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Characterization of cathepsin L secreted by *Sf*21 insect cells

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Abstract

*Sf*21 cells, derived from the *Spodoptera frugiperda* pupa, are commonly used for the heterologous expression of proteins. While purifying recombinant proteins from this system we encountered a protease, secreted at high levels by *Sf*21 cells, that readily degraded recombinant proteins and also tended to co-purify with histidine-tagged proteins from Ni^{2+} affinity columns. Purification and characterization of the protease revealed that it has many properties consistent with cysteine proteases of the papain family, including autoactivation under reducing conditions and acidic pH, and inhibition by E-64. Amino acid sequence analysis showed that the *Sf*21 enzyme may be identical to a putative insect procathepsin L cloned from the cotton bollworm. The subsite specificity of the *Sf*21 cathepsin and its inhibition profile by cystatins are consistent with the protease being an insect homologue of cathepsin L. Monoclonal antibodies useful for the detection and purification of the insect cathepsin L were developed.

Keywords: Cysteine proteases; Cathepsin L; Subsite specificity; Cystatins; Monoclonal antibodies

The majority of known cathepsins are peptidases that utilize a catalytic cysteine residue in their active site. These enzymes, structurally related to papain, belong to the C1 peptidase family [1]. While C1 cathepsins are generally endopeptidases, there are known examples of exopeptidases in the family (e.g., cathepsins C and X) [2]. Most cathepsins are present in lysosomes, where they play a major role in intracellular protein degradation [1,2]. Cathepsins are also present in other cellular compartments, or are secreted and associated with the plasma membrane, allowing them to perform additional functions. Cathepsins are known to be involved in antigen presentation [1,3,4], zymogen activation [5], and prohormone processing [6]. Most cathepsins are ubiquitously expressed, but others have a more restricted tissue distribution [1,2], resulting in specialized functions. For example, cathepsin K is secreted by osteoblasts and plays a major role in bone resorption. Cathepsin K deficiencies are responsible for the bone diseases pycnodysostosis and osteopetrosis [7,8].

Cathepsins of the C1 family are expressed by most multicellular eukaryotes, including insects. The majority of insect cathepsins are homologous to mammalian cathepsins B and L [9]. A number of secreted insect cathepsins may be involved in the protein degradation that occurs during food digestion [10], embryogenesis [11,12], and metamorphosis [13,14]. Insect cathepsins may also be a part of a defense system, eliminating foreign proteins by degradation [15].

*Sf*21 insect cells are derived from pupal ovarian cells of *Spodoptera frugiperda* (Fall Armyworm) [16]. These cells are frequently used for the heterologous expression of recombinant proteins. During several attempts at the expression of mammalian cathepsins in the *Sf*21 cell line, we observed that the cell line secreted a protease with properties similar to mammalian cathepsins that also co-purified with the human enzymes. The insect protease was purified and biochemically characterized. The pH/activity and inhibitor profiles showed that the insect protease is a cysteine protease of the C1 family. Substrate specificity studies and amino acid sequence data revealed that the protease is an insect homologue of mammalian cathepsin L. Monoclonal antibodies were raised against the pro and

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mature forms of the insect cathepsin L. These antibodies are useful for the detection of the enzyme, and possibly its purification by immunoaffinity methods.

Materials and methods

Protein purification

All purification steps were performed at 6–10 °C. *Sf*21 cells were grown in Ex-cell 401 serum-free insect medium from JRH Biosciences (Lenexa, KS) to a density of 1.4×10^7 cells/ml. Conditioned medium was harvested, concentrated 10-fold by tangential flow ultrafiltration, and the pH was adjusted to 7.5 with 1 N NaOH. Insoluble material was removed by centrifugation at 21,000*g*. The soluble protein was diluted 4-fold with cold 5 mM Hepes,[1] 0.1 M NaCl, pH 7.5, then loaded onto an SP Sepharose (GE Healthcare, Piscataway, NJ) column equilibrated in 25 mM Hepes, 0.1 M NaCl, pH 7.5 (buffer A). Bound proteins were eluted using a linear gradient of NaCl (0.1–1 M) in buffer A, with the fractions collected in tubes containing 1 M NaCl to minimize precipitation of the eluted protein. Fractions were assayed, after autoactivation, using the substrate benzyloxycarbonyl-Phe-Arg-4-methyl-7-coumarylamide (Z-FR-MCA) at 10 μM in 50 mM NaOAc, 1 mM DTT, pH 5.0. The fractions with high specific activity were pooled. A 5-ml chelating Sepharose column (GE Healthcare) was charged with Ni^{2+} ions, then equilibrated in 25 mM Hepes, 0.5 M NaCl, and 10 mM imidazole, pH 7.5 (buffer B). Protein pooled from the SP Sepharose column was diluted 4-fold in buffer B and loaded onto the chelating Sepharose column. The column was washed with buffer B before the elution of bound proteins with 0.2 M imidazole, 0.5 M NaCl, pH 7.5. The protein eluted from the chelating Sepharose column was concentrated to 4 ml by ultrafiltration using a 30-kDa NMWL Ultracel membrane in an Amicon stirred cell from Millipore (Billerica, MA), and insoluble material removed by centrifugation at 16,000*g*. The concentrated soluble protein was then subjected to gel permeation chromatography on a 2.5×90 cm Superdex 200 (GE Healthcare) column equilibrated in 25 mM Hepes, 0.5 M NaCl, and 0.1% Brij 35, pH 7.5. The Superdex 200 column was calibrated with molecular mass standards (ferritin, catalase, aldolase, albumin, ovalbumin, and chymotrypsinogen A) purchased from GE Healthcare to determine the molecular mass of the native enzyme. SDS–PAGE was performed using Tris–glycine gels with molecular mass standards purchased from Bio-Rad Laboratories (Hercules, CA).

[1] *Abbreviations used:* Boc, *tert*-butoxycarbonyl; DTT, dithiothreitol; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); MCA, 4-methyl-7-coumarylamide; LC–MS/MS, liquid chromatography nano-spray mass spectrometry/mass spectrometry; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NaOAc, sodium acetate; RFU, relative fluorescence unit; Z, benzyloxycarbonyl.

Amino acid sequence analysis

The purified enzyme in solution was submitted to the Molecular Structure Facility of the University of California at Davis for NH_2-terminal sequence analysis by the Edman degradation procedure. The resulting amino acid sequence was used to search protein databanks for sequence matches using the Blastp program [17]. The selected sequence matches were aligned using Clustal W [18]. The purified protein was also submitted to the Molecular Biology and Biophysics Proteomic Analysis Core facility at the University of Minnesota, St. Paul for internal peptide analysis involving in-gel trypsin digestion followed by liquid chromatography nano-spray mass spectrometry/mass spectrometry (LC–MS/MS).

Determination of carbohydrate content

The carbohydrate content of the purified *Sf*21 protease was measured using a glycoprotein carbohydrate estimation kit purchased from Pierce Biotechnology (Rockford, IL). The *Sf*21 protease and standard proteins of known carbohydrate content were first oxidized by sodium *meta*-periodate, then the resulting aldehydes were reacted with an aldehyde detection reagent as recommended by the manufacturer. The absorbance at 550 nm was then measured for the standard proteins and the *Sf*21 protease, and a standard curve was generated to relate the absorbance to percentage carbohydrate content. Lysozyme was used as a negative control. Positive controls were ovalbumin, apotransferrin, fetuin, and α_1-acid glycoprotein.

Enzyme autoactivation and assay optimization

To determine the optimal conditions for autoactivation of the cathepsin, purified cathepsin was diluted >20-fold (to 0.02 mg/ml) in 0.1 M NaOAc, and 10 mM DTT, pH 5.0, then incubated at 37 °C to permit autoactivation. At various time points aliquots of the incubation mixture were removed for immediate assay using the Z-FR-MCA substrate. At each time point, 2 μg aliquots of protein were removed and the protein was precipitated by the addition of trichloroacetic acid to a final concentration of 10%. The precipitated protein was saved for analysis by SDS–PAGE under reducing conditions.

The activity of the *Sf*21 protease was examined under conditions in which the pH and ionic strength of the reaction buffer were varied. Purified enzyme autoactivated in 0.1 M NaOAc, 10 mM DTT, pH 5.0, was diluted 20-fold in 10 mM DTT, then diluted an additional 10-fold into microplate wells containing NaOAc buffers (pH 3–5) or Mes buffers (pH 5.5–7). Buffer concentrations were 55 mM, and the final DTT concentration was 1 mM. NaCl concentration was varied at each pH from 0–1 M. Reactions were initiated by the addition of Z-FR-MCA substrate to 10 μM final concentration. Reaction rates were monitored by measuring the rates of liberation of free 7-