

Study of a Novel Antiosteoporosis Screening Model Targeted on Cathepsin K

JUN YANG*, GUANG-DONG SHANG[#], AND YUE-QIN ZHANG*

**Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, 100050, China; [#]Department of Chemistry and Chemical Biology, Cornell University, USA*

Objective To establish an effective assay to access the effects of natural products on cathepsin K for screening antiosteoporosis drugs. **Methods** To obtain the purified cathepsin K, we cloned the target fragment from the mRNA of human osteosarcoma cell line MG63 and demonstrated its correctness through DNA sequencing. Cathepsin K was expressed in a high amount in *E.coli* after IPTG induction, and was purified to near homogeneity through resolution and column purification. The specificity of the protein was shown by Western blotting experiment. The biological activity of the components in the fermentation broth was assayed by their inhibitory effects on cathepsin K and its analog papain. **Results** With the inhibition of papain activity as a screen index, the fermentation samples of one thousand strains of fungi were tested and 9 strains among them showed strong inhibitory effects. The crude products of the fermentation broth were tested for their specific inhibitory effects on the purified human cathepsin K, the product of fungi 2358 shows the highest specificity against cathepsin K. **Conclusions** The compounds isolated from fungi 2358 show the highest biological activity and are worth further structure elucidation and function characterization.

Key words: Cathepsin K; Inhibitor; Antiosteoporosis; Screening model

INTRODUCTION

Since Drake found that the selective expression of the mRNA of cathepsin K, cloning and tissue distribution experiments have shown that cathepsin K has a unique tissue distribution with the highest abundant expression in osteoblasts^[1,2]. A human genetic disorder with an osteopetrotic phenotype termed pycnodysostosis has been linked to a mutation in cathepsin K which results in its inactive form. A cathepsin K gene knock out mouse shows the same phenotype as pycnodysostosis patients and the bone resorption was greatly damaged. So the inhibition of cathepsin K activity provides a novel therapeutic approach to treat disorders such as osteoporosis.

Cathepsin K is a proteinase belonging to the papain family of cysteine proteinases. It plays an important role in the resorption of the bone matrix. Bone remodeling is a dynamic process that involves bone resorption and formation. The resorption phase of this process is carried out by osteoclast cells, which adhere to the bone surface, leading to the creation of an extracellular compartment, the resorption pit, which is maintained at low pH and into which the osteoclast secretes proteolytic enzymes. In this acidic environment, the mineral

Biographical note of the first author: Jun YANG, female, born in 1977, Ph. D., majoring in pathology of osteoporosis. Tel: 86-10-83162728. E-mail: yangjunimb@hotmail.com

region of the underlying bone is removed and the protein matrix is exposed to degradation by proteolytic enzymes^[3].

Cathepsin K is synthesized as a 37-kDa protein which contains a 15 amino-acid leader sequence, a 99 amino acid prodomain and a 215 amino-acid mature active enzyme. It secretes from the cell as an inactive form followed by converting to its mature active form by proteolytic cleavage of the prodomain from the amino-terminus at pH 4.0 *in vivo*.

Mature cathepsin K shares 46% sequence similarity with papain on the aminoacids level^[4]. Cysteine protease inhibitors such as E64 and Leupeptin were shown to be effective in inhibiting bone resorption. Based on this knowledge, we established a new antiosteoporosis drug screening model targeted on cathepsin K to search new inhibitors from microbial metabolic products.

MATERIALS AND METHODS

Materials

Oligonucleotide primers were synthesized at Shanghai Sangon Co. Ltd. DTT, Papain, Z-Phe-Arg-MCA, MCA, Leupeptin, Chymostatin were purchased from Sigma Chemical Co. Anti-Cathepsin K (rabbit) was purchased from CALBIOCHEM, horseradish peroxidase-linked anti-rabbit IgG (H+L) produced by Beijing Zhongshan Biotech. Co. Low molecular weight protein marker were purchased from HuaMei Co. Ltd. BD TALON Metal Affinity Resins was provided by Clontech. T-vector was from promega. ThermoScriptTM RT-PCR System was obtained from invitrogen.

Cloning and Sequencing of the Cathepsin K

The cDNA encoding the propeptide of human cDNA of cathepsin K containing amino acids 1-329 was amplified from the mRNA prepared from human osteosarcoma MG63 cells according to the method described by ThermoScriptTM RT-PCR System (invitrogen). The primers used in this experiment were: sense 5' -AAAGAATTCATGTGGGGGCTCAAGGTTCTGCTG-3' and antisense 5' -AAAAGCTTTCACATCTTGGGGAAGCTGGCCAG -3' with *EcoR* I, *Hind* III restriction enzyme sites engineered as underlined at the sense and antisense primer respectively. The 987 bp DNA fragment was amplified and cloned into the T-vector, the correctness of the insert was confirmed through DNA sequencing. The insert was then excised with *EcoR* I and *Hind* III and cloned into the same enzymes treated The *E.coli* expression vector pET30a, the construct was named pEK43 which was transformed into *E. coli* BL21(DE3) for protein expression. One colony was picked up and transformed into 2 mL LB medium with 50 µg/mL kanamycin and grew at 37°C over night. The culture was transferred to 100 mL LB medium with 50 µg/mL kanamycin till the OD₆₀₀ reached about 0.6. The expression of the protein was induced by adding 1 mmol/L IPTG as final concentration. The pellet was harvested after 3 hours.

Purification of Cathepsin K

Because nearly all the proteins exist as the inclusion body though a lot of expression conditions were tried, we have to render the denaturation and renaturation method to purify the protein. After 3 h induction, cells were pelleted by centrifugation at 5000 g for 10 min. Cells containing the recombinant propeptide in inclusion bodies were purified as denatured protein form following the protocol of BD TALON Metal Affinity Resins. The solubilized

propeptides were refolded by a slow drop-wise dilution (20-fold dilution) in stirred refolding buffer (prepared at room temperature): 50 mmol/L Tris/HCl pH8.0, 5 mmol/L EDTA, 10 mmol/L reduced glutathione and 1 mmol/L oxidized glutathione (plus 0.1 mol/L L-arginine). After stirring overnight at 4°C, the solution was concentrated using an Amicon ultrafilter system with a 3-kDa cut-off membrane. The concentrated solution was then dialyzed against 25 mmol/L Na₂HPO₄ and NaCl 0.5 mol/L, pH 7.0 for 24 hours with three changes of dialysis buffer.

Western Blotting Assay

Proteins were analyzed on a 12% SDS-PAGE gel and run at 100 mA/gel. For western blotting, proteins were transferred onto PVDF membrane, blocked with 5% nonfat dry milk in PBST, then probed with a anti-cat K antibody (1:1000) in PBST containing 0.1% BSA for 2 h. The membrane was washed three times with 15 min each using PBST and then stained with Luminol reagent A and B (provided by Beijing Zhongshan Biotech. Co.), and the band intensity was scanned with a phosphorimager (Alpha Innotech Corporation).

Enzymatic Activity Assay

The enzymatic activity of papain and purified cathepsin K (4 µg/mL) was determined from the rate of hydrolysis of the Z-Phe-Arg-Methyl-Coumarin. Assays were performed at 40°C, buffer A for papain and buffer B for purified cathepsin K, and the buffers were incubated with enzyme for 10 min before use. Substrate hydrolysis was monitored over a period of 10 min, and then the reaction was quenched by adding the stop solution. Substrate hydrolysis was monitored in fluorescent plate reader (BMG Co.), and all activity measurements were calculated as initial rates over the first 5 min of the reaction. Buffer/activator solution A: 0.4 mol/L NaH₂PO₄-Na₂HPO₄ buffer pH 6.8, 4 mmol/L EDTA, 8 mmol/L dithiothreitol was added prior use. Buffer/Activator Solution B: 50 MES buffer, pH 5.5, containing 2.5 mmol/L dithiothreitol, 10% DMSO. Papain solution: 10.4 mg papain/10 mL in 0.1% Brij35 (50 µmol/L), The working strength substrate solution (0.2 µmol/L) was prepared by diluting the stock solution with 0.1% Brij35. Substrate solution: 10 mmol/L Z-Phe-Arg-Methyl-Coumarin in DMSO, the working strength substrate solution (20 µmol/L) was prepared by diluting the stock solution with 1% DMSO. Standard solution: 7-Amino-4-Methyl-Coumarin was dissolved in 1% DMSO (10 µmol/L). Stop Solution: 100 µmol/L CH₃COOH-CH₃COONa Buffer, pH 4.3.

RESULTS

Expression of Human Cathepsin K in Escherichia coli BL (DE3) Cells

A full length cDNA encoding the prodomain and mature enzyme of human cathepsin K was PCR amplified from total mRNA of the cell MG63. The cathepsin K cDNA was cloned into pET30a and transfected into *E.coli* BL (DE3) cells. Stable cell lines containing pET30a vector with or without (control) the cathepsin K cDNA insert were obtained by selection with the kanamycin on culture plates (Fig. 1A).

The expression of cathepsin K was determined by immunoblot analysis. The extract of *E.coli*/control and *E.coli*/pEK43 stable cell lines were subjected to SDS-PAGE, and analyzed by immunoblotting using a monoclonal antiserum to cathepsin K. The extract of the *E.coli*/control cells contained no protein signal detectable by immunoblot with the cathepsin K, while the *E.coli*/pEK43 lane contained an immunoreactive protein of 43 kDa

corresponding to pro-cathepsin K confirming the expression of cathepsin K (Fig. 1B).

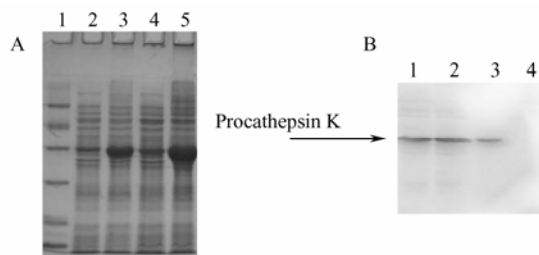


FIG. 1. A. SDS-PAGE analysis of the expression of *E.coli* BL (DE3)/ Cat K. 1. Low molecular weight protein marker (KD): 96, 66, 45, 30, 20.1, 14.4. 2. *E.coli* BL (DE3)/control with 2 h induction. 3. *E.coli* BL (DE3)/pEK43 with 2 h induction. 4. *E.coli* BL (DE3)/control with 3 h induction. 5. *E.coli* BL (DE3)/pEK43 with 3 h induction.

B. Western blotting analysis of expressed cathepsin K 1, 2, 3 the blots of *E.coli* BL (DE3)/pEK43 with 3 h induction 4. The blots of *E.coli* BL (DE3)/control with 3 h induction.

Enzymatic Activity of Papain and Cathepsin K

To confirm that the cathepsin K's activity measured for the Z-Phe-Arg-MCA substrate was due to proteinase, activity in the presence or absence of leupeptin (a wide-spectrum inhibitor of cysteine proteinase) was measured. Enzymes were preincubated for 10 min with various concentrations of leupeptin and assayed with Z-Phe-Arg-MCA substrate. We used this dipeptide as the substrate for the optimal measurement of papain and cathepsin K activity in the extracts. The results obtained in extract of the *E.coli*/pEK43 were comparable to those obtained for the papain. The IC_{50} of leupeptin on the purified cathepsin K was 0.395 $\mu\text{mol/L}$, while on papain was 0.5 $\mu\text{mol/L}$ (Fig. 2).

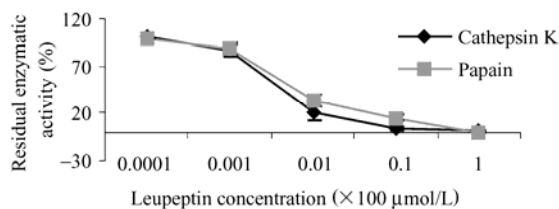


FIG. 2. The inhibition of leupeptin on the activities of cathepsin K and papain. x axial represent the concentration of leupeptin, y axial represents the residual activity of enzymes (cathepsin K and papain). Screen for the inhibitors of cathepsin K.

Papain was used for the initial screening of the inhibitors from the fermentation broths of 1000 fungal strains and 9 strains were obtained which showed strong inhibitory effects on the Z-Phe-Arg-MCA degradation of papain (Table 1).

TABLE 1

The Enzymatic Activities of the 9 Strains

Train	Reaction System Value			Residual Enzymatic Activity (%)	Inhibitor Activity (%)
	Raw-fluor Escence	Blank-fluo Rescence	Papain Enzymatic Activity (100%)		
x-2322	5823.1	3522.7	51496.0	4.500	95.50
2358	16064.0	2798.3	51039.2	27.50	72.50
3574	13629.3	4426.5	57564.8	17.96	82.04
3608	10579.3	3049.1	54218.8	14.70	85.30
3909	10183.5	3049.1	54218.8	13.92	86.08
3939	16161.9	3578.6	52871.2	24.55	75.45
3940	17859.7	3049.1	54218.8	28.90	71.10
3945	14511.7	2221.9	46787.8	27.58	72.42
3954	9202.8	4426.5	57564.8	8.860	91.14

Further selectivity assays were carried out with purified cathepsin K using two inhibitors leupeptin and chymostatin as control. Leupeptin is an aldehyde derivate of microbial origin and a wide-spectrum inhibitor of cysteine proteinases, it inhibits the biological activity of thiol proteinases as well as some serine proteinases, such as papain, cathepsin K and cathepsin B. The result showed that chymostatin was exhibiting stronger inhibition effect against cathepsin K than on cathepsin B. By examining the residual enzymatic activity of cathepsin K and cathepsin B with the different concentration of two inhibitors, we obtained the characteristic value, M ($IC_{50_{\text{cathepsin B}}} / IC_{50_{\text{cathepsin K}}}$), chymostatin was more sensitive to these two enzymes, with $M=34.47$, leupeptin with less sensitivity, with $M=2.65$. This fact coincided with the different selectivity in the inhibition of chymostatin and leupeptin (Fig. 3). So we chose chymostatin as a positive control, and leupeptin as a negative control.

Of the 9 strains screened out, # 2358 showed the highest inhibition activity with M is 32.51(Fig. 4).

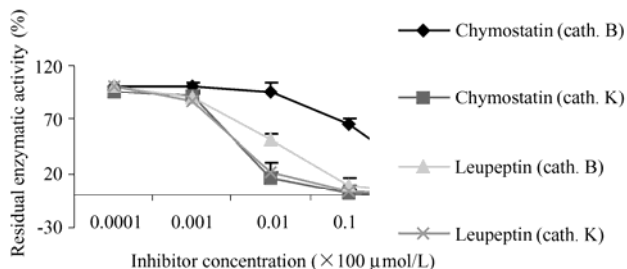


FIG. 3. The inhibition of chymostatin and leupeptin on the activities of cathepsin K and cathepsin B. x axial represents the concentration of inhibitors (leupeptin and chymostatin), y axial represents the residual activity of enzymes (cathepsin K and cathepsin B).

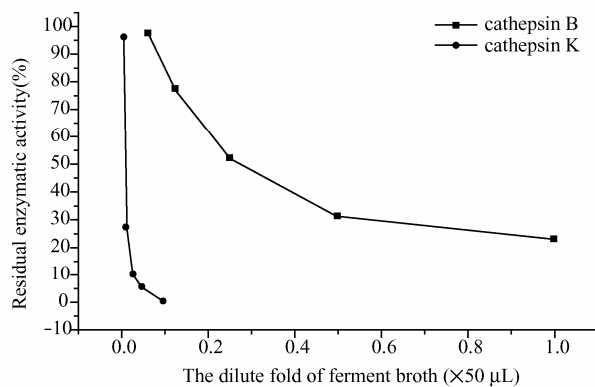


FIG. 4. The inhibition of the components of metabolite products of fungi 2358 on cathepsin K and cathepsin B. The line with circle represents the inhibition line of cathepsin K, the line with square represents the inhibition line of cathepsin B.

DISCUSSION

Searching for anti-osteoporosis drugs is an important task for world-wide researchers. cathepsin K belongs to a lysosomal cysteine proteinase family, and recently it was found to be the main enzyme in the bone collagen degradation process. It is selectively expressed in osteoclasts and plays an important role in the bone resorption. As bone resorption is essential to bone density maintenance and is also crucial for the treatment of osteoporosis, it is a good assumption to make use of *in vitro* cathepsin K activity as an index for bone resorption and further, for screening of novel anti-osteoporosis drugs. The specific inhibitors of cathepsin K exhibit their antiosteoporosis effects through inhibiting the degradation of the collagen I of bone supporting protein^[5,6].

Procathepsin K is a single chain polypeptide without glycosylation, and is suitable to be expressed in *E.coli*. In this study, *E.coli* high expression vector pET30a was used for the protein expression. With the same systems we have successfully got the active N-acetylneuraminidase to complete biotransformation in the prior experiment (paper submitted). So the cDNA of cathepsin K was cloned from MG63 cell line and expressed in *E.coli* BL21(DE3) following sequence confirmation. There are 6 cysteines residues in mature cathepsin K to form 3 pairs of disulfide bonds in molecular to attain the stable structure. The expression of cathepsin K is high up to 32% of the total protein. However, unfortunately, it resides in inclusion bodies. So we used the BD TALON Metal Affinity Resins to purify it to near homogeneity (more than 95%).

In acidic conditions, through the autocleavages of the prodomain, mature cathepsin K exposes the catalytic triad (cysteine, histidine and asparagine) and with which to catalyze the degradation of the substrates. The propeptide inhibits the hydrolysis through the interaction of hydrophobic bonds, hydrogen bonds and salt bridge^[7-9]. Purified pro-cathepsin K has been shown to be activated to the mature form by lowering the pH to 4.0. This decrease in pH is believed to bring a change in the conformation of the protein, which helps the active site cysteine to autocatalyze the activation to the mature form of the enzyme^[10,11]. We put a small portion of the purified protein in the buffer (pH=4) and treated it in 50°C for 15 min, placing it in room temperature for 30 min, and then adding it into a large volume of untreated sample (at least 20 folds), in 4°C for 6 days, and then assayed the enzymic activity with substrate Z-Phe-Arg-Methyl-Coumarin compared with papain.

Prior to the activity assay with cathepsin K, papain was chosen as a cheap surrogate for the screening of cathepsin K inhibitor because it has 46% amino acid sequence identity to cathepsin K and its biological activities are well characterized.

Type I collagen is one of the most abundant molecules in mammals, and its degradation is an important physiological issue. The specificity of the cleavage of collagen is determined by the type of proteinase rather than by the position of hyperactive sites in the collagen molecule. Unspecific proteinases including cathepsins L, B, and S cleave collagen molecules only in telopeptides site but not in the native triple helix, generating monomers of type I collagen. Cathepsin K cleaves collagen molecules both in the telopeptides and at multiple sites within the native triple helix, generating fragments of various sizes^[12-14]. A selective inhibitor to cathepsin K is very important for our work. After examination, we chose chymostatin as the positive selectivity control, and leupeptin as negative selectivity control. So we developed an enzyme based assay method to screen for specific antiosteoporosis screening model targeted on cathepsin K from metabolic products of fungal origin and found 9 fungal strains exhibiting positive effects, one of which showed strong selectivity on cathepsin K.

More and more evidences show the importance of cathepsin K in pathophysiological conditions such as osteoporosis, and provide increased impetus for the development of cathepsin K inhibitors. The long term goal of this research is to obtain the active, non-peptide compounds which can permeate the membrane directly to promote the research and development of new antiosteoporosis drugs. Considering the limited availability of osteoclasts and the lack of established cell lines, this assay will be useful for the rapid high through-out screening of anti-osteoporosis drugs as well as to promote the study of the mechanism of the occurrence and the treatment of osteoporosis. Still, the compounds isolated from the strain 2358 we got are worthwhile further studying^[15-17].

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