Technical paper

Efficient Removal of Cathepsin L from Active Cathepsin X using Immunoprecipitation Technique

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Abstract

Cathepsin X is a cysteine protease which is involved in various important physiological and pathological processes. For the purpose of biochemical and structural studies, pure and active cathepsin X is required without contamination with other related proteases. Recombinant cathepsin X was obtained by expression in methylotropic yeast *Pichia pastoris*. During purification, cathepsin X has to be activated with cysteine protease cathepsin L, however, separation methods, used so far, can not completely remove cathepsin L from the activated cathepsin X. Here we describe a new purification procedure which provides active recombinant cathepsin X without the presence of residual cathepsin L.

Keywords: Cathepsin X, cathepsin L, purification, immunoprecipitation

1. Introduction

Human cathepsin X is a lysosomal cysteine carboxymonopeptidase.[1] It is expressed in various cells of the immune system such as monocytes, macrophages and dendritic cells.[2] Additionally, it was also found in tumor and immune cells of prostate[3] and gastric carcinomas,[4] in macrophages of gastric mucosa after infection with *Helicobacter pylori*[5] and in cells of nervous system during the aging and neurodegenerative processes. Several studies on cathepsin X imply also its role in inflammatory processes.[5–8] Further, it was found that cathepsin X interacts with integrin receptors[9,10] influencing cell signaling and adhesion. Cathepsin X was also shown to promote T lymphocyte migration through 3D extracellular matrix barrier.[11] As shown recently by our group, β_2 integrin chain and enolase have been determined as natural substrates for carboxypeptidase activity of cathepsin X (N. Obermajer, submitted). Therefore, important physiological functions in regulation of immune response and neurodegenerative processes designate cathepsin X as a promising target in diagnostic and therapeutic applications.

To be used in further studies, in particular that determining enzyme specificity against natural substrates and in design of specific inhibitors, the preparation of pure recombinant enzyme is needed. Human cathepsin X has been expressed in yeast *Pichia pastoris* as an inactive proenzyme. Unlike other cathepsins, procathepsin X does not autoprocess and needs other proteases for its activation. The most efficient activation can be obtained at low concentrations of cathepsin L,[15] however, the removal of cathepsin L after activation could be a problem, since its small traces may change the enzymatic properties of the preparation.[1] The impurities may provide misleading results, as happened in studies, reporting that cathepsin X displays also dipeptidyl carboxypeptidase activity and that it can be inhibited by other cathepsin inhibitors, such as CA-074.[12, 13] Later it was found that dipeptidyl carboxypeptidase activity of purified cathepsin X from human liver could be attributed to the trace amounts of cathepsin B[1,14] and, actually, that CA-074 inhibits cathepsin B and not cathepsin X.[14] It was also shown that dipeptidyl carboxypeptidase activity of the recombinant human cathepsin X could be attributed to the contamination with cathepsin L which is used for the procathepsin X activation.[1]

In this study we show that significant amount of cathepsin L remains in recombinant cathepsin X sample after separation on SP Sepharose Fast Flow column at pH 4.4[1] and describe a method for effective removal of cathepsin L by immunoprecipitation.

2. Experimental

Human procathepsin X cDNA was amplified by PCR from human cathepsin X clone (RZPD German Resource Center for Genome Research, Germany) using primers 5´-CCGCTCGAGAAAAGAGAGGCTGAAG CTGGCCTCTACTTCCGCCGG-3´ and 5´-CA CTGCGG CCGCTTAAACGATGGGGTCCCCAAATGTACAGTGCTCC-3´. It was cloned and expressed in *P. pastoris* as an α-factor fusion construct as described previously.[15] The culture medium was centrifuged at 3000g for 10 min and the supernatant concentrated 10-fold using Amicon Ultrafilter membrane YM 10. The concentrate was applied to a CM-Sephadex column equilibrated with 50 mM sodium acetate buffer pH 5.0, and procathepsin X was eluted with 0.6 M NaCl in the same buffer. Procathepsin X was activated with 25 nM cathepsin L (versus 3–4 μM procathepsin X) in 50 mM sodium citrate, pH 5.5, containing 0.2 M NaCl, 1 mM EDTA and 2 mM DTT.[15] Cathepsins X and L were then separated on a SP Sepharose Fast Flow column.[1] Final sample was characterized by ELISA and Western blot using 3B10 monoclonal antibody to cathepsin X[2] and sheep anti-cathepsin L polyclonal antibody[16] to cathepsin L. Anti-mouse peroxidase conjugate and anti-sheep peroxidase conjugate (both from Sigma-Aldrich, St. Louis, MO) were used as secondary antibodies for visualization of the immune complex.

2. 1. Immunoprecipitation

After activation of procathepsin X with cathepsin L, pH of the sample was raised from 5.5 to 7.5 using 10 M NaOH to allow binding of the antibodies to cathepsin L. Sheep anti-cathepsin L polyclonal antibodies were added in the ratio 1:3 in favor of antibodies and the sample was incubated on a shaker for 2 hours at room temperature. 250 μl of nProtein A Sepharose 4 Fast Flow (GE Healthcare, Sweden) was washed four times in 50 mM sodium citrate, pH 7.5, containing 0.2 M NaCl, 1 mM EDTA and 2 mM DTT, resuspended in 50 μl of the same buffer and added to the sample. The suspension was incubated overnight on a shaker at 4 °C and subsequently centrifuged for 5 min at 10000 g. Supernatant was aliquoted and stored at –80 °C. Samples were analyzed with cathepsin L ELISA[16], SDS-PAGE using 10% Tris-Glycine gel and by Western blot.

2. 2. Deglycosylation

Different samples of cathepsin X were treated with peptide N-glycosidase F (Sigma-Aldrich) in order to assess the potential impact of changes in production and isolation procedure to the glycosylation profile. Concentration of cathepsin X during the deglycosylation was 0.1 mg/ml and reaction was performed according to the manufacturer's instructions. Samples were analyzed with SDS-PAGE using 10% Tris-Glycine gel and by Western blot.

3. Results and Discussion

In the first published purification procedure for isolation of recombinant active cathepsin X from *Pichia pastoris* ion exchange chromatography on DEAE column at pH 7.0 was used for separation of cathepsin L from active cathepsin X.[15] pH 7.0 might decrease the activity of cathepsin L, which was reflected in low k_{cat}/k_m values ($< 10^2 \cdot M^{-1} s^{-1}$) against Cbz-FR-MCA substrate.[14, 15] Later it was shown that the separation and/or inactivation was not complete,[1] since the residual cathepsin L was obviously the reason for confusing results on cathepsin X enzyme kinetics and specificities.[12, 13] Puzer and co-workers tried to improve the procedure by using SP Sepharose Fast Flow column at pH 4.4.[1] However, this purification step is still not sufficient to provide pure cathepsin X. On Western blot (Fig. 1a, lane 2) and ELISA (Fig. 1c) we clearly show that after separation on SP Sepharose Fast Flow there is still a significant amount of cathepsin L present in the final sample. The application of potent antibody, forming an immune complex with cathepsin L in a nanomolar concentration and its precipitation with Sepharose beads, coated with Protein A could be effective to remove contaminant. Indeed, using sheep polyclonal antibody in the immunoprecipitation procedure there was no cathepsin L in the final sample as detected by Western blot (Fig.1a, lane 3) and ELISA (Fig.1c). However, cathepsin L was clearly detected in the pull-down fraction (Fig. 1a, lane 6). In a control experiment cathepsin L was incubated at pH 7.5 for the same period of time as needed for antibody binding in the immunoprecipitation reaction to check whether higher pH could affect its stability.[17] After incubation cathepsin L was still detected by Western blot (Fig. 1a, lane 5). This is in line with the procedure of Nägler and coworkers who incubated cathepsin X sample for several hours at pH 7.0 while performing ion-exchange chromatography but were not able to remove cathepsin L contamination.

The molecular mass of cathepsin X isolated by our new procedure appears to be higher than the mass of the previously isolated cathepsin X used as a control (Fig. 1b). This could be attributed to the variation in glycosylation during the expression in *P. pastoris* rather than the changes

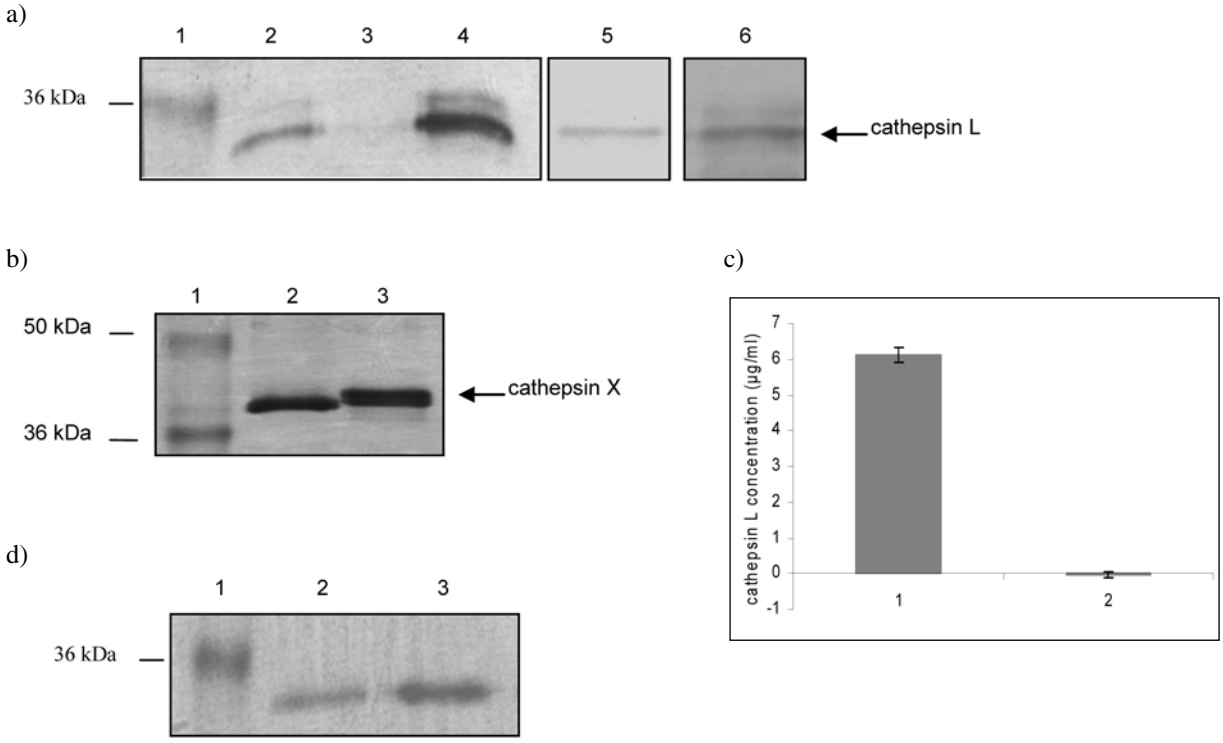

Fig. 1: Analysis of recombinant human cathepsin X

a) Western blot analysis using sheep polyclonal antibody against cathepsin L. 1 µg (lanes 2-4) or 5 ng (lane 5) of protein was loaded. Lane 1: protein standard (Invitrogen, SeeBlue® Plus 2 Prestained Standard); lane 2: cathepsin X isolated with SP Sepharose Fast Flow method; lane 3: cathepsin X after immunoprecipitation of cathepsin L; lane 4: cathepsin L, used for the activation of procathepsin X; lane 5: cathepsin L after 2 hours of incubation at pH 7.5; lane 6: cathepsin L pull-down fraction with sheep polyclonal antibody against cathepsin L.

b) Western blot analysis using 3B10 monoclonal antibody against cathepsin X. Lane 1: protein standard; lane 2: cathepsin X isolated with SP Sepharose Fast Flow method; lane 3: cathepsin X after immunoprecipitation of cathepsin L

c) The levels of cathepsin L in cathepsin X final sample, determined by ELISA (mean concentration of four parallels): after SP Sepharose Fast Flow method (1) and after immunoprecipitation (2).

d) Western blot of recombinant human cathepsin X after treatment with N-glycosidase F using 3B10 monoclonal antibody against cathepsin X. 1 µg of protein was loaded. Lane 1: protein standard; lane 2: deglycosylated cathepsin X isolated on SP Sepharose Fast Flow; lane 3 deglycosylated cathepsin X after immunoprecipitation of cathepsin L.

in isolation procedure. It is well known that the molecular mass of recombinant proteins may differ from one preparation to another when using *Pichia pastoris* expression system. In order to verify whether different glycosylation causes the changes in the molecular mass cathepin X samples were treated with peptide N-glycosidase F. Samples were separated with SDS-PAGE and detected by Western blot (Fig. 1d). The results show that after deglycosylation the samples of cathepsin X exhibited the same molecular mass. The enzymatic properties of cathepsin X are most likely similar since until now there was no report that different glycosylation could affect cathepsin X activity.

4. Conclusions

We developed a novel immunoprecipitation-based procedure which enables the complete purification of recombinant cathepsin X. Reported technique is time efficient and efficiently removes the residual cathepsin L, used in previous steps for the activation of procathepsin X. We believe that our finding may increase the reliability of the forthcoming studies on the physiological role of cathepsin X.

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6. References

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Povzetek

Katepsin X je cisteinska proteaza, ki je udeležena v različnih pomembnih fizioloških in patoloških procesih. Za potrebe biokemijskih in strukturnih raziskav je potreben čist in aktiven katepsin X brez vsebnosti sorodnih proteaz. Rekombinantni katepsin X smo izrazili v metilotropni kvasovki *Pichia pastoris*. V postopku izolacije je potrebno katepsin X aktivirati s cisteinsko proteazo katepsinom L, vendar z do sedaj objavljenimi metodami ni bilo mogoče popolnoma ločiti katepsina L in aktivnega katepsina X. V članku opisujemo novo metodo izolacije, s katero dobimo aktiven katepsin X brez prisotnosti katepsina L.