Preparation of recombinant lysosomal cysteine protease, cathepsin K in insect cells

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Abstract

Cathepsin K, which is localized in osteoclasts, plays an important role of bone resorption. Selective inhibitors of cathepsin K therefore can be promising therapeutic candidates for the treatment of diseases such as osteoporosis. Recombinant cathepsin K protein is necessary for screening the therapeutic candidates and elucidating the enzymatic functions because the amount of native cathepsin K is very low. Silkworm is one of the most attractive hosts for large-scale production of eukaryotic secretory proteins. The gene for fusion cathepsin K protein with *myc*-tag at the C-terminus was inserted into expression vector for silkworm and recombinant viruses were isolated by end-point dilution assay. Cathepsin K was expressed in the cell lysate, but not in the medium, and consequently we gave up the idea of cathepsin K expression in the silkworm.

Thus, we tried to isolate cathepsin K protein from the cultured cells, and purified it by sequential operations with DEAE-Sepharose column, Mono-S column, and anti-*myc* antibody affinity column. The purification fold of cathepsin K activity increased approximately 300 times in comparison with that in soluble fraction of cell lysate.

KEYWORDS : insect cell, recombinant enzyme, secretory enzyme, *myc*-tag
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INTRODUCTION

The papain-like cysteine protease, cathepsins represents a major component of the lysosomal proteolytic system. Cathepsins comprise a large number of members, which can be subdivided into two functional groups according to their tissue distribution. Cathepsins B, H, and L as well as the recently discovered cathepsins C, O, F, and Z show a ubiquitous expression[1-7], which mainly play an essential role in unspecific protein degradation. Cathepsins K, L2, S, and W show a tissue-restricted expression pattern[8-11]. These enzymes play important specific proteolytic roles. Bone remodeling includes the processes of bone resorption and rebuilding, and the resorption is carried out by osteoclasts, multinucleate giant cells[12]. Cathepsin K is localized in osteoclasts[13]. Pycnodysostosis, an autosomal recessive osteochondrodysplasia, has been reported to result form the cathepsin K deficiency[14]. These results demonstrate that cathepsin K plays an important role of bone resorption.

Selective inhibitors of cathepsin K therefore could be promising therapeutic candidates for the treatment of

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diseases such as osteoporosis, characterized by low bone mass and deterioration of bone tissue. To screen the therapeutic candidates and elucidate the enzymatic functions, the recombinant cathepsin K protein is needed because of the low amount of native cathepsin K protein in osteoclasts. Recombinant cathepsin K has been expressed in baculovirus-infected SF21 cells[15].

Silkworm is one of the most attractive hosts for large-scale production of eukaryotic proteins, and secretory proteins can be expressed at high concentration up to 1mg / mL. Since cathepsin K is the secretory protein, we tried to express cathepsin K protein in silkworm larvae due to obtain easily larger amount of the protein.

MATERIALS AND METHODS

General methods: The experiments using recombinant DNA techniques were performed under the permission of Recombinant DNA Adversary Committees in Toyama Medical and Pharmaceutical University.

Vector construction and co-transfection to insect cells : Cathepsin K gene were isolated by PCR from the cDNA library of human spleen, and the *myc*-tag (EQKLISEEDL) was added at the C-terminus of cathepsin K by PCR with the both primers, 5' -CCC GGG ATG TGG GGG CTC AAG GTT CTG C-3' and 5' -CTG CAG CTA CAG GTC CTC CTC GGA GAT CAG CTT CTG CTC CAT CTT GGG GAA GCT GGC CAG G-3' . The amplified DNA was inserted to pGEM-T vector (Promega, USA). *Sma* I-*Pst* I DNA fragment was prepared and inserted to the *Sma* I-*Pst* I site of pBm4-luc vector. The constructed expression vector was co-transformed with virus genome DNA of p6E strain into BoMo15A IIc cells. Co-transformation was performed with 0.9 μg vector DNA and 0.23 μg virus DNA. The recombinant viruses were isolated by end-point dilution assay. Luciferase assay was performed with Steady-Glo Luciferase Assay System (Promega).

Expression and purification of cathepsin K in the insect cells: The recombinant viruses were infected the BM-N4 cells at MOI 5, and culture for 36 h at 25 ℃. The cell were suspended in the buffer (25 mM sodium phosphate (pH7.0), 5 mM DTT, 2.5 mM EDTA) and disrupted by sonication. The supernatant of the cell lysate were separated by DEAE-Sepharose (10 mm in diameter × 100 mm in the length; Amersham Japan, Japan) and the pass through fraction was recovered. The collected fractions were further separated by Mono-S column (5 mm in diameter × 50 mm in the length; Amersham Japan) with gradient elution of NaCl (0 to 0.5 M in 100 min). The eluted fractions were collected, and the cathepsin K protease activity in the fraction was determined. The fractions containing cathepsin K protein were applied the anti-*myc* antibody affinity column (10 mm in diameter × 10 mm in the length) to purify the *myc*-fused cathepsin K protein. The absorbed proteins were eluted by 0.1 M citrate solution (pH 3.0).

*SDS-PAGE and Western blotting:*SDS-PAGE was performed with 13 % polyacrylamide gel according to the standard method. The separated proteins were blotted to PVDF membrane with semi-dry blotter at 16 V for 20 min. Blocking of the membrane was achieved by placing in PBS with 5 % non-fat milk for 1 h. Mouse anti-*myc* IgG (1000 × dilution) and HRP-rabbit anti-mouse IgG (2000 × dilution) were used as 1st and 2nd antibody. The cross-reacting protein bands were visualized by ImmunoStar Reagents (Wako, Japan).

*Enzyme assay with fluorogenic substrates:*The specimen was pre-incubated with 160 mM acetate buffer (pH 5.5) containing 1.6 mM EDTA and 8 mM cysteine at 37 ℃ for 10 min. Assays were initiated by the addition of Z-Phe-Arg-MCA as substrate that had been pre-incubated at 37 ℃ at a final concentration of 50 μM.

After 30-min incubation, the reactions were stopped by the addition of sodium monochloroacetic acid at a final concentration of 60 mM, and their fluorescent intensities were measured by a Hitachi 650-10 spectrofluorometer (Hitachi, Japan) using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Averages of the activity were shown (n=3).

RESULTS AND DISCUSSION

Vector Construction and Selection of Recombinant Virus: Cathepsin K gene was amplified by PCR among the human spleen cDNA library, and fused with *myc*-tag

(EQKLISEEDL) at the C-terminus. The fused gene was inserted into the pBm4-luc vector for expression in silkworm. The constructed vector was co-transformed with genome DNA isolated from the *Bombyx mori* nuclear polyhedrosis virus to the BoMo15A IIc cells, which are capable of high transfection efficiency. Recombinant viruses were isolated by end-point dilution assay with 96-well plates (Fig. 1). The transformed cells by recombinant virus should be lack of polyhedron formation and show luciferase luminescence. To isolate the recombinant virus, the procedures of end-point dilution assay were repeated three times.

Expression of cathepsin K in the BM-N4 cells: The recombinant virus was infected to the BM-N4 cells at MOI 5 and the cells were cultured for 60 h at 25 ℃. The collected cells were disrupted by sonication and then the supernatant and pellet were separated by a centrifugation. They were separated by SDS-PAGE and analyzed by a Western blotting with anti-*myc* antibody (Fig. 2A and B). The recombinant viruses expressed cross-reacting protein band of approximately 43 kDa, the molecular mass for the *myc* sequence + pro-enzyme from which the leader pre-sequence had been removed. This protein band was not detected in the sample prepared from uninfected BM-N4 cells (Fig. 2B). Although we examined the expression experiments at various conditions, the protein band was detected only in cell lysate, not in medium (data not shown). This result indicates that the expressed cathepsin K protein is not secreted in the *Bombyx* cells, and consequently we gave up the idea of cathepsin K expression in silkworm larvae. Thus, we tried to isolate the cathepsin K protein from the BM-N4 cells. Figure 2C shows that the cultured cells for 36 h are abundant in soluble form of cathepsin K protein.

Purification of cathepsin K from the infected cell: The cultured BM-N4 cells were infected with recombinant virus at MOI 5. The supernatant of the cell lysate was recovered at 36 h post-infection, and its pH was adjusted at 7.0. The specimen was separated with DEAE-Sepharose column and the pass though fraction was recovered. Then, the recovered fraction was separated with Mono-S column by gradient elution of NaCl (Fig.3A). Cathepsin K protease activity was assayed in each fraction indicated by number on the elution profile of Figure 3A. The fractions No. 7 and 8, which are eluted by approximately 150 mM NaCl, contained higher protease activity comparing other fractions (Fig. 3B). These fractions were analyzed by Western blotting with anti-*myc* antibody, and were confirmed containing the cathepsin K protein (Fig. 3A). The fractions No. 7 and 8 were further separated with anti-*myc* antibody affinity column. The elution profile was shown on Figure 3C and the Western blotting analysis with anti-*myc* antibody shows that the eluted fraction b contained the cross-reacting protein. Figure 3D shows that the eluted fraction b was also rich in the cathepsin K protease activity comparing to pass fraction a. Summary of purification of cathepsin K protein was shown on Table 1. The purification fold of cathepsin K activity increased approximately 300 times after the purification with anti-*myc* antibody affinity column comparing to soluble fraction of cell lysate.

Table 1 Summary of cathepsin K purification

Purification step	Total protein [mg]	Total activity [nmol / min]	Activity per protein [nmol / min / mg protein]
Soluble fraction	18.7	2.1	0.11
DEAE-Sepharose	10.2	1.2	0.12
Mono-S	0.072	0.03	0.35
Anti-*myc* affinity	0.004	0.12	31.25

In conclusion, we prepared the recombinant cathepsin K protein with cultured insect cells, but not with silkworm larvae because of lack of secretory function in recombinant cathepsin K protein. The cathepsin K with high specific activity in this study can be used for screening the inhibitors for cathepsin K. Further studies are required to obtain larger amount of recombinant cathepsin K using the cultured cells and to provide the secretory function to recombinant cathepsin K to prepare in silkworm larvae.,

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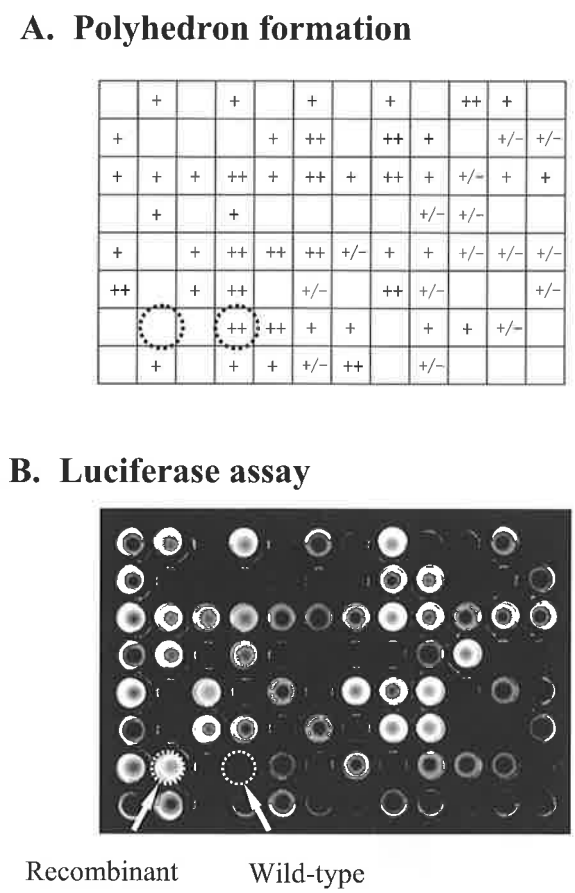

Fig. 1

Fig.1 *Selection of recombinant virus of cathepsin K.* A) Polyhedron formation was observed by microscopy.

The degree of polyhedron formation was indicated by "++", "+" and "+/-". The polyhedron formation was not observed in the well indicated by the blank column. B) The luminescence of expressed luciferase was observed by CCD camera.

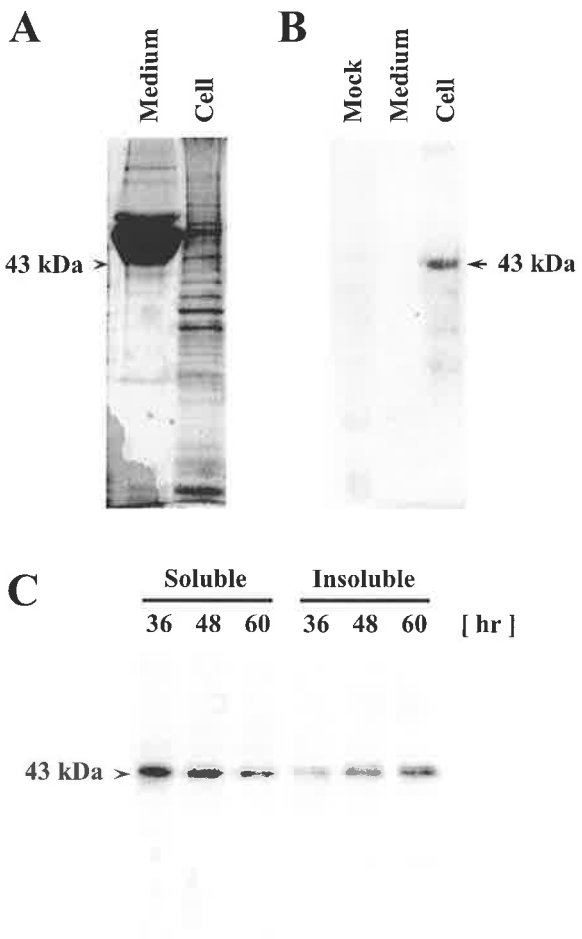

Fig. 2

Fig.2 *Western blotting analysis with anti-myc antibody.* A) Total proteins on 13 % polyacrylamide gel were visualized by staining with Coomassie Brilliant Blue, B) The medium and cell lysate were analyzed by Western blotting with anti-*myc* antibody and visualized by chemiluminescence. C) The soluble and insoluble fractions of cell lysate were analyzed by Western blotting with anti-*myc* antibody and visualized by chemiluminescence.

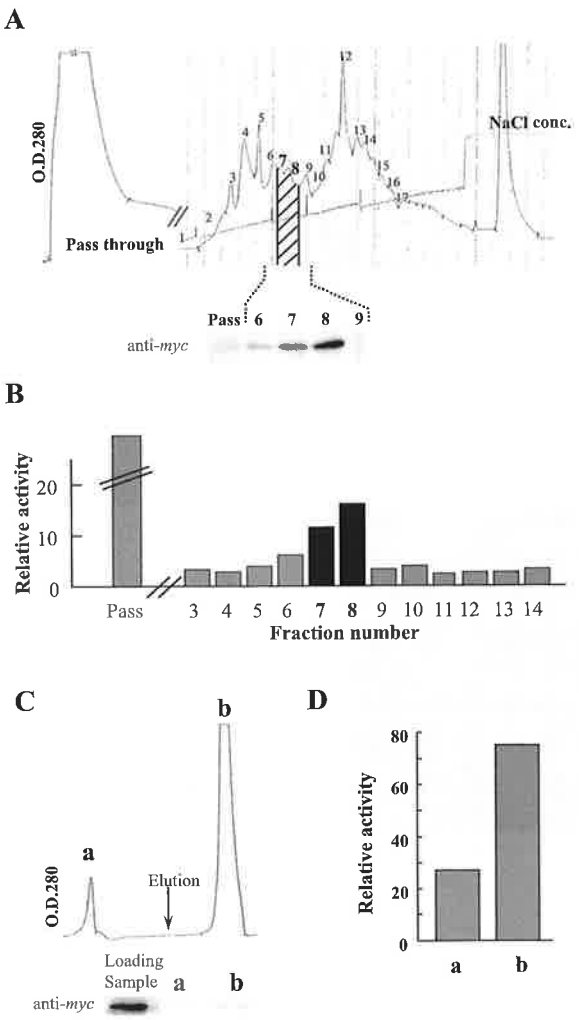

Fig. 3

Fig.3 *Purification of cathepsin K from insect cells.* A) Elution profile of DEAE-Sepharose column. Absorbance at 280 nm was measured during gradient elution of 0 to 0.5 M NaCl, and the fraction numbers were indicated on the profile. Western blotting analysis with anti-*myc* antibody was performed with the fractions 6 to 9, and the result was shown below the profile. B) Cathepsin K protease activity was measured in each fraction. C) Elution profile of anti-*myc* antibody affinity column. The pass-through and eluted fraction were indicated by a and b, respectively. Western blotting analysis with anti-*myc* antibody was performed with the fractions a and b, and the result was shown below the profile. D) Cathepsin K protease activity was measured in fractions a and b.

昆虫細胞を利用したカテプシンK 酵素の作製

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日本語要旨

破骨細胞に局在するシステインプロテアーゼであるカテプシンK は，骨の再吸収に関わる酵素である。カテプシンK の阻害剤は骨粗鬆症の治療薬の候補になり得る。スクリーニングや酵素の機能を調べるためには組換えカテプシンK を大量に作製することが必要である。我々はカイコ幼虫を用いた発現系が分泌型酵素であるカテプシンK の発現に適していると考えた。C 末端に*myc*タグを付けたカテプシンK 遺伝子をカイコ用の発現ベクターに挿入し，限界希釈法で組換えウイルスを得た。このウイルスをカイコ培養細胞に感染させてカテプシンK の発現場所を調べたところ，細胞内で発現していた。細胞外への分泌が観察されなかったのでカイコ幼虫での発現をあきらめ，培養細胞からの精製を試みた。DEAE－セファロースカラム，Mono-S カラム，抗*myc* 抗体アフィニティーカラムで順次精製した結果，比活性を細胞可溶性画分の約300 倍に高めることに成功した。

キーワード：昆虫細胞、組換え酵素、分泌型酵素、*myc* タグ