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# Expression, Purification and Characterization of Hexahistidine-Tagged Human Cathepsin K in High Five Cells

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## Abstract

Cathepsin K is a protease with high collagenolytic and elastinolytic activity. A full-length cDNA clone of human cathepsin K was used to construct an expression system in insect cells. The recombinant protein had a C-terminal tag of six histidine residues, which allowed purification of this protein by a one-step $Co^{2+}$-Sepharose affinity chromatography. Minimal amounts of pro-cathepsin K were secreted into the medium but most of the cathepsin K was present within infected cells. Very little processing of pro-enzyme to mature form occurred in High Five cells.

Spontaneous in vitro activation of pro-cathepsin K to mature-cathepsin K occurred at pH 4.0. The ~29 kDa mature-cathepsin K efficiently hydrolyzed the fluorogenic peptide substrate and r-headpin, a serine protease inhibitor down-regulated in head and neck cancer cell lines and tumor tissues, potently inhibited the r-catK activity *in vitro*. Taking cathepsin K as an example, we present a strategy, which should facilitate the expression of perhaps other cathepsins or proteases in general in insect cells.

**Key words:** CathepsinK, Sf9, metal affinity chromatography, HNSCC, headpin

## Introduction

In previous studies, we and others have cloned the headpin cDNA from oral keratinocytes and demonstrated that recombinant headpin inhibited lysosomal cysteine proteinases cathepsins K and L (1-5). Cathepsin K (catK[2], EC 3.4.22.38) is a cysteine proteinase that is selectively and highly expressed by bone-resorbing osteoclasts (6-13). The enzyme has the capacity to degrade type I and type II collagen (14-17). Besides osteoclasts and chondroclasts and their precursors, epithelial cells of various organs (brain, liver, lung, muscle, adult lung, heart, kidney, skin, small intestine, esophagus, trachea, thyroid, and bladder), breast cancer cells, and synovial fibroblasts expressed significant amounts of this enzyme (18-21). Because of its ability to degrade bone matrix proteins, inhibition of catK is expected to prevent osteoclast-mediated bone resorption or synovial fibroblasts mediated cartilage erosion in diseases such as post-menopausal osteoporosis and rheumatoid arthritis and osteoarthritis (13, 20).

In mammalian cells, cathepsin K is synthesized as a 39-kDa pre-pro-enzyme, which undergoes maturation to a catalytically active form of 27 kDa (21-23). The cDNA encoding this protease was cloned from human, rabbit, and mouse osteoclast libraries expressed and purified in baculovirus-infected Sf9 or Sf21 insect cells by several independent groups as an inactive secreted pro-enzyme (24-29). Since the baculo-expressed recombinant catK has no fusion tags, Bromme et al (28) purified the enzyme from a 1-liter Sf9 cell culture (~$2\times10^9$ cells) using conventional hydrophobic chromotography (butyl-Sepharose 4 Fast Flow) and ion exchange chromatography (Mono S). The average yield was ~2 mg of purified enzyme per 1-liter Sf9 cell culture (~$2\times10^9$ cells). Similarly, Bossard et al purified (29) the un-tagged enzyme from 10-liter growth media of infected Sf21 cells using S-Sepharose Fast Flow chromotography, AIC-Blue-2

Dye mimetic chromatography and gel filtration chromatography (Superdex 75 sizing column). Both Bromme (28) and Bossard (29) have demonstrated activation of the recombinant pro-enzyme in vitro by proteolytic degradation of the N-terminal 99-amino acid pro-peptide. Subsequently it was shown that the vitro activation of the pro-cathpsin K was an autocatalytic process (30).

Here, we sought to produce an active recombinant catK by affinity-tagging the enzyme and assess the merits of appending the tags to catK. Altogether, our results demonstrate that the use of six histidine at the C-termini of catK significantly accelerated the purification of this enzyme using a one-step metal-chelating affinity chromatograph. Using a small-scale culture, we were able to obtain 0.3 to 0.5 mg of active catK enzyme from 200 ml cell lysate. The strategy reported here could also be used perhaps for other cathepsins such as catS, catL, etc.

**Materials and Methods**

**Materials:** All restriction enzymes and $T_4$ DNA ligase were purchased from New England Biolabs. Insect cell culture medium was obtained from BioWhittaker and Invitrogen. Sera were obtained from Life Technologies. The BAC-To-BAC Baculovirus Expression System and CellFECTIN were obtained from Life Technologies. Anti-hcatK mAb (AF-9202) was obtained from Medicorp (Quebec, Canada). The human catK expression clone in pIND/GS (H-K1000) was purchased from Research Genetics. Precast SDS-PAGE gels and prestained markers were purchased from Bio-Rad. TALON resin was obtained from Clontech. Z-Phe-Arg-R110 was purchased from Molecular Probes. E64 and pepstain were purchased from Sigma.

**Cells and Virus**: Sf9, High Five insect cells and wild-type baculovirus were purchased from Invitrogen Corp. The Sf9 cells were cultured in Insect-XPRESS medium (BioWhittaker) supplemented with 10% bovine fetal serum (Life Technologies, Inc.), 1 × penicillin-streptomycin (Life Technologies, Inc.), and 1 × amphotericin B (Boehringer Mannheim) as described previously (31). The High Five cells were cultured in Ultimate Insect serum free medium (Invitrogen Corp).

**Construction of the Expression Vector pFASTBAC1-hCatK**: A GeneStrom hORF Expression Vector (H-K1000) containing the cDNA fragment encoding, the signal sequence, pro-domain

and mature enzyme of human cathepsin K fused with V5 epitope and six-His at its carboxyl-terminus, was cleaved with BamHI and PmeI. The resulting 1.1-kilobase pair fragment was gel purified and ligated into the baculovirus transfer vector pFASTBAC1 that had been digested with BamHI and StuI, generating the plasmid pFASTBAC1hCatK.

**Transfection and Isolation of Recombinant catK baculovirus:** Recombinant catK composite bacmids were generated and transfected into Sf9 cells using the CellFECTIN-mediated transfection technique as described (31). The supernatants from the transfection were collected, amplified, tittered, and stored as virus stocks for subsequent experiments. This construct was designed to express a full-length catK protein (329 amino acids), including the first 15 $NH_2$-terminal amino acids which constitute the signal peptide plus 45 additional amino acids consisting of a V5 epitope and 6 × His, fused in frame to the carboxyl-terminus of catK. The expected molecular mass of the recombinant human cathepsin K is 41790.25 Da.

**Analysis of Proteins from Cathepsin K Recombinant Virus-infected Cells:** Sf9 or High Five insect cells were separately infected with about 2.5 plaque-forming units of recombinant catK virus per cell at 27 ºC in a serum containing or serum free medium, respectively. Seventy-four hours after infection, the cells were pelleted, resuspended in insect cell lysis buffer and cytoplasmic extract was prepared as described before (31-32). Aliquots of the supernatant collected from the infected cells were adjusted to contain 2 % SDS and 10 % 2-mercaptoethanol and boiled for 5 min prior to electrophoresis in a 0.1 % SDS, 12 % polyacrylamide gel. Western blots were carried out as described previously (33-34) using a 1:2000 dilution of anti-human cat K mAb or 1:2500 dilution of penta-His mAb. For time course expression of catK, aliquots were collected 48, 72, or 96 h after infection with the recombinant viruses. For small scale production of the protein, High Five cells at a density between 1 and 2 × $10^6$ /ml were infected with the recombinant catK virus.at 2.5 MOI for 96 h.

**Immunstaining of Recombinant Cathepsin K in High Five Cells**: The ABC staining kit (Vector Laboratories, Burlingame, CA) was used for immunocytochemical protein localization, according to the recommendations of the manufacturer. Briefly, 2,000~4,000 High Five cells incubated with catK recombinant virus were seeded on to glass chamber slide and incubated for

at least 72 h. The medium was discarded and cells were fixed with 10% formalin for 30 minutes. To retrieve the target antigen, cells were treated with PBS buffer containing 0.5% Triton X-100 then washed with PBS alone twice at room temperature. Cells were incubated with biotin blocking solution at room temperature for 30 min. After washing the slides twice with PBS buffer, cells were incubated with the protein blocking solution at room temperature for 30 min. Protein blocking solution was discarded and slides were incubated with catK mAb (0.6 µg/ml) at 4°C over night. After washing the chambers with PBS solution, cells were incubated with biotinylated secondary antibody at room temperature for 30 min. After extensive washing with PBS, the alkaline phosphatase-catalyzed product was visualized with BCIP/NBT substrate in 100 mM Tris-Cl, pH 9.5. Staining experiments with secondary antibodies alone were also carried out as controls. All slides were evaluated using a Nikon labophot-2 microscope.

**Purification of Recombinant Cathepsin K from Cell Extract:** High Five cells in spinner flasks were cultured at 27 °C in ultimate serum-free insect medium (Invitrogen) containing only half-strength antibiotics and without fetal bovine serum. The rcatK was purified from the cell lysate by TALON affinity chromatography (31-32). Typically, $3.5 \times 10^8$ High five cells were infected with P3 recombinant viruses using a MOI of 2.5 plaque-forming units/cell. The cell pellet, collected 96 h post-infection, was resuspended at a ratio of $1.0\text{-}1.5 \times 10^7$ cells/ml in a lysis buffer containing 50 mM Tris-HCl (pH 8.0), 130 mM NaCl, 1.0% (v/v) Triton X-100, 10% glycerol, and 1.0 µg/ml each of, pepstatin and PMSF. The suspension was kept on ice for 1 h. The lysed cell suspension was sonicated (15-s pulses at 80 Watt for 3 times) and the cell debris was removed by centrifugation. The cell-free extract was diluted with 2x binding buffer containing 40 mM Tris-HCl (pH 8.0), 260 mM NaCl, 2.0% (v/v) Triton X-100, and 10% (v/v) glycerol and loaded onto a 1.0 × 5-cm column containing 0.6 ml of TALON Superflow (CLONTECH) equilibrated in binding buffer. The column was washed with binding buffer until the $A_{280}$ was less than 0.005. The protein was then step eluted with 150 mM imidazole in binding buffer. The fractions (eluates 2 through 6) containing homogeneous catK proteins were pooled, concentrated using the YM3 Centriplus (Millipore) and finally stored in binding buffer containing 15% glycerol at –80 °C. The average yield of rpro-catK was 2.0 to 3.0 mg per 200 ml

cell lysate.

**In vitro Activation and Activity Assay of Cathepsin K:** In vitro activation and activity assay of Cathepsin K were done as described previously (29-30) with minor modifications. Briefly, imidazole eluates (2 through 6) containing homogeneous pro-cathepsin K were combined and concentrated using a Millipore YM3 concentrator. The buffer was exchanged twice to activation buffer consisting of 200 mM sodium acetate, 20 mM L-cysteine-HCl, and 10 % glycerol, pH 4.0. The catK protein (~3.0 mg/ml) in activation buffer was incubated at 4ºC and aliquots were removed every 30 or 60 min. The extent of activation was assessed by SDS-PAGE. For large-scale isolation of recombinant mature cathepsin K, the in vitro activation reaction was carried over 3 h and buffer was exchanged to TALON binding buffer. The tagged mature catK present in the activation assay mixture was purified by TALON metal affinity chromatography as described above. The catK activity was determined using a fluorogenic substrate in a microtiter plate format. The reactions consisted of 100-200 nM of catK, 5 µM Z-Phe-Arg-R110, in 50 mM sodium acetate, pH 5.5, 4 mM dithiothreitol, 1 mM EDTA, and 0.1% CHAPS. Reactions were initiated by the addition of substrate in DMSO to the enzyme sample. The assay followed Z-Phe-Arg-R110 hydrolysis over time (velocity) using the FluorImager 575 (Molecular Dynamics, Sunnyvale, CA).

**N-terminal Amino Acid Sequence Analysis:** For amino acid sequence analysis, the ~42-kDa or ~29-kDa rcatK were isolated from the SDS-PAGE gel and digested with 1.5µg of trypsin and peptides were analyzed by nanospray MS/MS at the Baylor College of Medicine-Protein Chemistry Core Laboratory (Houston, TX).

**CatK Inhibition Assay by Headpin, E64, and Leupeptin:** Assays were performed in a volume of 100 µl in low-binding microtiter plates (Costar 9017, Costar, Cambridge, MA). CatK inhibition by headpin, E64, and leupeptin was assayed by mixing catK with inhibitors and incubating for 20 min at room temp. Residual enzyme activity was measured by adding Z-Phe-Arg-R110 and reading its hydrolysis over time (velocity) using the FluorImager 575 (Molecular Dynamics, Sunnyvale, CA)

## Results and Discussion

**Optimization of human CatK expression.** High Five cells infected with catK composite virus exhibited very strong staining with catK mAb and no staining with control antibody (Figure 1A). To follow the time course of expression and to determine the optimum time to harvest the recombinant catK, daily aliquots of the expression medium and cells were removed beginning with the second day of expression until the time of harvesting. Extracellular and intracellular catK expression was determined by immunoblot analysis (Figure 1B). Preliminary Western blot analyses using cell lysates (50 to 100 µg/well) have shown that penta-His mAb is more sensitive than the catK mAb in reacting to the recombinant cat K bands. We used penta-His mAb in the initial optimization experiments (Figure 1B). The medium of the high five/catK infected cells contained no protein signal detectable by immunoblot with penta-His mAb at 48 h, 72 h, or 96 h post infection. (Figure 1B, lanes 3, 5, and 7). However, with long expression times at 144 h, the medium of the high five/catK infected cells contained a very weak immunoreactive band of ~42 (Figure 1B, lane 10). In contrast, the cytoplasmic extract of the high five/catK infected cells contained two immunoreactive bands of ~42 (very strong) and ~29 kDa (very weak) (Figure 1B, lanes 2, 4, and 6). The high molecular weight protein increased with longer expression (Figure 1B, lanes 2 vs 6). We presume that the immunoreactive bands of ~42 (very strong) and ~29 kDa (very weak) correspond to pro-cathepsin K and mature-cathepsin K, respectively, as previously reported for human untagged cathepsin K in Sf9 or Sf21 cells (28, 29). Finally, the medium and extract of the high five/mock cells harvested after 48 h, 72 h, or 96 h (96 h shown) contained no protein signal detectable by immunoblot with penta-His mAb (Figure 1B, lanes 8 and 9) suggesting that the penta-His mAb is very specific in reacting with His-tagged cathepsin K. These observations further suggest that maximal expression of catK reached at about 96 h and most of the total cathepsin K expressed by the high five cells was in the pro-form and retained in the cell pellet. Routinely, the proteins were harvested after 96 h infection. These observations are consistent with the previoulsy reported results for human catK expression in Sf9 and Sf21 insect cells (28, 29).

**Purification of recombinant CatK by TALON metal affinity chromatography.** As we mentioned in the "Introduction", the untagged human catK expressed in these cells was previously purified by more than two chromatographic steps (28-30). Since the recombinant catK we engineered in the present studies was tagged with six histidines at its C-terminus, we sought to purify this enzyme using a one-step TALON metal chelating affinity chromatography method. The results obtained from a typical purification scheme are shown in Figure 2A. To demonstrate purity of the isolated pro-catK, samples were evaluated on 12% SDS-PAGE stained with Coomassie blue (Figure 2A). The elution profiles obtained from a typical purification procedure are shown in Figure 2B. The imidazole eluates migrated as a prominent band of 43 kDa. (Figure 2A, lanes 5, 6, 7, and 8).

**Immunoblot of CatK during TALON purification**. The identity of TALON-affinity purified cathepsin K was confirmed by Western blotting and detection with a cathepsin k and penta-His specific mAbs (Figigure 3A and 3B). Both mAbs reacted with the 43 kDa protein despite that the catK mAb is less sensitive ((Figure 3A) than penta-His mAb (Figure 3B). Based on the predicted size of the pro-cathepsin K protein, its ability to react with human catK and penta-his mAbs, the 43-kDa protein was identified as pro-cathepsin K. In some preparations (see below) we observed a 29 kDa protein co-purifing with the 43-kDa protein. However, this band reacted faintly in Western with both catk and penta-his mAbs, suggesting that some of this protein band contains mature catK.

In a typical purification scheme, a 200 ml cell pellet contained 268 mg of soluble protein by protein assay with an albumin standard (Table 1). Using this one step metal affinity purification chromatography, about 3.0 mg of purified pro-catK was recovered from the soluble protein (Table 1). Since eluate one contained a higher molecular weight protein contaminant in addition to the prominent pro-cathepsin K band, it was excluded from pooled eluates 2 through 8. Moreover, no attempts to further purify this protein was made since one-step affinity purification yielded greater than 90% pure pro-cathepsin K. In addition, the following phenomena were observed. (1) Internal amino acid sequence of this protein confirmed the molecular identity of this protein to be cathepsin K. (2) The pro-cathepsin K protein in eluate buffer was stable at

4ºC. (3) When the pro-cathepsin K was concentrated and exchanged against activation buffer no pellet or degradation was found in the above process.

**In Vitro CatK activation and purification**. In initial experiments, no cathepsin K activity was detected in infected High Five cells using fluorogenic peptide. Similarly, no activity was detected in TALON-affinity purified cathepsin K sample when analyzed with the fluorogenic peptide (Table 1). It was concluded that High Five cells were not efficient in converting the proform to mature form and in vitro activation of one step purified cathepsin K would be required in demonstration of catK hydrolysis of the fluorogenic peptide. Therefore, eluates containing pure pro-catK were pooled (2.5 mg/ml), concentrated, and resuspended in activation buffer (2.0 mg/ml). The treatment of pro-cathepsin K at pH 4.5 lead to its generation of the approximate size about 29 kDa is shown in Figure 4A. In this particular experiment, there is also substantial amount of a lower molecular size of about 29 kDa, corresponding to the size of mature catK was present in the purified procatK during the in vitro activation. Upon incubation at 4 ºC for 30 min, most of the pro-cathepsin K was converted to the mature cathepsin size of 29 kDa (Figure 4A, lane 2). By 2 to 3 h of incubation, almost all pro-cathepsin K was activated to the mature cathepsin K (Figure 4A, lanes 4 and 5). Western blot analysis of a sample incubated at 4 ºC for 3 h gave a homogeneous protein of 29 kDa, which co-migrated with the commercially obtained mature-catK and reacted strongly with the catK mAb (Figure 4B, lane 1). Finally, this sample (2.0 mg/ml) was passed through a small TALON-affinity column and the mature cathepsin K was eluted with imidazole buffer with a yield of about 0.5 mg/ml. N-terminal analysis of the 29-kDa protein demonstrated the presence of one amino-terminal sequences, GRAPDSVDYRKK, confirming the molecular identity of the 29-kDa protein to be that of mature catK.

**Recombinant mature CatK is active and rheadpin inhibits its activity**. Cathepsin K is a cysteine protease with substrate $S_2P_2$ specificity of a positively charged residue such as arginine in $P_1$ and a large hydrophobic residue in $P_2$. We assayed the in vitro activity of H5 cells purified and activated rCatK activity using a fluorogenic peptide substrate in the presence and absence of different protease inhibitors including r-headpin protein (Table 2). Our results demonstrated that

the ~29 kDa recombinant mature-cathepsin K efficiently hydrolyzed the fluorogenic peptide substrate and r-headpin, a serine protease inhibitor down-regulated in head and neck cancer cell lines and tumor tissues, potently inhibited the r-catK activity *in vitro*.

**Conclusions**

Hexahistidine-tagged human cathepsin K was successfully produced in an insect cell/baculo-virus expression system. This required fusing a six-histidine sequence in frame with the carboxyl-terminus methionine of mature cathepsin K. Through a single purification step using a $Co^{2+}$ column, 0.5 mg of the activated mature cathepsin K was obtained from 200 ml cell lysate. There was no detectable effect of tagging histidines on the enzymatic properties of the recombinant cathepsin K. The ability to produce milligram quantities of protein in small culture, using a one-step purification scheme, makes this system useful for expressing native human cathepsin K and perhaps for expression of other proteases in general.

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**Table 1.** Purification of Hexahistidine-Tagged Human Cathepsin K from High Five Cell Lysate

| Purification step | Volume (ml) | Total Protein(mg) | Specific activity (fluorescence units/µg) |
|---|---|---|---|
| Total cell lysate | 18 | 298 | ND |
| Soluble | 17.5 | 268 | ND |
| Flow-through | 18 | 207 | ND |
| TALON (pooled eluates) | 1 | 2.0 | 0 |
| TALON (post in vitro activation) | 0.5 | 0.8 | 4500 |

Note: Specific activity was determined as described under Materials and Methods and is expressed in fluorescence units per microgram of protein per min. ND, not determined.

**Table 2. r-Headpin inhibits r-Mature cat K *in vitro***

| Assay conditions | RFU/min/0.1µg mature catK |
|---|---|
| Z-Phe-Arg-R110 (5 µM) | 6,500 |
| +r-Headpin (500 nM) | 100 |
| +E-64 (0.2 µM) | 50 |
| +Pepstain A (0.5 µM) | 6,200 |

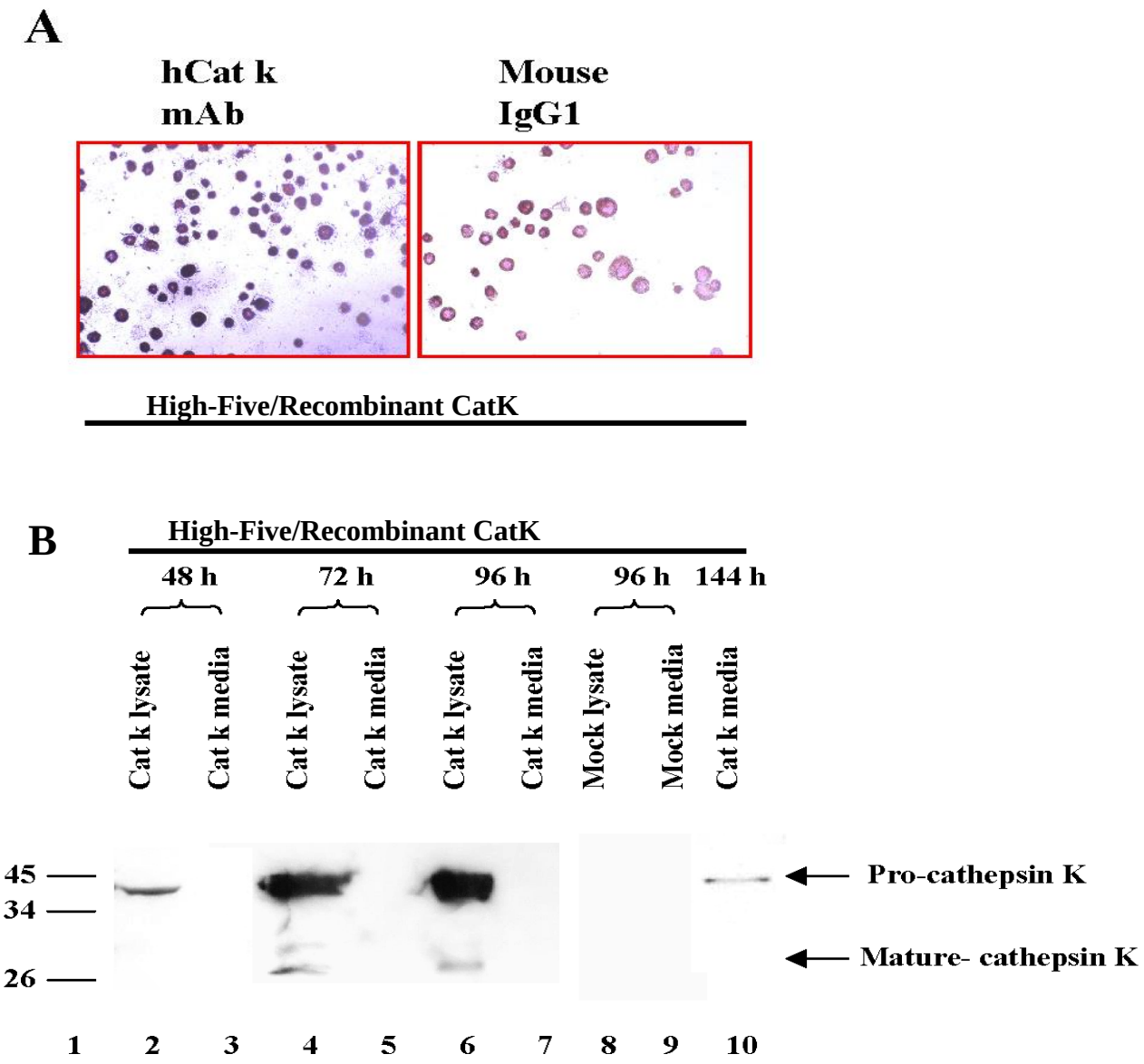


**Figure 1: Optimization of CatK expression in High-five cells.** (A) Immunocytochemical analysis of CatK expression. Mock and cathepsin K expressing (2.5 MOI) high five cells were grown in chamber slides. 96 h post infection, cells were stained with catK mAb or mouse IgG1, ×200). (B) Time course of cathepsin K expression analyzed by immunoblot. CatK was expressed by baculovirus-infected (2.5 MOI) high five cells. 30-µl aliquots of medium or 50 µg of the cell lysate were mixed with 10 µl of 4 × SDS sample buffer and heated at 100ºC for 5 min. The samples were separated by SDS-PAGE, transferred to a membrane and stained with penta-His mAb. The numbers above each lane are the hours after infection. The molecular size of the markers are given to the left of the blot. The arrow marks the position of the recombinant pro-cathepsin K and mature cathepsin K.

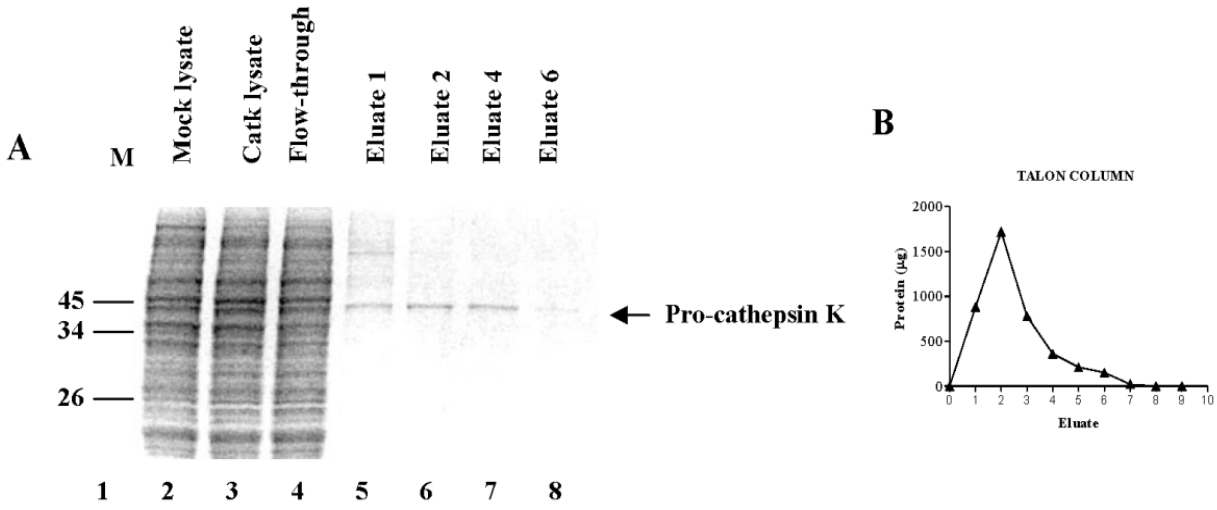


**Figure 2**: **Purification of recombinant CatK by TALON metal affinity chromatography**. CatK was purified from 200 ml cell lysate as described under "Materials and Methods" using 0.6 ml TALON Superflow resin. The arrow marks the position of the recombinant pro-cathepsin K. (A) SDS-PAGE analysis. lane 1, molecular size markers; lane 2, mock extract (58 μg); lane 3, Cat K extract (58 μg); lane 4, CatK extract TALON flow-through (50 μg); lane 5, eluate 1 (2.0 μg); lane 6, eluate 2, (1.0 μg); lane 7, eluate 4 (1.0 μg); lane 8 eluate 6 (0.2 μg). (B) Elution profiles. Total yield was 2.9 mg.

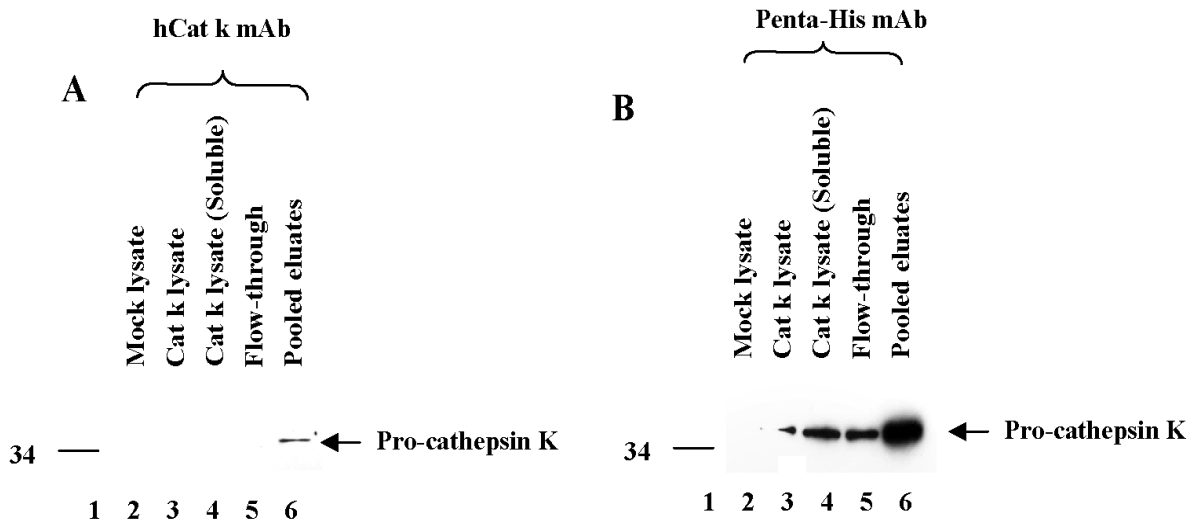


**Figure 3: Immunoblot of CatK during TALON purification.** Fractions were analyzed in 12% SDS-PAGE and the gel was transferred to a membrane and stained with human catK mAb (A) or penta-His mAb (B). Lane 1, molecular size markers; lane 2, mock extract (total, 58 μg); lane 3, Cat K extract (total, 58 μg), lane 4, CatK extract (soluble, 50 μg); lane 5, CatK extract TALON flow-through (50 μg); lane 6, pooled eluates (2 through 6, 0.5 μg).

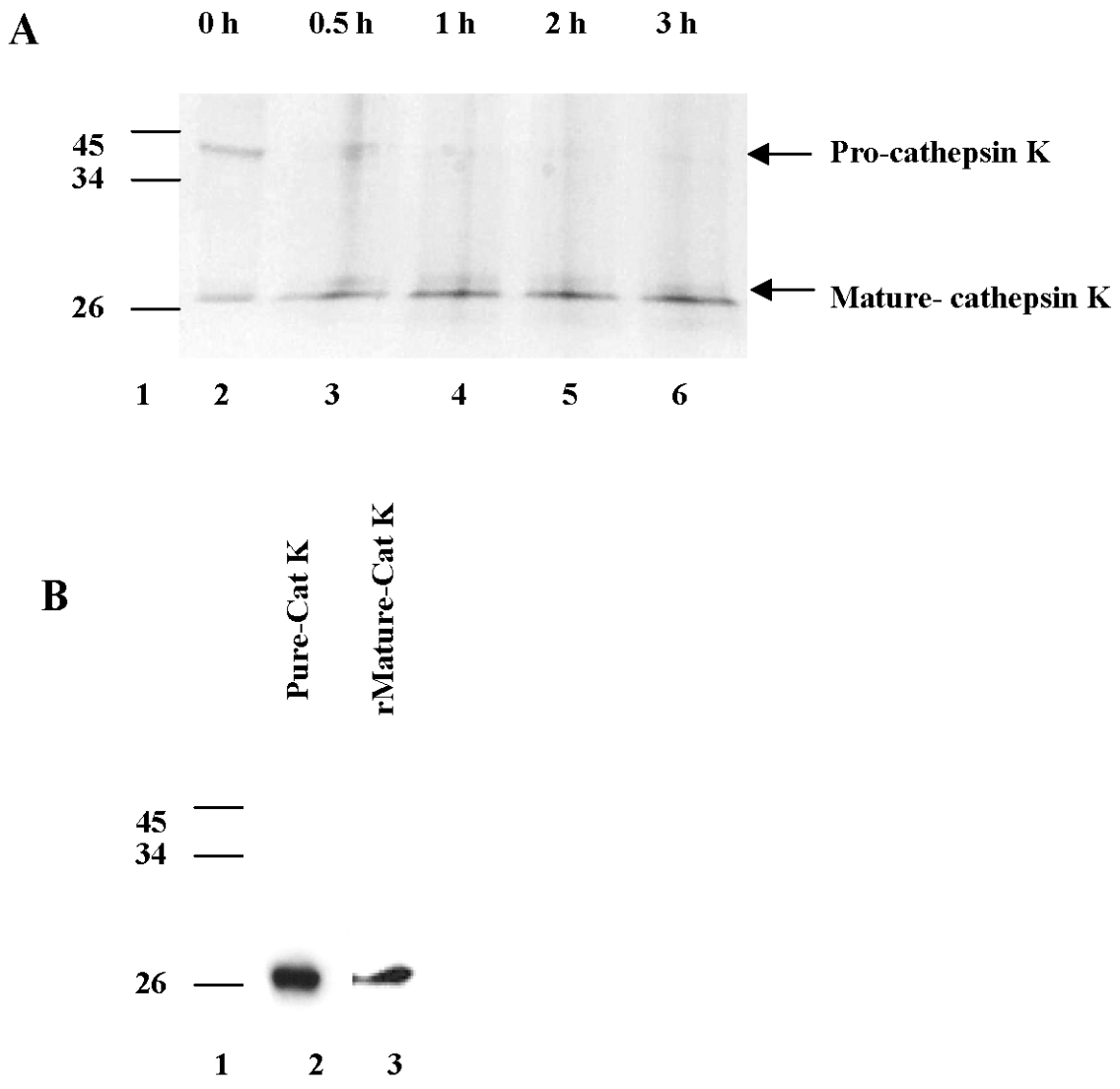


**Figure 4: Recombinanat pro-CatK activation at 4ºC.** CatK was purified from 200 ml cell lysate and using 0.6 ml TALON Superflow resin as described under "Materials and Methods". Pro-cathepsin K (~3.0 mg/ml) in activation buffer was incubated at 4 ºC. The numbers above each lane are the hours of activation. (A) Aliquots were analyzed by 12% SDS-PAGE and stained with Coomassie Brilliant Blue R-250. Lane 1, molecular size markers; lane 2, TALON purified pro-cathepsin K (1.0 µg); lanes 2-6, samples of pro-cathepsin K (0.5 µg ) after incubation at pH 4.0, 4 ºC for 0.5, 1, 2, 3, and 4, respectively. (B) Western blot analysis of in vitro activated, and TALON purified mature catK with catK mAb. Lane 1, molecular size markers; lane 2, pure mature catK (0.4 µg); lane 3, recombinant mature catK (0.6 µg).

## Authors Column

**Arumugam Jayakumar** received his Ph.D. in membrane biochemistry from JNU, New Delhi, India. He moved to US in 1980 and spent 2 years at NIH, 18 years at Baylor College of Medicine and currently working as a research scientist at M.D. Anderson Cancer Center since 2000. He authored or co-authored 65 peer-reviewed publications and written 2 book chapters. He has co-built and co-managed an extensive research portfolio funded by NSF/NCI/NIH/HGC, American Academy of Otolaryngology-head and neck surgery, Welch Foundation and Viragh Foundation. He has trained/mentored 30 pre/post-doctoral trainees/junior faculty/clinical fellows/summer students/technicians. He is a co- inventor for 1 issued patent and 2 licensing agreement. He serves as an editorial member for Web Med Central plus, Asian Journal of Medical Sciences, J Cancer Metastasis Treat, and MOJ Proteomics & Bioinformatics.

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