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Subcloning and Expression of Functional Human Cathepsin B and K in *E. coli*: Characterization and Inhibition by Flavonoids

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1. Introduction

1.1 Cathepsins

Cathepsins, originally identified as lysosomal proteases, play a fundamental role in intracellular protein turnover in lysosomes. However, several cathepsins and variants of cathepsins can also be found on the cell membrane, in the cytosol, nucleus, mitochondria, and extracellular space. These cathepsins are involved in a variety of important physiological and pathological processes [reviewed in: (Brix et al., 2008; Frlan and Gobec, 2006; Lutgens et al., 2007; Mohamed and Sloane, 2006; Nomura and Katunuma, 2005; Obermajer et al., 2008; Reiser et al., 2010; Stoka et al., 2005; Turk et al., 2001; Vasiljeva et al., 2007; Victor and Sloane, 2007)]. Cathepsins are classified mechanistically into groups which include serine (cathepsins A and G), aspartic (cathepsins D and E), and cysteine cathepsins (cathepsins B, C, F, H, L, K, O, S, V, W, and X). This classification is based on the nucleophilic residues present on their active sites responsible for proteolytic cleavage (Rawlings et al., 2006; Turk et al., 2001).

Cathepsins are synthesized as zymogens composed of a signal peptide, a propeptide, and mature protein of distinct length and substrate specificity for individual cathepsins (Rawlings et al., 2006). The signal peptide is cleaved in the Endoplasmic Reticulum and the pro-protein is activated by proteolytic removal of the N-terminal pro-peptide either by autocatalysis in acidic environments, or by other proteases. The pro-peptide region of the cathepsin plays multiple roles. It can act as an inhibitor to block access to the active site that regulates cathepsin activity. In addition the propeptide can act as an intramolecular chaperone that assists in protein folding, or as a trafficking signal that targets the protein to its destination (Turk et al., 2002). Cathepsins exhibit a broad range of functions and tissue expression (Brix et al., 2008; Turk et al., 2001). Some of the cathepsins are ubiquitously expressed and others are tissue or cell-type specific.

Cathepsins have been shown to be involved in the process of tumor invasion and metastasis (Białas and Kafarski, 2009; Lindeman et al., 2004; Nomura and Katunuma, 2005; Obermajer

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et al., 2008) and have been linked to many types of cancer including melanoma (Matarrese et al., 2010; Quintanilla-Dieck et al., 2008), hepatocellular carcinoma (Leto et al., 1997; Leto et al., 1996), breast cancer (Foekens et al., 1998; Laurent-Matha et al., 1998; Masson et al., 2011; Vashishta et al., 2007), lung cancer (Ledakis et al., 1996; Schweiger et al., 2000), prostate cancer (Brubaker et al., 2003; Kishore Kumar et al., 2010; Podgorski et al., 2009; Podgorski et al., 2007; Steffan et al., 2010), nasopharyngeal cancer (Cheng et al., 2008; Xu et al., 2009), thyroid cancer (Mikosch et al., 2008; Tedelind et al., 2010), bone cancer (Podgorski et al., 2009; Podgorski et al., 2007) as well as osteoporosis (Bone et al., 2010; Deal, 2009; Stoch and Wagner, 2007; Yasuda et al., 2005), rheumatoid arthritis (Skoumal et al., 2005; Skoumal et al., 2008), Alzheimer's Disease (Hook et al., 2007; Hook et al., 2009; Urbanelli et al., 2008), cardiovascular disease (Bengtsson et al., 2008; Lutgens et al., 2007), and obesity (Li et al., 2010; Naour et al., 2010; Podgorski et al., 2007; Yang et al., 2008).

1.2 Cysteine cathepsins

Cysteine cathepsins belong to papain-like enzyme family sharing similar amino acid sequences and tertiary structures (Turk et al., 2001). Eleven cysteine cathepsins B, C, F, H, L, K, O, S, V, W, and X have been identified in the human genome (Turk et al., 2001). Cysteine cathepsins have been documented to play a vital role in a variety of biological processes and pathological processes (Brix et al., 2008; Joyce et al., 2004; Lecaille et al., 2002; Obermajer et al., 2008; Reiser et al., 2010; Stoka et al., 2005; Turk et al., 2001; Victor and Sloane, 2007). The correct sorting and trafficking of the members of cysteine cathepsins are critical in their proteolytic actions to maintain homeostasis (Brix et al., 2008). The dysregulation of protease activity has resulted in numerous pathologies. Several cysteine cathepsins have been recognized as relevant drug targets in the development of many disease therapies (Deal, 2009; Le Gall et al., 2008; Mohamed and Sloane, 2006; Podgorski, 2009; Turk, 2006; Vasiljeva et al., 2007).

1.2.1 Cathepsin B and K

Human cathepsin B precursor is a protein of 339 amino acids that consists of a signal peptide at amino-terminal end of 17 amino acids (1-17). The proprotein is then activated as a single-chain form of 254 amino acids (80-333) or double-chain form of 47 amino acids (80-126) and 205 amino acids (129-333) (Pungerčar et al., 2009; Rozman et al., 1999). Unlike other cysteine proteases cathepsin B is unique due to its ability to act both as an endopeptidase and a peptidyl dipeptidase. It contains a unique occluding loop which is characterized by two adjacent histidine residues (His 110 and His 111) and are responsible for the dipeptidyl carboxypeptidase activity (Illy et al., 1997). Cathepsin B is the most abundant in all of the cysteine proteases (Kirschke et al., 1995).

Cathepsin B participates in many diverse cellular processes including protein degradation, antigen processing (Zhang et al., 2000), and apoptosis (Bien et al., 2010; Chwieralski et al., 2006; Roberts et al., 1999). It has been implicated in a variety of diseases including cancer invasion and metastasis (Ledakis et al., 1996; Matarrese et al., 2010; Roshy et al., 2003; Sinha et al., 2001; Szpadarska and Frankfater, 2001; Yan et al., 1998), angiogenesis (Im et al., 2005; Kruszewski et al., 2004; Malla et al., 2011; Sinha et al., 1995), inflammation (Hashimoto et al., 2001; Kakegawa et al., 2004), and Alzheimer's Disease (Gan et al., 2004; Hook, 2006; Hook et al., 2008). Thus, cathepsin B is a promising target for anti-cancer drug design (Lim et al., 2004; Palermo and Joyce, 2008) and a potential target for Alzheimer's Disease (Hook et al., 2008).

Human cathepsin K precursor is a protein of 329 amino acids that consists of an amino-terminal signal peptide of 15 amino acids (1-15), a propeptide of 99 amino acids (16-114), and a catalytic region of 215 amino acids (115-329) (Lecaille et al., 2008).

Cathepsin K is highly expressed in osteoclasts (Drake et al., 1996) but also occurs in lung epithelia cells (Bühling et al., 1999), cultured primary neonatal skin fibroblasts activated chondrocytes, and in synovial fibroblasts of patients suffering from rheumatoid arthritis (Lecaille et al., 2008; Ruettinger et al., 2008; Skoumal et al., 2005). Because of its strong collagenolytic activity cathepsin K has been described as the major enzyme responsible for the degradation of organic bone matrix, and is believed to play a fundamental role in bone resorption (Gowen et al., 1999; Saftig et al., 1998; Salminen-Mankonen et al., 2007; Stoch and Wagner, 2007). Cathepsin K is also involved in lung matrix homeostasis (Bühling et al., 2004), dermal extracellular matrix homeostasis (Rünger et al., 2007), and atherosclerotic plaque remodeling (Guo et al., 2009). Circulating serum cathepsin K has been found to play a significant role in both prostate cancer and breast cancer related bone metastasis (Tomita et al., 2008; Valta et al., 2008).

Cathepsin K has become an established drug target for osteoporosis (Deal, 2009; Stoch and Wagner, 2007; Vasiljeva et al., 2007). Two cathepsin K inhibitors have progressed to Phase II and Phase III clinical trials for osteoporosis. Ono's ONO-5334 (Eastell et al., 2011; Manako, 2011) is in Phase II while Merck's odanacatib (Pérez-Castrillón et al., 2010) is in Phase III clinical trials.

Cathepsin K has also been found to play roles in atherosclerosis (Guo et al., 2009), inflammation (Asagiri et al., 2008; Lecaille et al., 2008), and obesity (Podgorski et al., 2007; Yang et al., 2008). These findings suggested that cathepsin K may be one of the common biological links connecting low bone density to cardiovascular disease (Lutgens et al., 2007; Podgorski, 2009; Podgorski et al., 2007). Thus pointing to possible future anti-cathepsin K drug applications toward dual therapy for skeletal disease and atherosclerosis (Podgorski, 2009)

1.3 Research objectives

Specific proteinase inhibitors are useful in investigations of the mechanisms and pathways of intracellular protein degradation and could lead to the development of therapeutic agents for treatment of many types of carcinomas, skeletal disease and atherosclerosis, as well as Alzheimer's Disease. In the present communication, we report the successful production of functional human recombinant cathepsin B and K. The active enzymes have been used successfully in screening flavonoids for effective inhibitors against human cathepsins B and K enzymes.

2. Experimental

2.1 Materials

E. coli strains JM109 and BL21(DE3)pLysS were used as host cells. The pET-15b was used for the expression vector. Antibiotics ampicillin and chloramphenicol were purchased from Sigma and Fisher Scientific, respectively. Yeast extract and bactotryptone were from BD Biosciences. Isopropyl thio- β -galactoside (IPTG) was from Calbiochem. FidelityTM PCR master mix was from United States Biochemical Corp and PCR master mix (2x) from Promega corporation. XhoI, alkaline phosphatase, and T4 DNA ligase were from New

England Biolabs. HisTrap FF column was from GE Healthcare. Z-Phe-Arg-pNA (Carbobenzoxycarbonyl-L-Phenylalanyl-L-Arginine para-nitroanilide) was from Enzo Life Sciences. Oligonucleotides were synthesized by Integrated DNA Technology. Glutathione (reduced) and GSSG (oxidized) were from Sigma-Aldrich. Precision Plus Protein Kaleidoscope Standards were from Bio-Rad Laboratories.

2.2 Subcloning of recombinant human procathepsin B and K

Full-length cDNA encoding of the human pre-pro-cathepsin B (GenBank accession number BC095408) and the human pre-pro-cathepsin K (GenBank accession number BC016058) were purchased from Open Biosystems. Plasmid DNA isolated from each clone was used as templates for amplification of coding region of procathepsin B and K (excluding signal peptide) using gene-specific primer pairs (see Table 1). Each primer was appended with XhoI restriction enzyme recognition site (underlined) to facilitate cloning. The XhoI restriction site was chosen because both cathepsin genes lack XhoI recognition site. The extra six nucleotides (selected at random) at the 5' end of each primer were to facilitate cleavage by restriction enzymes.

	Primers
ProCatB forward primer	ATA TAA <u>CTC GAG</u> CGG AGC AGG CCC TCT TTC C
ProCatB reverse primer	ATA TAA <u>CTC GAG</u> TTA GAT CTT TTC CCA GTA CTG ATC GGT G
ProCatK forward primer	ATG CGA <u>CTC GAG</u> CTG TAC CCT GAG GAG ATA CTG G
ProCatK reverse primer	ATG CGA <u>CTC GAG</u> TCA CAT CTT GGG GAA GCT GG

Table 1. Synthetic primers for amplification of coding sequences of procathepsin B and K.

Each PCR solution (50 µl) consisted of 25 µl Fidelity™ PCR Master Mix (2x), 1 µM of each forward and reverse primer, and 10 ng plasmid template. The reaction was pre-denatured at 94 °C for 5 min. Then 25 cycles were conducted, which consisted of denaturation at 94 °C for 45 sec, annealing at 55±10 °C for 45 sec, and extension at 68 °C for 1.5 min. A final extension was done at 68 °C for 10 min. The cycling process was accomplished by the Eppendorf Mastercycler. The PCR amplified DNA fragments were extracted once with equal volume of phenol/chloroform (1/1), once with chloroform/isoamyl alcohol (24/1), and DNA precipitated with ethanol (Sambrook and Russell, 2001). Each PCR fragments of procathepsin B or K was digested with XhoI to generate sticky ends and the digested products were separated on a 1.2% agarose gel. The desired bands were purified from agarose gel slice (Kim, 1992) and ligated to pET-15b which had been treated with XhoI and alkaline phosphatase. Each ligation reaction was then transformed into JM109 competent cells according to the method of Chung et al. (Chung et al., 1989) and plated on ampicillin containing plates. The transformants were screened to find recombinant DNA containing colonies by Quick Screening (Huang et al., 2004). In Quick Screening, a toothpick was used to isolate a colony and the cells were resuspended in 25 µl of STE solution (100 mM NaCl, 20 mM Tris-Cl pH 7.5, and 10 mM EDTA). The suspension was extracted with phenol/chloroform and the aqueous layer was analyzed on a 1% agarose gel. The chimeric DNA (with DNA insert) migrates slower than the vector alone. The selected chimeric plasmids were isolated using boiling method (Sambrook and Russell, 2001).

The orientation of the insert in the chimeric plasmids was checked by PCR using the pET 5' sequencing primer and the gene specific reverse primer. Promega's 2x PCR master mix was used because proofreading was not required in this case. PCR products are expected only for clones harboring an insert in the correct orientation. The correct recombinant plasmids (confirmed by DNA sequencing) were each transformed into BL21(DE3)pLysS host cells for protein expression.

2.3 Overexpression of recombinant human procathepsin B and K

The chosen transformants of BL21(DE3)pLysS harbored the chimeric plasmid (procathepsin B or K) in the correct orientation and reading frame was induced for protein expression in the presence of IPTG. Several growth conditions (varying growth media, induction temperatures, induction time, and inducer concentration) were tested and the one yielded the best results is described below. Each clone was grown in 10 mL terrific broth (1.2 % bacto-tryptone, 2.4 % bacto-yeast extract, 0.4 % glycerol, 0.017 M KH_2PO_4 and 0.072 M K_2HPO_4) containing 100 $\mu\text{g}/\text{ml}$ ampicillin and 20 $\mu\text{g}/\text{ml}$ chloramphenicol overnight on a shaker at 37 °C. Next day, the culture was diluted 1:20 in terrific broth containing ampicillin and chloramphenicol in a baffled Erlenmeyer flask. The culture was grown until A_{600} reached 0.8–1.2. At this point IPTG inducer was added to a final concentration of 0.5 mM along with 25% fresh terrific broth. The incubation temperature was dropped to 25 °C and cells were harvested 4–16 hours after IPTG induction. Overexpression of the target protein was checked by comparing total protein patterns before and after IPTG induction by SDS-PAGE.

2.4 Purification of recombinant procathepsin B

Recombinant procathepsin B was obtained from purified inclusion bodies of IPTG induced cells by a procedure described by Kuhej, et al, 1995 with minor modifications. The IPTG induced cell pellet (4.6 g from 500 mL culture) was re-suspended in 46 mL of 50 mM Tris-HCl, pH 8.0 containing 5 mM EDTA and 5% sucrose, and was sonicated. The homogenate was divided into two centrifuge tubes and centrifuged at 6,000 \times g for 10 min. The pellets containing inclusion body were washed twice by homogenizing each in 15 mL of 50 mM Tris-HCl, pH 8.0 containing 5 mM EDTA and 0.1% Triton X-100 using a generator sawtooth followed by centrifugation at 6,000 \times g for 10 min at 4 °C. Each inclusion body pellet was consecutively washed twice with 10–15 mL of 50 mM Tris-HCl, pH 8.0 containing 5 mM EDTA and 2 M Urea. The content was centrifuged as before. The purified inclusion bodies were solubilized in approximately 10 mL of 8 M Urea/0.1 M Tris-HCl, pH 8.0/10 mM DTT by stirring for 1.5 h at room temperature. The protein concentration was approximately 10.8 mg/mL.

The procathepsin B in the solubilized inclusion body was refolded and re-oxidized by dilution and dialysis according to a reported procedure (Kuhelj et al., 1995) with minor modification. An aliquot of the solubilized inclusion body was diluted to approximately 30 $\mu\text{g}/\text{mL}$ with 8 M urea in 0.1 M Tris-HCl, pH 8.0 containing 10 mM DTT and dialyzed against 4 L of 0.1 M sodium phosphate pH 7.0 containing 5 mM EDTA and 5 mM L-Cysteine overnight. (It took about 2 hours of occasional stirring with a glass rod to diffuse some urea so that the dialysis tubing could float). Dialysis was then carried out for approximately 24 hours. Fresh buffer was changed once during dialysis. The dialyzed procathepsin B solution was collected and centrifuged at 10,000 \times g for 10 min to remove precipitated protein. The protein was stored at -70 °C until use. Protein purification progress and purity were checked by 12% SDS-PAGE.

2.5 Activation of the procathepsin B

Procathepsin B was autoactivated to its mature cathepsin B form by lowering the pH to 3.6 (Kuhelj et al., 1995) with 1 M formic acid. The reaction was terminated by increasing the pH to 6 with addition of 1 M sodium phosphate pH 6.0. The autoactivation was carried out at 4 °C for various time intervals. The successful activation/cleavage was monitored by cathepsin B activity assay and 15% SDS-PAGE analysis.

2.6 Activity assay of cathepsin B

Cathepsin B was assayed using chromogenic substrate, Z-Phe-Arg-pNA. The reaction consisted of 20 mM sodium acetate, pH 5.0, 1 mM EDTA, 5 mM L-Cysteine, 0.9-1.8 µg/mL cathepsin B, and 0.015-0.6 mM Z-Phe-Arg-pNA in a total volume of 500 µl. The release of the para-nitroaniline (pNA) chromophore was monitored at 405 nm at 25 °C for 3 min, and the reaction rate was calculated from the slope of the trace showing the increase in absorbance over time.

2.7 Purification of recombinant procathepsin K

The recombinant procathepsin K was purified from IPTG induced *E. coli* cells according to a procedure reported by Hwang and Chung (Hwang and Chung, 2002) with slight modifications. Briefly, the cell pellet (from 250 mL culture) was sonicated in 25 mL of buffer A (50 mM Tris-HCl, pH 8.0, 0.5 M NaCl). The homogenate was centrifuged at 10,000 g for 20 minutes at 4 °C. The pellet (containing inclusion body) was washed in buffer A plus 2 M urea. The content was centrifuged at 10,000 g for 20 minutes at 4 °C. The pellet was re-suspended in 12.5 mL of buffer B (50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 6 M guanidine hydrochloride, 5 mM imidazole) to solubilize the inclusion body. The suspension was homogenized using a generator sawtooth and stirred at 4 °C for 1 hour. This was centrifuged at 10,000 g for 30 minutes. The supernatant was filtered using a 0.8 µm syringe filter. The filtrate (approximately 12 mL) was loaded onto a Ni column (5 mL HisTrap™ FF from GE Healthcare) that had been equilibrated with buffer B. The column was washed with buffer B until A₂₈₀ reached baseline. The bound protein was eluted with buffer B plus 1 M imidazole and ten 1.3 mL fractions were collected. Protein assay was performed using Bio-Rad protein binding assay. Fractions #4 and #5 which contained the most protein were used in refolding. The purified procathepsin K was observed on 12 % SDS-PAGE.

2.8 Refolding and activation of recombinant procathepsin K

The refolding of procathepsin K was performed by dilution and dialysis (Hwang and Chung, 2002). The eluted protein (2 mL) in guanidine-HCl was diluted in 100 mL of refolding buffer A (5 mM EDTA, 10 mM GSH, 1 mM GSSG, 0.7 M L-Arg, 1 % CHAPS). The dilution was achieved by adding the denatured protein drop by drop at 1 mL/minute into the refolding buffer followed by stirring overnight at 4 °C. The content was dialyzed (MWCO 8,000 - 14,000) against 4 L of 25 mM Tris-HCl, pH 8.0, 0.5 M NaCl overnight at 4 °C. Fresh buffer was changed once during dialysis. Dialyzed protein was clarified by centrifugation and then concentrated using Vivaspin 20 with MWCO 10,000. The volume was reduced to one third of the original volume. The procathepsin K protein was then activated in 0.2 M sodium acetate, pH 4.0, 5 mM DTT, 5 mM EDTA, and porcine pepsin (20 µg/mL). The progress of activation was monitored by activity assay and 15% SDS-PAGE. Activation was terminated by raising the pH to 5.5 with addition of sodium acetate (pH 5.5) to 0.1 M as pepsin is inactive at this pH (Tauber and Kleiner, 1934).

2.9 Cathepsin K activity assay

Cathepsin K was assayed using chromogenic substrate, Z-Phe-Arg-pNA. The reaction mixture consisted of 100 mM sodium acetate buffer, pH 5.5, 2.5 mM EDTA, 2.5 mM DTT, various amounts of Z-Phe-Arg-pNA, and appropriate amount of mature cathepsin K in a total volume of 500 μ l using a procedure reported by Hwang and Chung (Hwang and Chung, 2002) with minor modifications. The release of the pNA chromophore was monitored at 405 nm at 25 °C for 3 min, and the reaction rate was calculated from the slope of the trace showing the increase in absorbance over time.

2.10 Inhibition of active cathepsin B and K with flavonoids

Amentoflavones have been shown to inhibit cathepsin B and K (Pan et al., 2005; Zeng et al., 2006). Therefore, several different commercially available flavonoids were utilized, including baicalin, amentoflavone, celastrol, fisetin, kaempferol, luteolin, rutin, limonin, myricetin, and apigenin. Each flavonoid was tested for its activity as cathepsin B or K inhibitors. The enzyme reaction was monitored for three minute intervals by spectrophotometric assay at 405 nm using the chromogenic substrate described above.

3. Results and discussion

3.1 Subcloning of human procathepsin B and K

The full-length human cDNA clone encoding cathepsin B purchased from Open Biosystems was in pBluescriptR vector and the cathepsin K cDNA was in pOTB7 vector. Each of the procathepsin B or K genes was amplified by PCR as described in the Experimental section. The PCR products were analyzed by 1% agarose gel (Figure 1). The reaction worked well at all three annealing temperatures (57.1 °C, 54.4 °C, 51.7 °C for procathepsin B and 54.4 °C, 51.7 °C, 49.3 °C for procathepsin K) as a strong band of about 900 bp was observed in every lane.

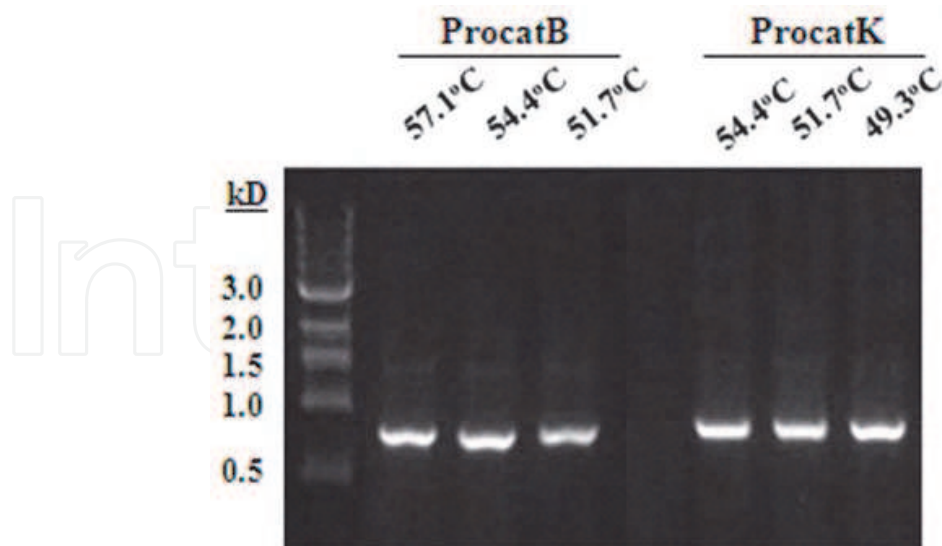


Fig. 1. Agarose gel electrophoresis of PCR products of procathepsin B and K DNA inserts. The plasmid pBluescriptR containing human cathepsin B cDNA was used as template and gene specific primer pair shown in Table 1 to amplify procathepsin B gene. Similarly, the plasmid pOTB7 containing human cathepsin K cDNA was used as template and its gene specific primer pair to amplified procathepsin K gene. PCR cycles were described in the Experimental section.

The XhoI-treated procathepsin B and K fragment from each gel was purified, inserted into the expression vector pET-15b, and transformed into JM109 competent cells. Transformants were treated with ampicillin to induce ampicillin resistance. All transformants were screened for the presence of a chimeric plasmid by the quick screening method as described in the Experimental. Since the cDNA was inserted into the vector at a single restriction site, XhoI, ligation of the insert is possible in either orientation. Only the chimeric plasmid that contains the procathepsin B or K cDNA insert in the correct orientation is useful. Therefore, several insert-containing clones were checked for the orientation of the insert by PCR. Plasmids isolated by boiling method from each selected colony were used as template in the PCR. When the insert is in the correct orientation, a PCR product of about 1050 bp appears with pET 5' primer and procat B or K reverse primer (Figure 2). This PCR product cannot be formed unless the insert is in the correct orientation. A positive PCR control was included using Forward and Reverse primer pair of procatB or K which yields about a 900 bp PCR product regardless of the insert orientation. Clones 3, 12 and 13 of procathepsin B candidates appeared to contain the DNA insert in the correct orientation, while clone 5 contained a deletion and clones 1 and 14 contained the DNA insert in the wrong orientation (Figure 2 left panel). Clones 6, 7, 11, 12, 14 and 15 of procathepsin K candidates appeared to contain the DNA insert in the correct orientation, while clones 8, 10 and 13 had the DNA insert in the wrong orientation (Figure 2 right panel).

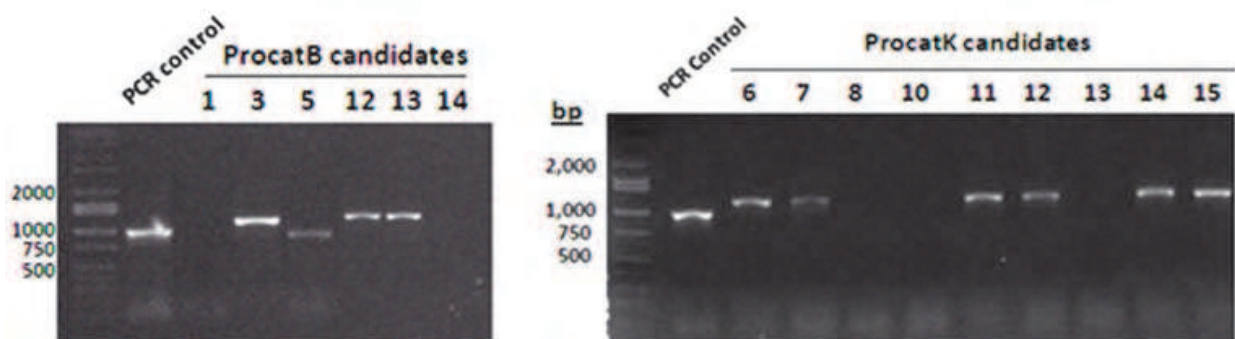


Fig. 2. Orientation check of putative pET-15b-procat B and K clones by PCR. Template used: isolated plasmids from clones #1, 3, 5, 12, 13, and 14 of procatB candidates; isolated plasmids from clones #6, 7, 8, 10, 11, 12, 13, 14, and 15 of procatK candidates. Primers used: for the PCR control, procatB or K forward and reverse primer pairs were used; for procatB candidate clones pET 5' primer and procatB reverse primer were used; for procatK candidate clones pET 5' primer and procatK reverse primer were used. Promega's 2x PCR mix was used. PCR cycles were described in the Experimental section.

3.2 Overexpression of recombinant procathepsin B and K

The chimeric plasmids with DNA inserts in the correct orientation and correct reading frame were transformed into BL21(DE3)pLysS for protein expression. Overexpression of the procathepsin B had been achieved according to the SDS-PAGE which showed overexpression of the proenzyme at about 35 kDa (Figure 3. left panel). Expression level of the recombinant procathepsin B after 4 h IPTG induction was greater than 40% of total bacterial protein and the procathepsin B level remained high after overnight induction. Overexpression of the recombinant procathepsin K was clearly visible after 4 h IPTG induction and the expression level increased with increasing induction time up to 22 hours

(Figure 3. Right panel). The recombinant procathepsin K showed an approximate molecular weight of 38 kD on SDS-PAGE gel.

3.3 Purification of recombinant procathepsin B and K

3.3.1 Purification of recombinant procathepsin B

The recombinant procathepsin B was expressed largely as insoluble inclusion bodies as very little procathepsin B was found in the soluble protein fraction (Figure 4. lane 3). Procathepsin B was purified from the inclusion bodies as described in the Experimental section. The progress of purification was monitored by SDS-PAGE (Figure 4). As shown in Figure. 4 lane 4, the inclusion bodies contained greater than 90% of procathepsin B. Overall yield of the procathepsin B enzyme was about 1.5 mg per 100 ml of bacterial culture.

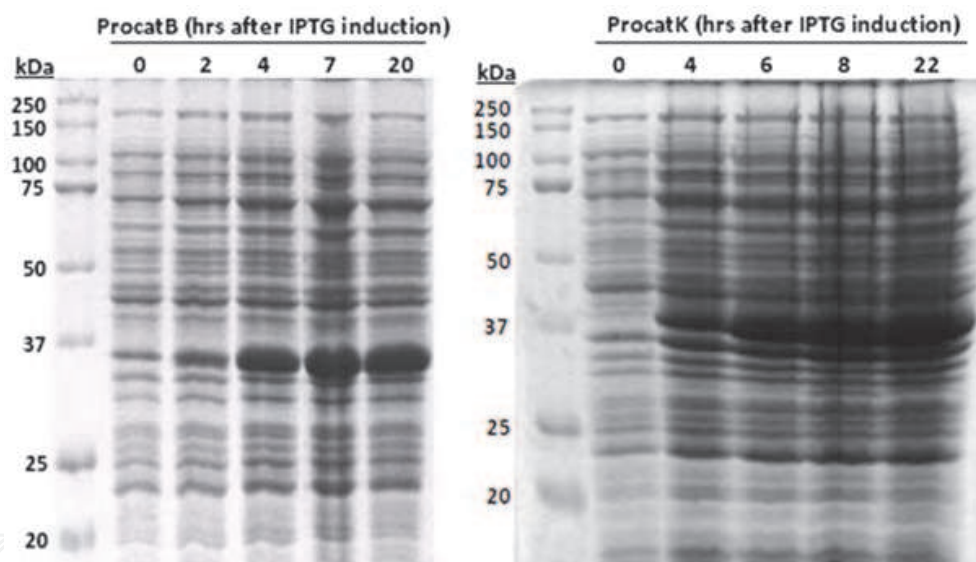


Fig. 3. SDS-PAGE showing induction progress of recombinant procathepsin B and K after IPTG addition. BL21 (DE3)pLysS cells containing pET-15b-procathepsin B or pET-15b-procathepsin K were cultured and protein expression induced by IPTG as described in the Experimental section. An aliquot of each culture was collected at various time intervals after IPTG addition. The cells were lysed in SDS-PAGE loading buffer by repeated vortexing and heating. The cell lysate was analyzed by 12% SDS-PAGE.

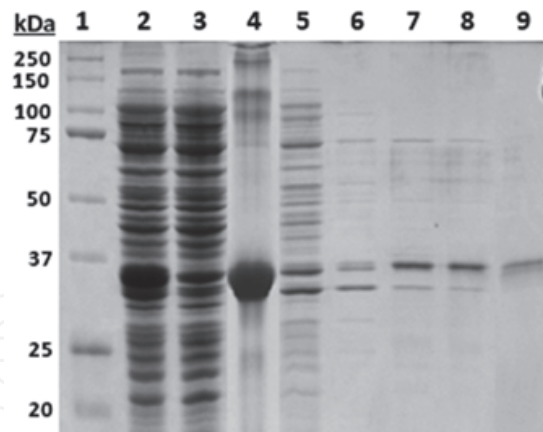


Fig. 4. SDS-PAGE showing the progress of purification of procathepsin B. The gel contained 12% acrylamide. Lane 1: Precision Plus Protein Kaleidoscope Standards; lane 2: Total IPTG induced *E. coli* cell lysate; Lane 3: Soluble proteins in the cell-free extract; Lane 4: Crude inclusion bodies before being subjected to washes; Lanes 5 & 6: First and second washes of the inclusion bodies with 50 mM Tris-HCl pH 8.0 containing 5 mM EDTA and 0.1% Triton X-100; lane 7 & 8: First and second washes of the inclusion bodies with 50 mM Tris-HCl, pH 8.0 containing 5 mM EDTA and 2 M Urea; Lane 9: Purified and refolded procathepsin B.

3.3.2 Purification of recombinant procathepsin K

The procathepsin K was also expressed as insoluble proteins in inclusion bodies as the procathepsin K band was missing from the soluble protein fraction (Figure. 5. lane 5). His-tagged procathepsin K protein was purified from the solubilized inclusion bodies by affinity chromatography on a HisTrap™ FF column to near homogeneity. The protein was then refolded and concentrated (Figure. 5 lane 7). Overall yield of the procathepsin K enzyme was about 1 mg per 100 ml of bacterial culture.

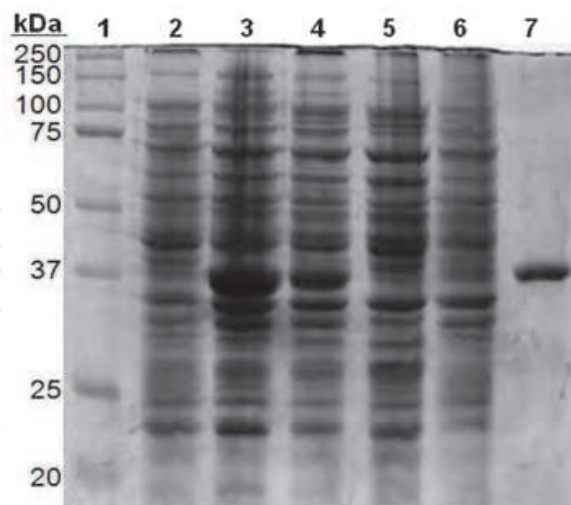


Fig. 5. SDS-PAGE showing overexpression of the human procathepsin K and the purification process. The gel contained 12% acrylamide. Lane 1: Precision Plus Protein Kaleidoscope Standards; lane 2: total lysate of the uninduced cells; lane 3: total lysate of 8 hours IPTG induced cells; lane 4: homogenate of the IPTG induced sample; lane 5: soluble proteins in cell-free extract of the IPTG induced sample; lane 6: Ni column wash; lane 7: purified procathepsin K.

3.4 Activation of the procathepsin B and K proteins

3.4.1 Activation of procathepsin B and functional cathepsin B assay

The recombinant procathepsin B activation was carried out by autoactivation as described in the Experimental section. The success of the activation was demonstrated by the cathepsin B activity assay and SDS-PAGE. As observed in the SDS-PAGE (Figure 6) clear bands are observed and the proteolytic cleavage was evident (lane 2).

The cathepsin B activity was demonstrated by its ability to cleave the Z-Phe-Arg- ρ NA. These results show a linear relationship between the formation ρ NA products with increasing cathepsin B levels from 0.024 to 0.19 μ M (Figure 7).

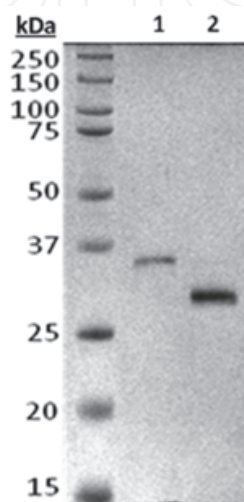


Fig. 6. SDS-PAGE showing autoactivation of procathepsin B. The gel contained 15% acrylamide. Lane 1: purified procathepsin B protein; Lane 2: autoactivated cathepsin B protein.

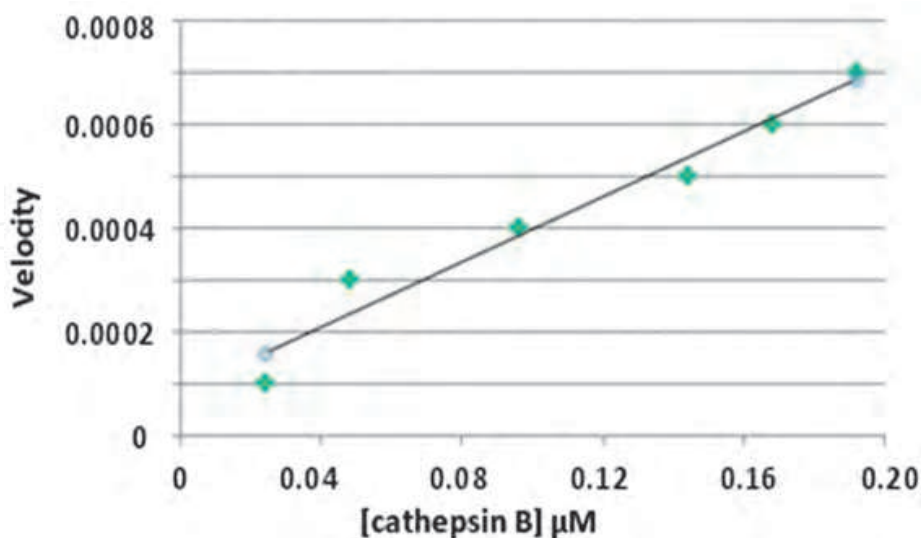


Fig. 7. Cathepsin B demonstrated enzyme concentration-dependent activity. The product formation linearity was verified by the use of increasing amounts of cathepsin B from 0.024 to 0.19 μ M. The activity was assayed using a chromogenic substrate, Z-Phe-Arg- ρ NA, and the rate was monitored by the increase in absorption at 405 nm due to the release of the ρ NA chromophore.

3.4.2 Activation of procathepsin K and functional cathepsin K assay

The refolded procathepsin K protein was activated to mature cathepsin K with the aid of porcine pepsin. Figure 8 shows successful cleavage of the procathepsin K (~38 kDa) into 2 fragments: mature cathepsin K (~26.5 kDa) and propeptide fragment (~11.5 kDa).

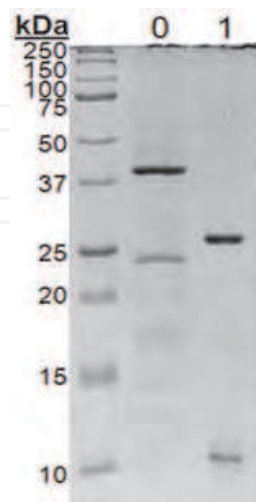


Fig. 8. SDS-PAGE showing activation of procathepsin K with pepsin. The gel contained 15% acrylamide. Lane 0: procathepsin K untreated; Lane 1: procathepsin K treated with pepsin for 1 hr. After 1 hr of pepsin activation, the procathepsin K (~38 kDa) was successfully cleaved into 2 fragments: mature cathepsin K (~26.5 kDa) and propeptide fragment (~11.5 kDa).

The cathepsin K activity is demonstrated by its ability to cleave the Z-Phe-Arg- ρ NA. The results show a linear relationship between the formation ρ NA chromophore product with increasing cathepsin K levels from 0.09 to 0.72 μ M (Figure 9).

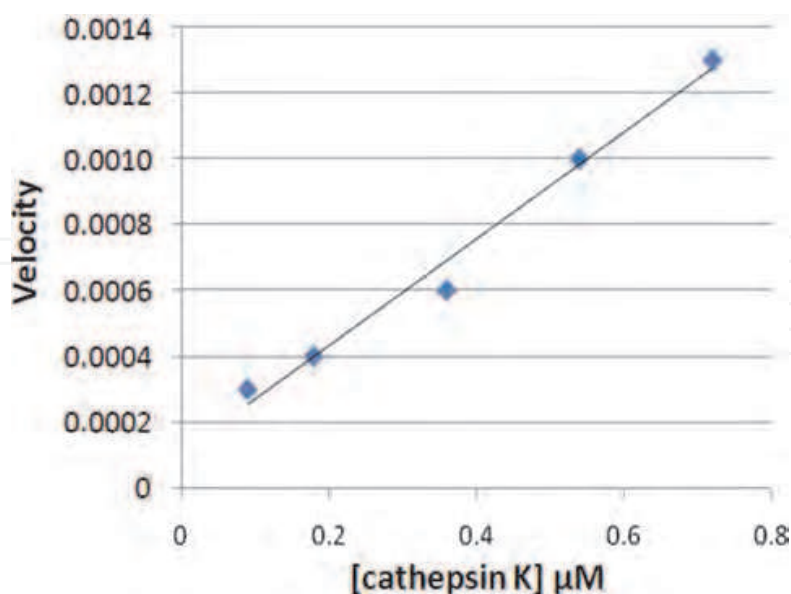


Fig. 9. Cathepsin K demonstrated enzyme concentration-dependent activity. The product formation linearity was verified by the use of increasing amounts of cathepsin K from 0.09 to 0.72 μ M. The activity was assayed spectrophotometrically by monitoring the increase in absorption at 405 nm as described in the text.

3.5 Screening of flavonoid inhibitors of cathepsin B and K

Flavonoids, polyphenolic compounds predominantly found in colorful fruits and vegetables, have been regarded of as possessing “antiviral, anti-allergic, antiplatelet, anti-inflammatory, antitumor, and antioxidant (Arora et al., 1998; Rice-Evans and Miller, 1996)” properties. Flavonoids have been known to have anti-cancer effects and could potentially inhibit cathepsin B. In particular, amentoflavones have been shown to inhibit cathepsin B and K (Pan et al., 2005; Zeng et al., 2006). Therefore a search for other flavonoid inhibitors of cathepsins was undertaken. Several commercially available flavonoids were tested (amentoflavone, apigenin, baicalin, celastrol, fisetin, kaempferol, limonin, luteolin, myricetin, and rutin), for their ability to inhibit cathepsin B or K. The inhibition of cathepsin B activity by each of the flavonoid at 40 μM is shown in Figure 10. Amentoflavone, celastrol, and luteolin demonstrated the best flavonoid inhibitors of cathepsin B of those screened. The IC_{50} value for celastrol was 125 μM .

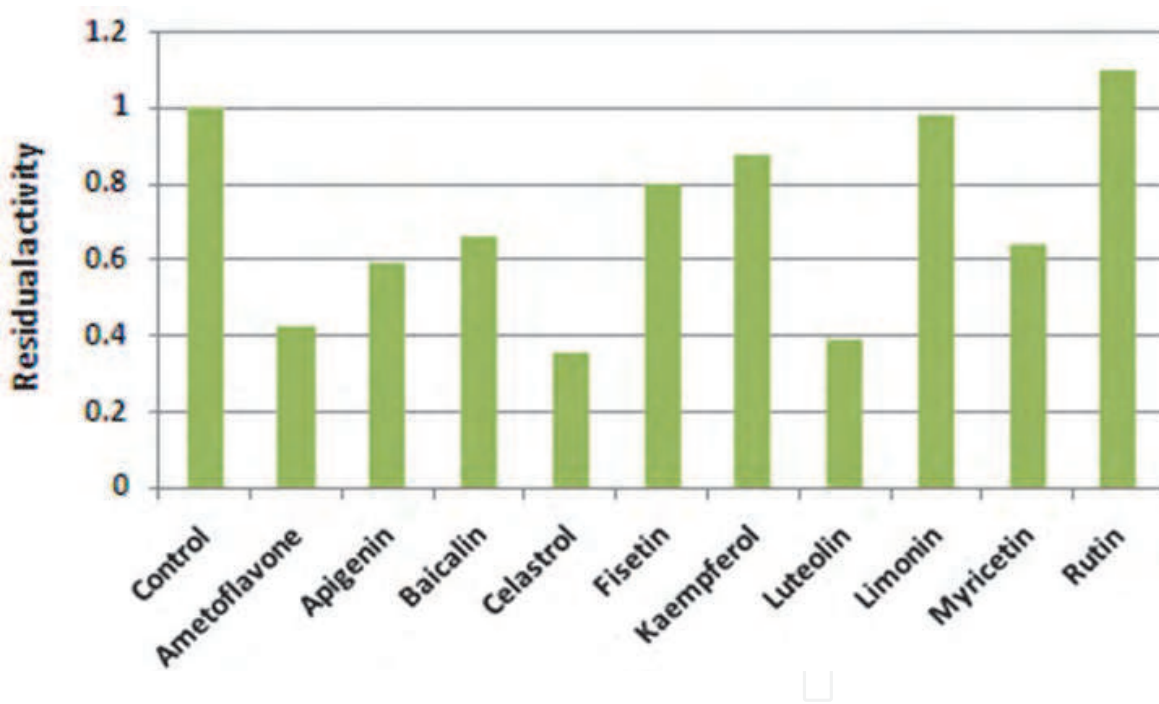


Fig. 10. Inhibition data of cathepsin B by flavonoids. Cathepsin B was incubated with 40 μM flavonoid. The residual activity was determined using chromogenic substrate, Z-Phe-Arg-pNA. The enzymatic reaction was monitored by the increase in absorption at 405 nm due to the release of the pNA chromophore.

Most of these flavonoids were also screened for their inhibition of cathepsin K activity (Figure 11). Results show that apigenin, celastrol, and myricetin are among the strongest inhibitors of cathepsin K of those tested. Their IC_{50} values of the cathepsin K inhibitors were determined to be 8.7 μ M, 135.5 μ M, and 100.5 μ M for apigenin, celastrol, and myricetin, respectively.

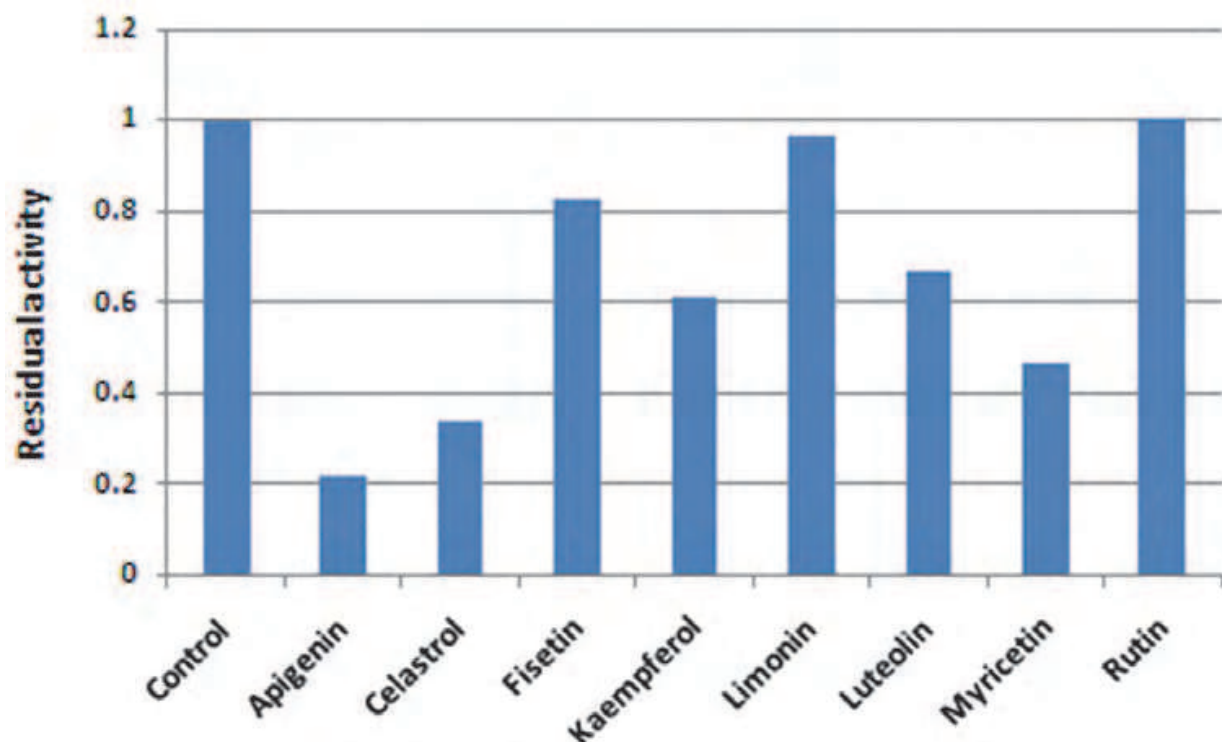


Fig. 11. Inhibition data of cathepsin K by flavonoids. Cathepsin K was incubated with 40 μ M flavonoid. The residual enzyme activity was determined using chromogenic substrate, Z-Phe-Arg-pNA, by monitoring the increase in absorption at 405 nm due to the release of the pNA chromophore.

4. Conclusion

In conclusion, the subcloning, expression, and purification of recombinant procathepsins B and K has been accomplished. Each of the proenzyme has been activated and shown to be functional as demonstrated by the enzyme's ability to cleave the chromogenic substrate, Z-Phe-Arg-pNA. The enzymes have been used successfully to screen potent flavonoid inhibitors. The availability of substantial amounts of active cathepsin B and K will greatly facilitate the identification of small molecule inhibitors. The information obtained from studies such as this will greatly facilitate drug discovery programs in the efforts to develop effective therapies for osteoporosis, cancer, and Alzheimer's Disease.

5. Acknowledgment

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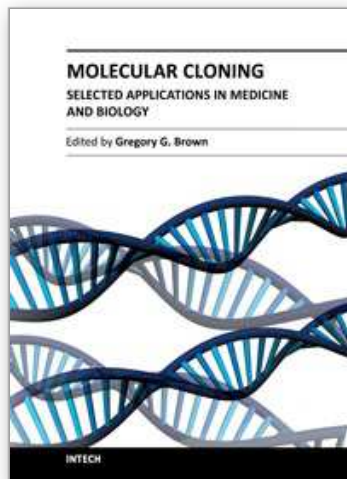
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The development of molecular cloning technology in the early 1970s created a revolution in the biological and biomedical sciences that extends to this day. The contributions in this book provide the reader with a perspective on how pervasive the applications of molecular cloning have become. The contributions are organized in sections based on application, and range from cancer biology and immunology to plant and evolutionary biology. The chapters also cover a wide range of technical approaches, such as positional cloning and cutting edge tools for recombinant protein expression. This book should appeal to many researchers, who should find its information useful for advancing their fields.

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