Preparation of recombinant lysosomal cysteine proteases highly expressed in mouse placenta, cathepsin-P/J and -6.

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Abstract

More than twenty cathepsins, a major component of the lysosomal proteolytic system, are identified. In the last decade, the cathepsins which show tissue-restricted expression pattern have been discovered. In placenta, eight novel genes related to cathepsin L have been recently identified. To characterize the cathepsins highly expressed in placenta, we prepared two recombinant cathepsins, cathepsins-P/J (Cat-P/J) and -6 (Cat-6) by *E. coli* expression system with pET-3 vector. Cat-P/J hydrolyzed both Z-Phe-Arg-MCA, a typical substrate for cathepsins, and Boc-Val-Pro-Arg-MCA, a substrate for α-thrombin, while Cat-6 hydrolyzed only Boc-Val-Pro-Arg-MCA. Though these activities are very weak, this is the first report describing the difference of substrate specificity between Cat-P/J and -6. Next we tried preparing the recombinant Cat-P/J by a baculovirus expression system because it had several advantages to produce functional soluble proteins. The soluble recombinant Cat-P/J was secreted into the culture medium, and its maximum amount was observed at 4 days post-infection. However, the purification of Cat-P/J was not achieved because of small amount of the recombinant proteins.

Key words : baculovirus expression system, refolding, peptide substrates, HPLC, His-tag

Introduction

Cathepsins, the papain-like cysteine protease, 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. Cathepsin K is localized in osteoclasts and is responsible for regulating bone remodeling[12]. Pycnodysostosis, an autosomal recessive osteochondrodysplasia, has been reported to result form the cathepsin K deficiency[13]. Cathepsin S is localized in lymphatic tissues and plays an essential role for the degradation of invariant chain (Ii) in peripheral antigen presenting cells[14], which is also required for the action of cathepsin L[15]. These show that the tissue-restricted expressed cathepsins are responsible for

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physiologically important roles.

Recently, cathepsins highly expressed in placenta were searched in the databases of rodent genome and placental EST. Cathepsin P[16] and J[17] (Cat-P/J), both genes had the identical amino acid sequence, were the first gene isolated in mouse placenta by the above approach. Subsequently, three novel cathepsins Q[18], M[19], and R[20] were also identified as placenta-specific ones by the same approach. Alternative approach, i.e. mouse chromosome analysis or cDNA subtraction cloning revealed the other placenta-specific cathepsins 1, 2, 3 and 6 were also identified[21-23]. The sequence homologies between Cat-P/J and the other seven cathepsins are 62 ~ 72 % and all of eight mouse genes are located on chromosome 13, which suggests that gene duplication event causes the placenta-specific cathepsins[22]. However, neither screening human genome database nor RT-PCR with degenerated primers in human placental tissue identified the Cat-P/J-like gene in human placenta[24].

The placenta is a special organ that provides the nutrients from the maternal blood during gestation to growing fetus. The amino acids incorporated into embryonic proteins are derived from the protein hydrolyzed in placenta[25]. This shows that certain proteases are involved in the placental functions. The cathepsin inhibitor, leupeptin was reported to have a teratogenic effect in rat embryos[26]. The gene disruption of cathepsin B and L did not affect growth and development of embryos during gestation[15,27]. These suggest that other cathepsins are responsible to the placental functions. Among eight placenta-specific cathepsins, both Cat-P/J and -6 showed that these transcripts were detected in the mouse placenta at 14.5 ~ 17.5 days post-coitus corresponding to the later stage of gestation, and localized on the labyrinthine layer of placenta[21]. In this study, we prepared the recombinant Cat-P/J and -6 using *E. coli* and baculovirus expression system, which should be available for understanding functions of placenta-specific cathepsins.

Materials and Methods

General methods: Experimental protocols operating animals met the "Guidelines for Animal Experimentation" approved by the Japanese Association of Laboratory Animal Science and the Japanese Pharmacological Society. The experiments using recombinant DNA techniques were performed under the permission of Recombinant DNA Adversary Committees in Toyama Medical and Pharmaceutical University.

Expression and purification of recombinant mouse cathepsin-P/J and -6 in E. coli: Placental tissues were surgically obtained from pregnant mouse (C57BL/6 Cr Slc: Sankyo Lab, Tokyo, Japan) at 13 days post-coitus. The placental RNA was isolated from these tissues and the first stranded cDNAs were generated by SuperScript Preamplification System (Gibco BRL, MD, USA). DNA fragment containing mouse procathepsin-P/J was obtained by PCR amplification with the combinations of sense primer 5'-cca tgg cac atg atc cca aat tgg atg ctg agt gg-3' and antisense primer, 5'-gga tcc tca aaa tat att ggg ata gct gg-3'. These primers were designed to include the *Nco* I site in sense primer and *Bam* HI sites in antisense primer. The underlines on nucleotide sequences showed the additive restriction sites. Since the procathepsin-6 DNA has *Nco* I site in its cording region, the DNA sequence of the internal *Nco* I site was mutated by site-directed mutagenesis using megaprimer PCR method[28]. The first PCR was performed with the combination of sense primer, 5'-cca tgg cac ttg atc c-3' and antisense primer, 5'-ctt cct cca tAg tgt atg-3', which amplified the double-stranded megaprimer. The second PCR was done with combination of the generated megaprimer as sense primer and antisense primer, 5'-gga tcc tac aat ggg ata atg tgc ata tgt agc aat gcc aca gtg gtt gtt ctg gtc tct gat aat ctt c-3'. The underline on the primers showed the additive restriction sites *Nco* I and *Bam* HI site, and the capital letter "A" in the antisense primer for preparing megaprimer showed the site of point mutation, which did not influence the amino acid sequence. The both PCR products (~1000 bp) were gel-purified and subcloned by pGEM T vector system (Promega, USA). The *Nco* I - *Bam* HI digestion product of the DNA fragments were ligated in the *Nco* I - *Bam* HI site of the T7 expression vector pET-3d (Novagen, WI, USA). The resulting constructs were transformed into the *E. coli* strain BL21 (DE3) pLysS (Novagen).

The transformed *E. coli* cells were grown at 37℃ in Lenox broth medium containing 50 μg/mL ampicillin and 34 μg/mL chloramphenicol for several hours. The full-grown cells (2 mL) were inoculated into 200 mL of same medium, and were cultured at 37 ℃ until an A_{600} of 1.0 was reached. Isopropyl thio-β-D-galactoside (IPTG) to a final concentration of 3 mM and were cultured for 3 h at 37℃ . The *E. coli* cells were collected by centrifugation at 5000 g for 10 min, resuspended and washed with an extraction buffer (EB; 50 mM Tris/HCl (pH 7.8), 30 mM NaCl and 5 mM EDTA). The final volume of the suspensions was adjusted to 10 mL, and the suspension was frozen and thawed 3 times to disrupt the cells, then centrifuged at 20,000 g for 20 min. The pellet was recovered and homogenized by glass homogenizer with EB containing 0.5 % Triton X-100. After 5-min incubation with gentle shaking, the pellets were recovered by centrifugation at 20,000 g for 10 min. Then, the specimen was homogenized with EB containing 2 M urea, incubated at 37℃ for 5 min with gentle shaking, and centrifuged at 20,000 g for 10 min. This procedure was repeated twice. The final recovered pellets were resuspended thoroughly with EB containing 8 M urea and 10 mM dithiothreitol, and incubated at 37℃ for 1 h. After the centrifugation at 20,000 g for 20 min, the supernatants were recovered and these protein concentrations were determined by Bradford method[29] with bovine serum albumin as a standard.

Refolding of the denatured cathepsins: Refolding the proteins were examined in several conditions. The optimal condition was described as follows. The final suspensions above were diluted with EB containing 8 M urea and 10 mM dithiothreitol to a final protein concentration of 50 μg/mL. The first dialysis of the diluted samples were performed against 100 volume of dialysis buffer (DB; 100 mM Tris/HCl (pH 8.0), 5 mM EDTA) containing 5 mM cysteine for 6 h at 4℃. The second dialysis was done against the same buffer for 18 h at 4℃, then the final dialysis against DB for 18 h at 4 ℃. The dialyzed samples were stored at - 80℃ until use.

Preparation of baculovirus transfer vector expressing His-tag fusion protein: To obtain His-tag fused cathepsin proteins, we altered the baculovirus transfer vector of pVL1392 (in BaculoGold Transfection Kit from PharMingen, CA, USA). Two oligonucleotides, 5'-cta gaa gag gat cgc atc acc atc acc atc act gac tcg aga-3' and 3'-ttc tcc tag cgt agt ggt agt ggt agt gac tga gct ctc tag-5' were prepared and annealed to prepare double-stranded DNA that encodes the short peptide sequence of "Arg Gly Ser His His His His His His plus stop codon" Both terminus of DNA were the 5'-protruding ends of restriction sites for *Xba* I and *Bgl* II, which were designed to insert into the *Xba* I - *Bam* HI restriction sites of pVL1392. The resultant vector, named pVL1392-His has no *Bam* HI site because the *Bam* HI site of pVL1392 was ruined by the insertion of 5'-protruding ends of *Bgl* II.

Expression of recombinant human cathepsin-P/J in baculovirus expression system: The first stranded cDNA generated with mouse placental RNA was used as a template for PCR-amplifying the mouse procathepsin-P/J with sense primer 5'-caa gat ctt gaa aca tga ctc cta ctg tcc ttc tg-3' and antisense primer, 5'-gct cta gaa aat ata ttg gga tag ctg gca agt g-3'. These primers were designed to include the *Bgl* II site in sense primer and *Xba* I sites in antisense primer, which are indicated by underlines. The PCR product (~1000 bp) was gel-purified and subcloned by pGEM T vector system. The *Bgl* II - *Xba* I digestion product of the DNA fragments were ligated in the *Bgl* II - *Xba* I site of the pVL1392-His vector. The resultant transfer vector and the DNA of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) were co-transformed into *Spodoptera frugiperda* (*Sf*) -21 cells to generate the recombinant virus expressing procathepsin-P/J. Single recombinant virus was obtained by end-point dilution assay with the transfected supernatant, and was amplified by the sequential transfection into *Sf*-21. Large-cultured *Sf*-21 cells were infected with the amplified virus as the MOI was 10, and cultured for 4 days in TC-100 insect medium with 10% FBS (both from Gibco BRL, USA). The cells were centrifuged at 2,000 g, and then the culture medium was recovered for the following purification procedure.

Purification procedures of the His-tagged Cat-P/J: The

His-tagged Cat-P/J was purified by the affinity chromatography with Ni-CAM HC RESIN (Sigma-Aldrich) from the 4-days cultured medium described above. Bound material was eluted with the buffer that consisted of 50 mM Tris/HCl (pH 7.4), 250 mM imidazole and 0.3 M NaCl, and recovered under the monitoring the absorption at 280 nm. The recovered fractions were concentrated by the dialysis against solid sucrose at 4℃ for 6 h, re-dialyzed against excess volume of EB, and used as a sample for immunoblotting. The pass-through fractions were also concentrated and dialyzed as same procedures as bound fractions, and then re-loaded onto the column packed with Ni-CAM HC RESIN. The bound material, which was recovered as described above, and the pass-through fraction were used as samples for immunoblotting.

Electrophoretic separation and detection of the His-tagged Cat-P/J: The samples were separated by SDS-PAGE carrying 8 or 10 % acrylamide slab gel, and stained with Coomassie brilliant blue G-250 (CBBG-250) or with silver stain kit (Wako, Japan). Some were transferred onto PVDF membrane using the semi-dry type apparatus. His-tagged Cat-P/J was detected with anti-His HRP conjugates (QIAGEN, USA), which was visualized with ImmunoStar Reagent (Wako, Japan) according to the manufacture instruction.

Amino acid sequence analysis: The amino acid sequences were determined using Edman degradation on a pulsed-liquid protein sequencer, equipped with an on-line phenylthiohydantoin amino acid analyzer (Shimadzu PPSQ-21, Japan).

Enzyme assay with fluorogenic substrates: The refolded recombinant Cat-P/J or 6 generated by *E. coli* expression system 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. Stock solutions of fluorogenic peptide substrates, Z-Phe-Arg-MCA, Pro-Phe-Arg-MCA, Boc-Val- Pro-Arg-MCA, Boc-Ala-Gly-Pro-Arg-MCA, Z- Arg-Arg-MCA, Lys-MCA, Boc-Gly-Ala-Arg-MCA, and Suc-Leu-Leu-Val-Tyr-MCA (all from Peptide Institute Inc, Japan) were prepared in Me_2SO. Assays were initiated by the addition of the substrates 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.

Peptidolytic assay with insulin B-chain: Insulin B-chain for "HPLC-purified and use-tested as a protease substrates" (Phe-Val-Asn-Gln-His-Leu- Cys-(SO_3H)-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-(SO_3H)-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala) was purchased from Sigam-Aldrich. The insulin at final concentration of 20 μM was incubated with the refolded Cat-P/J or 6 under the same condition as described above. After 1-h or 24-h incubation at 37℃, the samples were frozen to stop the reaction. The treated insulin was analyzed by reversed-phase HPLC using a Shimadzu HPLC system LC-VP with a C18 column, Develosil ODS-UG-5 (4.6 mm i.d. x 150 mm, Nomura Chemical Co. Ltd, Japan). A linear gradient of 0 - 40 % acetonitrile containing 0.1 % trifluoroacetic acid was performed over 20 min at a flow rate of 1 mL/min with monitoring at 215 nm.

Results and Discussion

Expression of recombinant procathepsin-P/J and -6 in E. coli: For expressing mouse procathepsin-P/J and -6 in *E. coli*, the Gln^{19} in Cat-P/J or Leu^{19} in Cat-6 was replaced with Met by using a PCR primer mutagenesis. The cDNA generated from RNA of mouse placenta at 13 days post-coitus was used as a template in both cases. Both DNAs were inserted into pET3-d *E. coli* expression vector downstream from the T7 promoter. The construction of both vectors was confirmed by DNA sequencing analysis (data not shown). The vectors were transformed into *E. coli* strain BL21(DE3) pLysS, and the IPTG was added to the growing medium for inducting recombinant cathepsin proteins. Abundant proteins with the molecular weights of 34 and 37 KDa, appeared in *E. coli* cells for Cat-P/J and Cat-6 expression, respectively (Fig. 1). Each molecular weight

of the generated protein is almost corresponded with the presumable one from amino acid sequences of procathepsin-P/J (35 KDa) or -6 (37KDa).

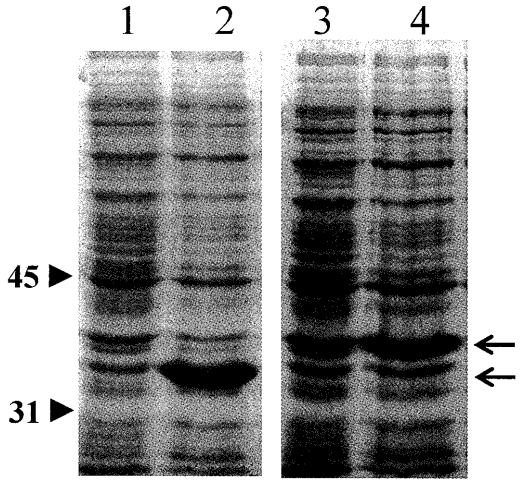

Figure 1. SDS-PAGE analysis of recombinant Cat-P/J and -6 expressed in *E. coli*. Recombinant Cat-P/J (lanes 1,2) and Cat-6 (lanes 3,4) were obtained by *E. coli* pET expression system. Cell lysate was prepared before (lanes 1,3) or after (lanes 2,4) IPTG induction. Arrows indicate the expressed proteins. The molecular mass markers were indicated in the left margin.

After disruption of the cells, recombinant proteins localized in the pellet fraction were washed several times, and dissolved with the buffer containing 8 M urea. In this purification step, densitometric analysis of SDS-PAGE stained with CBBG-250 revealed that both purities of Cat-P/J and -6 were found to be over 90 % (data not shown). The N-terminal sequences analysis using amino acid sequencer revealed that the N-termini of Cat-P/J and -6 were "Ala-Xaa-Asp-Pro-Lys-Lue-Asp-Ala-Glu-Trp-Lys-Asp-Trp" and "Ala-Leu-Asp-Pro-Asn-Leu-Asn-Ala-Glu-Trp-His-Asp-Trp", respectively. Each determined sequence was identical to the 20th -33rd amino acid sequence of Cat-P/J or -6, respectively. These results show that both cathepsin proteins were designed to start translating at the inserted Met[19] and the initiation Met was removed after the translation. This observation corresponds well the report that the initiation Met followed by an Ala residue is removed by processing mechanism in *E. coli*[30]. Taken together, we succeeded in obtaining the both purified procathepsin-P/J and -6 by *E. coli* expression system.

Refolding conditions for procathepsin-P/J and -6. Since placenta-specific cathepsins are highly relative to cathepsin L[24], the refolding condition for the denatured Cat-P/J and -6 started to be examined according to the procedures for cathepsin L[31]. The denatured proteins were dialyzed against the excess refolding buffer that consisted of 50 mM potassium phosphate (pH 10.7), 5 mM EDTA, 1 mM reduced glutathione, 0.1 mM oxidized glutathione and 0.7 M L-arginine. The arginine was stabilizing agent reported to be resulted in significantly improved yields of soluble protein[32]. However, a large amount of proteins precipitated during dialysis in both cathepsins. Next, we examined the alternative refolding condition that was used to obtain mature cathepsin B[33]. The denature proteins were dialyzed against the excess refolding buffer that consisted of 100 mM Tris/HCl (or sodium phosphate), 5 mM EDTA, 5 mM cysteine. The recovery yield of soluble proteins was examined at pH 6, 7 (using 100 mM sodium phosphate), 8 and 9 (using 100 mM Tris/HCl). For preparing both Cat-P/J and -6, the highest recovery yield was carried out in pH 8 (data not shown).

Characterization of recombinant Cat-P/J and -6 expressed in E. coli. The ability hydrolyzing fluorogenic peptide substrates was examined in the refolded Cat-P/J and -6, which had been prepared by *E. coli* expression system (Table 1). The reaction conditions according to determination of the cathepsin B activity were used[33]. Among the substrates, Cat-P/J hydrolyzed Z-Phe-Arg-MCA, a typical substrate for cathepsins, and Boc-Val-Pro- Arg-MCA, a substrate for α-thrombin, while Cat-6 hydrolyzed only Boc-Val-Pro-Arg-MCA. These showed that the substrate specificity is different between Cat-P/J and Cat-6. Both cathepsins hydrolyzed Boc-Val-Pro-Arg-MCA, and did not Boc-Ala-Gly-Pro-Arg-MCA, the substrate for γ ANP precursor processing enzyme. This shows importance of the amino acid in the P-3 position (indicated by underlines). Cat-P/J and -6 preferred Val, side chain of which is bulky group, to Gly in P-3 position for peptidolytic activity. The both substrates, Boc-Ala-Gly-Pro-Arg-MCA and Boc-Val-Pro-Arg-MCA were reported to be hydrolyzed by the recombinant cathepsin

K generated by *E. coli* expression system, while not at all by cathepsin L[34]. The other substrates such as Pro-Phe-Arg-MCA (a substrate for pancreatic or urinary kallikerin), Z-Arg-Arg-MCA (a substrate for cathepsin B), Lys-MCA (a substrate for aminopeptidase), Boc-Gly-Ala-Arg-MCA (a substrate for trypsin) and Suc-Leu-Leu-Val-Tyr-MCA (a substrate for calpain, ingensin or proteasome) were not significantly hydrolyzed by Cat-P/J and -6. Thus, the substrate specificity of Cat-P/J and -6 is different from the other three cathepsins, B, L and K.

Table 1 Characterization of recombinant Cat-P/J and -6

Substrates	Fluorescent intensity	
	Cat-P	Cat-6
Z-Phe- Arg-MCA	2.6	0.5
Pro-Phe-Arg-MCA	0.7	0.6
Boc-Val-Pro-Arg-MCA	2.8	2.8
Boc-Ala-Gly-Pro-Arg-MCA	0.5	0.5
Z-Arg- Arg-MCA	0.7	0.5
Lys-MCA	1.1	1.1
Boc-Gly-Ala-Arg-MCA	1.1	0.4
Suc-Leu-Leu-Val-Tyr-MCA	0.1	0.4

Since the insulin chain has been used as a general substrate for determining whether an enzyme has a peptidolytic activity, we examined the hydrolytic activity of Cat-P/J and -6 with insulin B-chain. The insulin chain (20 μM) was incubated with Cat-P/J or -6 at 37℃ for 1 or 24 h in the same reaction condition as examining with fluorogenic substrate (Fig. 2). The insulin B-chain was eluted at 21.3 min on HPLC analysis, which were indicated by arrows in Fig. 2A~E. Comparing Fig. 2A and 2C, or 2A and 2E, the amount of insulin was not affected by the 24-h incubation of Cat-P/J or -6, and any peaks did not appear during incubation. This shows that both cathepsins did not hydrolyze the insulin B-chain, which may be caused by the reason that insulin B-chain did not have suitable amino acid sequence for hydrolysis.

Expression of recombinant Cat-P/J and -6 in baculovirus expression system: Since Cat-P/J and -6 prepared by *E. coli* just showed very weak activity on the assays with the fluorogenic substrates in this study, we tried to prepare the Cat-P/J in the baculovirus expression

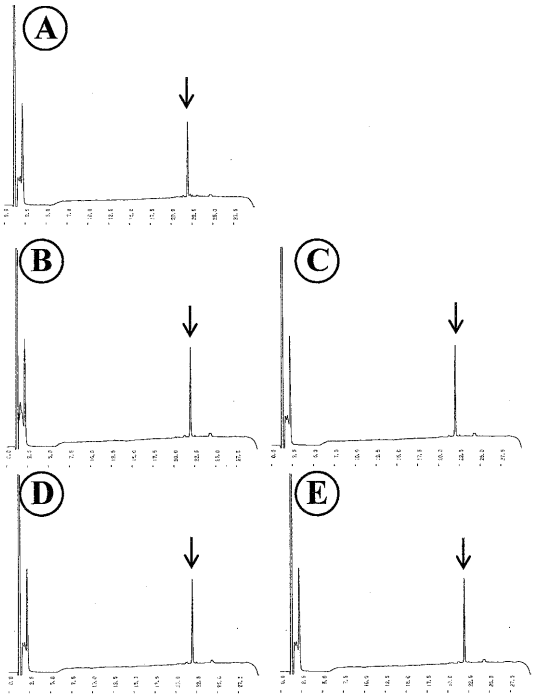

Figure 2. HPLC profiles of insulin B-chain incubated with recombinant Cat-P/J and -6. An initial insulin B-chain (A) and the ones incubated with recombinant Cat-P/J for 1h (B) or for 24 h (C), or Cat-6 for 1h (D) or for 24h (E) were analyzed by reversed phase HPLC. Arrows indicate the peaks of insulin B-chain.

system, which had several advantages over *E. coli* system for obtaining functional protein. We constructed the transfer vector pVL1392-His, which could express the protein possessing His-tag, for purifying recombinant proteins. For obtaining preprocathepsin-P/J, the PCR primers were designed for amplifying whole DNA encoding Cat-P/J. The PCR was performed with the cDNA generated from RNA of mouse placenta at 13 days post-coitus, and the amplified DNA was inserted into the appropriate site of pVL1392-His vector. The recombinant transfer vector and the AcNPV genome DNA were co-transformed into *Sf*-21 cells, and the cells undergoing homologous recombination were isolated by the procedures of end-point dilution. Culture medium of the isolated cells was used as a recombinant baculovirus suspension.

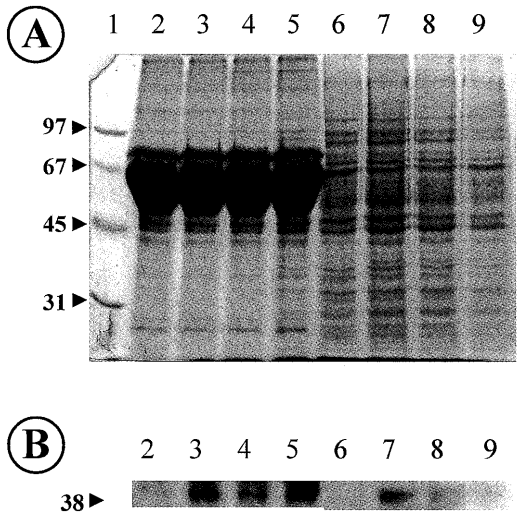

Figure 3. SDS-PAGE and immunoblotting analysis of recombinant Cat-P/J expressed in baculovirus system. Recombinant Cat-P/J was expressed in baculovirus expression system. The culture medium (lanes 2~5) and cell lysate (lanes 6~9) were recovered at 1st (lanes 2,6), 2nd (lanes 3,7), 3rd (lanes 4,8), or 4th day (lanes 5,9) after post-infection. These samples were separated by SDS-PAGE and stained with CBBG-250 (A). Immunodetection was performed with anti-His tag antibody (B). Molecular mass markers were indicated in lane 1 (A) and the left margin (A,B).

Cultured *Sf*-21 cells were infected with the recombinant virus, and the cultured medium and the cell lysate was recovered at 1st, 2nd, 3rd, and 4th day post-infection. The recombinant Cat-P/J was not distinguished in SDS-PAGE stained with CBBR-250 (Fig. 3A) during 4-days culture, which shows a little amount of recombinant protein in baculovirus expression system. Then, the recombinant Cat-P/J was analyzed by an immunoblotting with anti-His tag antibody because the recombinant Cat-P/J should have a detection tag of 6 x His (Fig. 3B). The majority of immunoreactive material was detected at around 38 KDa, the molecular weight of which was corresponded with the presumable one (38.6 KDa) of His-tagged preprocathepsin-P/J. The recombinant Cat-P/J accumulated in the cells until 2 days post-infection, and disappeared in the cell lysate, while it stared to accumulate at 2nd day post-infection in the culture medium, and the maximum amount of Cat-P/J in the medium was observed at 4th day post-infection. This expression profile was quite similar to that reporting a human preprocathepsin K expression in baculovirus system[35]. In the case of expressing the preprocathepsin K, the signal peptide corresponding to 15 amino acids in N-terminus was auto-processed during the secretion[35]. We could not determine by the electrophoresis whether the Cat-P/J secreted in the medium had the signal peptide because of small difference of molecular weight between the presence and absence of the signal peptide of Cat-P/J corresponding to 17 amino acids in N-terminus. It is necessary for the determination to analyze the N-terminal amino acid sequence using the purified recombinant protein.

Purification of the recombinant Cat-P/J from the culture medium of baculovirus expression system: The purification steps were illustrated in Fig. 4A. Since the maximum accumulation was achieved at 4th day post-infection, the culture medium (34 mL) was prepared from a large culture of *Sf*-21 cells cultured for 4 days after the infection, and loaded on the affinity column for His-tagged proteins. The pass-through fraction were recovered and concentrated by dialysis (7 mL). The bound material was eluted by high concentration of imidazole, and the removing imidazole and concentrating the sample were performed by dialysis (0.4 mL). The concentrated pass-through fraction was re-loaded on the affinity column, and the pass-through fraction and eluted fraction were recovered and concentrated to 3 mL and 0.5 mL, respectively. These samples were separated by SDS-PAGE, and visualized with silver staining (Fig. 4B). We could not, however, identify the recombinant Cat-P/J as the bound material (Fig. 4B, lanes 3 and 5). Thus, the immunodetection with anti-His antibody was performed on its analysis (Fig. 4C). Though the immunoreactive material was identified at around 38 KDa, recombinant Cat-P/J was not detected in the elute fractions (Fig. 4C, lanes 3 and 5). Since the recombinant Cat-P/J was recognized by anti-His antibody, the protein should have His-tag, which could bind nickel resin. The failure in the purification may be caused by a small amount of His-tagged protein, the concentration of which is not enough to bind each other.

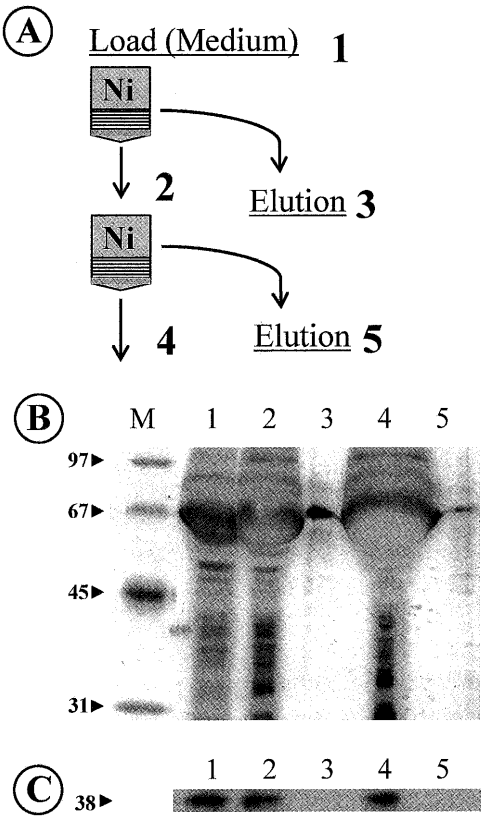

Figure 4. Purification of the recombinant Cat-P/J expressed in baculovirus system. The purification steps were illustrated (A). The culture medium at 4th day post-infection (1) was loaded onto affinity column for His-tag. Pass-through fractions (2,4) and eluted fractions (3,5) were recovered for analyses on SDS-PAGE visualized with silver stain (B) and immunodetection with anti-His tag antibody (C). The lane numbers in both (B) and (C) corresponded with the ones in (A), i.e. lanes 2,4 and 3,5 show the pass-through and eluted fractions, respectively. Molecular mass markers were indicated in lane M in (B) and the left margin (B,C).

Conclusion

Using *E. coli* expression system, we prepared placenta-specific cathepsin-P/J and -6, and showed that substrate specificity was different in each other. Though the hydrolytic activity was confirmed with fluorogenic peptide substrates, the activity was very weak in both cathepsins. Quite recently, Cat-P/J prepared by the *Pichia pastoris* expression system is reported to show high peptidolytic activity[36]. Further studies are required to obtain higher peptidolytic activity in the recombinant Cat-P/J and -6 prepared in this study.

Abbreviations: Boc, *t*-butyloxycarbonyl; EDTA, ethylenediaminetetraacetic acid; EST, expression sequence tag; FBS, fetal bovine serum; MCA, (7-methoxycoumarin-4-yl) acetyl; PCR, polymerase chain reaction; PVDF, Polyvinylidine fluoride; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tris, 2-Amino-2-hydroxymethyl-1,3-propanediol; Z, carbobenzoxy.

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マウス胎盤に高発現しているリソソーム・システインプロテアーゼ、カテプシンーP/Jおよびー6の組換えタンパク質の製作

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要　約

胎盤組織に局在するカテプシン（システインプロテアーゼ）は、ここ数年で8種類も同定されたが、いずれも遺伝子レベルの発現が確認されているだけである。本研究では、妊娠後11.5日から15.5日までのマウス胎盤でのみ発現するカテプシンーP/J（Cat-P/J）およびー6（Cat-6）に注目し、組換えタンパク質の製作による機能解明を目指した。両組換えタンパク質は大腸菌pET発現系で製作した。蛍光基質での解析により、Cat-Pはカテプシンの典型的基質であるZ-Phe-Arg-MCAとαスロンビンの基質であるBoc-Val-Pro-Arg-MCAの両者を、Cat-6は後者のみを切断することが明らかになった。これらの活性は非常に弱いものであったが、両者の基質特異性の差が初めて明らかになった。次に高活性の組換え体を得る目的で、昆虫細胞発現系での製作を試みた。組換えCat-P/Jの発現は、感染開始から4日目の培地中で最大となることを確認したが、精製するには至らなかった。

キーワード：バキュロウイルス発現系、リフォールディング、ペプチド基質、HPLC、ヒスチジンタグ