efficiency with TPL, we found the preference of the hMECD for expressing activity at pH 8.0 was consistent with the previous report about full-length MMP-12. Besides its ability to degrade elastin, MMP-12 also degrades a range of matrix and non-matrix substrates. Therefore, it is considered as an MMP with broad substrate specificity. hMECD can cleave the thiopeptolide and fluorogenic peptides substrates which were designed to assay MMP activity in our study also indirectly manifested this conclusion. hMECD cleave a substrate that was designed for stromelysin with highest catalytic efficiency (k_{cat}/K_m = 100 710 mol·L^{-1}·s^{-1}). This result is consistent with the report that MMP-12 has the highest degree of homology to stromelysin-1 (MMP-3); Moreover, their capabilities to hydrolyze a broad variety of extracellular matrix components are the most similar[19]. MMP is not the enzyme responsible for the cleavage of TNF from the cell surface. However the ability of hMECD cleaves a synthetic substrate of TACE indicated that MMP-12 maybe implicated in TNF-driven inflammation under appropriate conditions.

The screening of our chemical collection with hMECD as target has led to identification of ten

ISSN 1671-4083 Acta Pharmacol Sin 中国药理学报 2002 Feb; 23 (2)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

compounds with IC_{50} better than 1 μmol/L, and one natural product mixture with IC_{50} better than 1 g/L. These results combined with the fact that reference MMP inhibitors effectively inhibited the catalytic activity of hMECD, indicated that hMECD was an efficient enzyme for drug screening. More importance is that the catalytic domain inhibitors often inhibit corresponding full-length enzyme with the same potency and selectivity. These compounds and natural products that we obtained from our screening offer the clues for further development of lead compounds.

REFERENCES

1 Bode W, Fernandez-Catalan C, Tschesche H, Grams F, Nagase H, Maskos K. Structural properties of matrix metalloproteinases. Cell Mol Life Sci 1999; 55: 639 - 52.
2 Matrisian LM. The matrix-degrading metalloproteinases. Bioessays 1992; 14: 455 - 63.
3 Halpert I, Sires UI, Roby JD, Potter-Perigo S, Wight TN, Shapiro SD, *et al*. Matrilysin is expressed by lipid-laden macrophages at sites of potential rupture in atherosclerotic lesions and localizes to areas of versican deposition, a proteoglycan substrate for the enzyme. Proc Natl Acad Sci USA 1996; 93: 9748 - 53.
4 MacDougall JR, Matrisian LM. Contributions of tumor and stromal matrix metalloproteinases to tumor progression, invasion and metastasis. Cancer Metastasis Rev 1995; 14: 351 - 62.
5 Hautamaki RD, Kobayashi DK, Senior RM, Shapiro SD. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. Science 1997; 277: 2002 - 4.
6 Shapiro SD, Kobayashi DK, Ley TJ. Cloning and characterization of a unique elastolytic metalloproteinase produced by human alveolar macrophages. J Biol Chem 1993; 268: 23824 - 9.
7 Chandler S, Cossins J, Lury J, Wells G. Macrophage metalloelastase degrades matrix and myelin proteins and processes a tumour necrosis factor-alpha fusion protein. Biochem Biophys Res Commun 1996; 228: 421 - 9.
8 Kannan R, Ruff M, Kochins JG, Manly SP, Stoll I, El Fahime M, *et al*. Purification of active matrix metalloproteinase catalytic domains and its use for screening of specific stromelysin-3 inhibitors. Protein Expr Purif 1999; 16: 76 - 83.
9 Ye QZ, Johnson LL, Nordan I, Hupe D, Hupe L. A recombinant human stromelysin catalytic domain identifying tryptophan derivatives as human stromelysin inhibitors. J Med Chem 1994; 37: 206 - 9.
10 Ye QZ, Johnson LL, Yu AE, Hupe D. Reconstructed 19 kD a catalytic domain of gelatinase A is an active proteinase. Biochemistry 1995; 34: 4702 - 8.
11 Ye QZ, Johnson LL, Hupe DJ, Baragi V. Purification and characterization of the human stromelysin catalytic domain expressed in *Escherichia coli*. Biochemistry 1992; 31: 11231 - 5.
12 George HJ, Marchand P, Murphy K, Wiswall BH, Dowling R, Giannaras J, *et al*. Recombinant human 92-kDa type IV collagenase/gelatinase from baculovirus-infected insect cells: expression, purification, and characterization. Protein Expr Purif 1997; 10: 154 - 61.
13 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248 - 54.
14 Ye QZ, Nan FJ, Hu LY, Qian Z, Qian J. High-throughput screening for human collagenase 1 inhibitors. Acta Pharmacol Sin 2000; 21: 1021 - 6.
15 Shapiro SD, Senior RM. Macrophage Elastase (MMP-12). In: Parks WC, Mecham RP, editors. Matrix metalloproteinases. Academic Press; 1998. p 185 - 97.
16 Parkar AA, Stow MD, Smith K, Panicker AK, Guilloteau JP, Jupp R, *et al*. Large-scale expression, refolding, and purification of the catalytic domain of human macrophage metalloelastase (MMP-12) in *Escherichia coli* Protein Expr Purif 2000; 20: 152 - 61.
17 Suzuki K, Enghild JJ, Morodomi T, Salvesen G, Nagase H. Mechanisms of activation of tissue procollagenase by matrix metalloproteinase 3 (stromelysin). Biochemistry 1990; 29: 10261 - 70.
18 Nagase H. Activation mechanisms of matrix metalloproteinases. Biol Chem 1997; 378: 151 - 60.
19 Gronski TJ Jr, Martin RL, Kobayashi DK, Walsh BC, Holman MC, Huber M, *et al*. Hydrolysis of a broad spectrum of extracellular matrix proteins by human macrophage elastase. J Biol Chem 1997; 272: 12189 - 94.

表达和纯化人巨噬细胞弹性蛋白酶的催化区以高通量筛选酶抑制剂

程东航，沈 强，钱 静，钱 蓁，叶其壮[1]
(中国科学院上海生命科学研究院药物研究所国家新药筛选中心，上海 201203，中国)

关键词 基质金属蛋白酶；人巨噬细胞弹性蛋白酶；基因表达；高通量筛选

目的：获得具有催化活性的人巨噬细胞弹性蛋白酶的催化区（hMECD），并建立有效的高通量筛选方法筛选其抑制剂。**方法**：在大肠杆菌中表达 hMECD，并根据酶活性以比色法建立高通量筛选模型。对一批共 8560 个纯化合物和混合物进行了高通量筛选。**结果**：构建了有效的大肠杆菌表达系统。存在于包涵体中的表达蛋白在体外重折叠复性，经阴离子交

换柱层析的方法纯化，1 L 大肠杆菌培养物可得到 23 mg 纯化的活性蛋白．该蛋白的重折叠复性和酶活性需要钙锌离子，但高浓度的锌离子则抑制其重折叠复性和活性．hMECD 剪切合成底物包括一个硫酯和几个荧光发生的肽类底物，并在 pH 8.0 显示最强的活性．对 8560 个化合物和混合物的高通量筛选发现了 27 个纯化合物和 14 个天然产物在 20 mg/L 的浓度下具有大于 80 % 的抑制活性．**结论**：建立了有效的人巨噬细胞弹性蛋白酶的催化区表达和纯化的方法．该重组蛋白抑制剂的高通量筛选模型具有有效、可信和快速的特点．

（责任编辑　吴民淑）

Corrigendum

Acta Pharmacologica Sinica 2001 Dec; 22 (12): 1063.

In **line 6**: left side: 1.5 g/mL should be 1.5 mg/mL.