An aspartic protease that is significantly produced by baculovirus-infected Spodoptera frugiperda Sf9 insect cells was purified to homogeneity from a growth medium. To monitor aspartic protease activity, an internally quenched fluoresce (IQF) substrate specific to cathepsin D was used. The purified aspartic protease showed a single protein band on SDS–PAGE with an apparent molecular mass of 40 kDa. The N-terminal amino acid sequence of the enzyme had a high homology to a Bombyx mori aspartic protease. The enzyme showed greatest affinity for the IQF substrate at pH 3.0 with a $K_m$ of 0.85 $\mu$M. The $k_{cat}$ and $k_{cat}/K_m$ values were $13 \pm 1$ and $15 \pm 1$ $\mu$M$^{-1}$ $s^{-1}$ respectively. Pepstatin A proved to be a potent competitive inhibitor with inhibitor constant, $K_i$, of 25 pm.

Key words: aspartic protease; Sf9 insect cell; baculovirus; protease inhibitor

Insect cells can post-translationally fold and modify eukaryotic proteins in a manner similar to mammalian cells. When a recombinant baculovirus carrying a specific gene is used to infect insect cells, the cells produce large quantities of post-translationally modified recombinant proteins in a short period of time.1,2 This baculovirus expression vector system (BEVS) is used to produce recombinant proteins that can be applied in a number of fields, relating not only to basic life science research but also therapeutics and pharmaceuticals. Endogenous proteases are concomitantly produced during the production of recombinant proteins.3–6 Some endogenous proteases convert expressed precursor proteins into beneficial mature proteins, such as active human renin7,8 and functional hepatocyte growth factor.9 In many cases, however, these proteases can degrade recombinant expressed proteins before harvest.10–13 Human renin proteolytically activated from its precursor, prorenin, is also partially fragmented at a very late stage of infection culture.14

To improve the productivity of expressed recombinant proteins, characterization of proteases concomitantly produced in baculovirus-infected insect cell cultures is necessary. A mammalian-cathepsin L-like cysteine protease was identified in a gene of Autographa californica multiple nucleopolyhedrovirus (AcMNPV).9 The cysteine protease was recently determined to be responsible for the activation of expressed human prorenin into active rennin.10 A similar cysteine protease was found within the genome of the Bombyx mori nuclear polyhedrosis virus (BmNPV), and its enzymatic properties were analyzed.15 To minimize the proteolytic degradation of recombinant proteins due to BEVS, recombinant strains of baculovirus with deletions of the cysteine protease genes were generated.16,17 Besides cysteine proteases, aspartic proteases are also produced in significant amounts by baculovirus-infected Spodoptera frugiperda Sf9 insect cells throughout infection cultures.18 In addition, three types of protease activities have been found in the BEVS by zymography.19

The present study was conducted to purify an aspartic protease encoded in a gene of Sf9 insect cells from a culture medium of baculovirus-infected Sf-9 cells. Some enzymatic properties were examined using an internally quenched fluoresce (IQF) substrate that is specific to a typical aspartic protease, cathepsin D.

Sf9 insect cells were grown in serum-free SF-900 II medium (Invitrogen, Carlsbad, CA) at 28°C, using a 1-liter spinner flask equipped with bubble-free aeration tubing and a rubber heating pad. Agitation of the medium was done at a rate of 100 rpm, and a dissolved oxygen concentration was maintained at 5% of air saturation. At a cell density of $1 \times 10^6$ cells ml$^{-1}$, the cells were infected with a recombinant strain (vhpR) of AcMNPV containing human preprorenin cDNA under the control of a polyhedrin promoter at a multiplicity of infection (MOI) of 1.0 plaque-forming unit per cell (pfu cell$^{-1}$). Culture samples (1 ml) were periodically withdrawn. After centrifugation, cell pellets were sonicated in 1 ml of phosphate-buffered saline (PBS) and centrifuged to remove cell debris. For protease assay, a synthetic IQF substrate, [(7-methoxycoumarin-4-yl) acetyl (MOCAc)-Gly-Lys-Pro-Ile-Leu-Phe$^*$-Phe-Arg-Leu-Lys (2,4 dinitro-
phenyl (Dnp)-d-Arg-NH₂ (Peptide Institute, Osaka, Japan), designed for cathepsin D, was used; the asterisk represents a scissile peptide bond by cathepsin D. The resulting cell fractions and medium were incubated in a 50-μl assay mixture containing 100 mM sodium citrate at pH 3.0, 100 mM NaCl, 0.02% Tween 20, 0.02% NaN₃, 10 μM leupeptin, and 20 μM IQF substrate at 37°C for 10 min. Following this, the protease reaction was terminated by the addition of 200 μl of 100 mM triethanolamine. The fluorescent intensity of the hydrolyzed substrate was measured at an emission wavelength of 440 nm on excitation at 340 nm. One unit of protease activity was defined as the amount of enzyme that produced 1 μmol of the reference compound, MOCAC-Pro-Leu-Gly (Peptide Institute) per min.

Figure 1 shows the protease activity determined in the infection culture of Sf9 using a synthetic IQF substrate specific to cathepsin D. Intracellular enzyme activity was measured during the initial stages of infection at a certain level, whereas extracellular activity gradually increased, rapidly at the end of culture. These results suggest that aspartic protease is encoded in the gene of Sf9 insect cells.

To identify aspartic protease activity, we tried to purify the Sf9 derived aspartic protease enzyme (SAP), which is active against the synthetic IQF substrate. At the completion of culture, the medium was dialyzed against 20 mM citrate buffer, pH 4.5, containing 0.2 mM NaCl and 2 μM E64. It was then loaded on a pepstatin-aminohexyl Sepharose column (2.5 × 7.0 cm) previously equilibrated with the same buffer. After it was thoroughly washed with the same buffer, the SAP was eluted with 200 μl of 100 mM Tris-HCl, pH 7.5, and 8.0 ml of the eluate was collected in each tube. Then 300 μl of 10% acetic acid and 0.02% Tween 20 were added to each of the tubes to prevent inactivation. The eluted fractions were dialyzed against 20 mM sodium phosphate, pH 7.0, and 0.02% Tween 20, and then chromatographed on a DEAE Sepharose Fast Flow column (4.5 × 5.0 cm) previously equilibrated with the same buffer. SAP was eluted with a linear gradient of 0–0.3 M NaCl. The active fractions were concentrated with Amicon YM-10 and applied to a Sephadex 200 column equilibrated with 20 mM phosphate buffer, pH 7.0, containing 0.15 M NaCl, 0.02% Tween 20, and 0.02% NaN₃. The column was calibrated with blue dextran and standard molecular weight markers: catalase (232 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), aprotinin (6.5 kDa), and vitamin B12 (1.35 kDa). SAP was eluted from the column at a retention time corresponding to a molecular mass of 37 kDa. The eluate fractions were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) following Laemmli(18) using a 10–20% gradient polyacrylamide gel (E-T1020L, Atto, Tokyo). The fraction having the highest protease activity showed a single protein band on electrophoresis at a molecular mass of 42 kDa (Fig. 2). When a culture medium of uninfected Sf9 grown to the stationary phase, having a higher SAP activity, was used for SAP purification in the way described above, SAP was found to be eluted from a Sephadex 200 column at a retention time corresponding to a molecular mass of 81 kDa in addition to 37 kDa.

Fig. 1. Time Course of Aspartic Protease Activity Determined in Cultures of Baculovirus-Infected Sf9 Insect Cells with Synthetic IQF Substrate.

Protease activity was determined as described in the text. Cell density was determined by counting in a hemacytometer. Symbols: ●, intracellular activity; ○, extracellular activity; △, cell density.

Fig. 2. SDS–PAGE of SAP Purified from a Baculovirus-Infected Sf9 Insect Cell Culture.

Proteins were stained with Coomassie Brilliant Blue R-250. Lane M, protein molecular standard; lane 1, SAP purified from virus-infected Sf9 (0.5 μg).

Table 1. Purification Summary for Aspartic Protease Produced by Baculovirus-Infected Sf9 Insect Cells

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Spec. activity (U/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>760</td>
<td>22.8</td>
<td>0.04</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Pepstatin aminohexyl Sepharose</td>
<td>13.1</td>
<td>2.65</td>
<td>0.20</td>
<td>5</td>
<td>9.2</td>
</tr>
<tr>
<td>DEAE Sepharose</td>
<td>0.34</td>
<td>2.11</td>
<td>6.27</td>
<td>161</td>
<td>7.3</td>
</tr>
<tr>
<td>Superdex 200</td>
<td>0.03</td>
<td>0.40</td>
<td>13.8</td>
<td>354</td>
<td>1.4</td>
</tr>
</tbody>
</table>
B. mori aspartic protease (Cath D)

Drosophila pseudoobscura Q292G7

Drosophila melanogaster Q7K485

Table 2. Comparison of Selected Amino Acid Sequences of SAP and Aspartic Proteases from Several Insect Cells

<table>
<thead>
<tr>
<th>Aligned Sequence</th>
<th>Homology</th>
</tr>
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<tbody>
<tr>
<td>FLVYRLV RMKTVRKHFQEVGETLDVQ</td>
<td>—</td>
</tr>
<tr>
<td>LYRVPYVRMKTARTFHEVGETEEEL</td>
<td>72%</td>
</tr>
<tr>
<td>LERYPLNR FQSARRHEADVGETEQO</td>
<td>60%</td>
</tr>
<tr>
<td>LERYPLHR FQSARRHEADVGETEQO</td>
<td>56%</td>
</tr>
</tbody>
</table>

Fig. 3. Dixon Plot of SAP Using Synthetic IQF Substrate.

SAP was assayed with a synthetic IQF substrate at concentrations of 1 μM (○) and 4 μM (●) in the presence of the indicated amounts of pepstatin A (MW, 686).

Both the SAP fractions gave a single protein band on the SDS–PAGE at the same position as that for virus-infected Sf9 cells. This indicates that SAP is present as a homodimeric protein as well as a monomeric protein of approximately 40 kDa. Table 1 summarizes the purification procedure for SAP from the culture medium of virus-infected Sf9 cells. It was purified to homogeneity at approximately 350 fold, and it had a specific activity of 13.8 units/mg-protein.

The purified SAP was analyzed by N-terminal amino acid sequencing of up to 25 amino acids. The sequence of 13.8 units/mg-protein.

pepstatin A, but this was not accomplished by inhibitors against serine proteases, aspartic proteases, or metalloproteases.

We examined the effect of the pepstatin A concentration on the rate of reaction (v) of the enzyme at two different substrate concentrations. Figure 3 illustrates a plot of 1/v against the concentration of the inhibitor (Dixon plot). The lines converge above the horizontal axis, indicating that pepstatin A competitively inhibits SAP. Based on the point of intersection of the lines, the inhibitor constant, K_i, of pepstatin A to SAP was estimated to be 25 μM.

In the present study, we examined a cathepsin D-like aspartic protease whose activity is maintained at a certain level in both virus-infected and uninfected Sf9 insect cells and increases in the medium. The enzyme was purified from a medium and characterized for several properties. It is encoded in the gene of Sf9 insect cells, and has an amino acid sequence similar to that of cathepsin D of B. mori. Peptatin A was a potent enzymatic inhibitor. By incubating the purified SAP with rh-renin, SAP was found not to be the enzyme responsible for proteolytic degradation of rh-renin expressed in BEVS (data not shown). However, as far as the proteolysis by SAP is concerned, supplementation with pepstatin A in BEVS is expected to be useful in protecting expressed recombinant proteins from proteolytic degradation.

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References
