Efficient, low-cost protein factories: Expression of human adenosine deaminase in baculovirus-infected insect larvae

(recombinant proteins/*Trichoplusia ni*)

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ABSTRACT Human adenosine deaminase (EC 3.5.4.4), a key purine salvage enzyme essential for immune competence, has been overproduced in *Spodoptera frugiperda* cells and in *Trichoplusia ni* (cabbage looper) larvae infected with recombinant baculovirus. The coding sequence of human adenosine deaminase was recombined into a baculovirus immediately downstream from the strong polyhedrin gene promoter. Approximately 60 hr after infection of insect cells with the recombinant virus, maximal levels of intracellular adenosine deaminase mRNA, protein, and enzymatic activity were detected. The recombinant human adenosine deaminase represented 10% of the total cellular protein and exhibited a specific activity of 70 units/mg of protein in crude homogenate. This specific activity is 70–350 times greater than that exhibited by the enzyme in homogenates of the two most abundant natural sources of human adenosine deaminase, thymus and leukemic cells. When the recombinant virus was injected into insect larvae, the maximum recombinant enzyme was produced 4 days postinfection and represented about 2% of the total insect protein with a specific activity of 10–25 units/mg of protein. The recombinant human adenosine deaminase was purified to homogeneity from both insect cells and larvae and demonstrated to be identical to native adenosine deaminase purified from human cells with respect to molecular weight, interaction with polyclonal anti-adenosine deaminase antibody, and enzymatic properties. A pilot purification yielded 8–9 mg of homogeneous enzyme from 22 larvae. The production of large quantities of recombinant human adenosine deaminase in insect larvae is inexpensive and rapid and eliminates the need for specialized facilities for tissue culture. This method should be applicable to large-scale production of many recombinant proteins.

Adenosine deaminase (ADA; adenosine-aminohydrolase, EC 3.5.4.4) catalyzes the conversion of adenosine and deoxyadenosine to inosine and deoxyinosine. In humans deficiency of this purine salvage enzyme is associated with severe immunodeficiency and lymphopenia in both B- and T-cell lineages (1, 2). Pharmacologic agents that inhibit ADA induce lymphopenia and have been employed in the treatment of certain leukemias. The enzyme has been isolated as a single polypeptide from erythrocytes (M_r 36,000; ref. 3) and from leukemic cells and thymus (M_r 41,000; ref. 4). In lung and kidney, ADA is associated with a large "complexing protein" (M_r 298,000; ref. 5) of unknown function. While much is now known about the enzymatic activity of human ADA, including its interaction with various substrates (4) and inhibitors (6–8), no direct information is available concerning the enzyme active site. Although a preliminary analysis of crystals of murine ADA has been reported (9), the three-dimensional fine structure of the human enzyme is not known.

It has been suggested that the enzyme undergoes a conformational change upon inhibitor binding (6, 7) and that a reactive histidine group participates in catalysis of calf intestinal ADA (10). However, lack of sufficient quantities of human ADA has precluded detailed structural studies of important enzyme domains.

Human thymus contains relatively abundant quantities of ADA (7), but the scarcity of this tissue has rendered impractical the purification of the large quantities of protein required for structural and mechanistic studies. We have therefore explored production of ADA through recombinant DNA technology. In this paper, we describe high-level expression of human ADA from recombinant baculovirus in insect cells and larvae and a rapid procedure for purification of the recombinant protein.

MATERIALS AND METHODS

Materials. Restriction endonucleases *Hinf*I and *Nco*I were obtained from New England Biolabs. Endonuclease *Bam*HI, T4 DNA ligase, and Klenow fragment of DNA polymerase I were purchased from Promega. [14 C]Adenosine and [α - 32 P]-dATP were DuPont/NEN products. Human ADA was purified from thymus (7). The polyclonal antibody used to detect ADA by immunoblotting was raised in rabbits against human ADA and purified by antigen-affinity chromatography. All other chemicals were reagent grade.

Plasmids, Viruses, Cells, and Larvae. The transfer vector pAcC4 was kindly provided by E. Kawasaki (Cetus). *Autographa californica* nuclear polyhedrosis virus (AcMNPV strain Li) and *Spodoptera frugiperda* (Sf9) cells were generously provided by Marijo Wilson (University of Kentucky). The Sf9 cells were cultured in TMN-FH (11) or TC-100 (GIBCO) medium at 27°C. Ken Haynes (University of Kentucky) provided cabbage looper (*Trichoplusia ni*) eggs. The insect larvae were grown on a diet (modified from ref. 12) consisting of 450 g of dry pinto beans (soaked overnight in distilled H₂O), 9 g of ascorbic acid (Sigma), 2.5 g of sorbic acid (ICN), 6 g of methylparaben (Sigma), 20 g of Alphacel (ICN), 55 g of vitamin diet fortification mix (ICN), 5 ml of formaldehyde (Fisher), 150 g of brewers' yeast (ICN), and 600 ml of H₂O. These ingredients were blended and mixed with 6.2 g of agar dissolved in 500 ml of boiling H₂O. The diet mixture (4 fl oz, $\approx$ 110 ml) was poured into 8-fl-oz Thompson cups (Capital Paper, Louisville, KY) and solidified in the open containers at room temperature.

Construction of Recombinant Baculovirus. Human ADA cDNA, the *Eco*RI-*Eco*RI fragment of an ADA clone (from a λ gt10 library that was kindly provided by D. A. Wiginton, Childrens Hospital Research Foundation, Cincinnati, OH), was first inserted into the *Eco*RI site of pBR322, generating plasmid pBR8. pBR8 was digested with *Hinf*I, filled in with

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Abbreviations: ADA, adenosine deaminase; AcMNPV, *Autographa californica* nuclear polyhedrosis virus.

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FIG. 1. Construction of the pAcC4ADA transfer vector containing the polyhedrin gene promoter and the human ADA cDNA that encompasses the coding region for the protein. This vector was used along with the baculovirus AcMNPV Li to cotransfect Sf9 cells. Kb, kilobase(s); Kbp, kilobase pairs.

DNA polymerase Klenow fragment, and then digested with *Nco* I. This *Nco* I-blunt fragment was isolated from a low-melting-temperature agarose gel and ligated into pAcC4 vector DNA that had been prepared as illustrated in Fig. 1. The vector DNA was digested with *Bam*HI, extended at the restriction sites with Klenow fragment, and then digested with *Nco* I to create a directed cloning site. The resulting construct was termed pAcC4ADA and was designed to encode a nonfusion protein after homologous recombination with AcMNPV, the insect baculovirus. AcMNPV strain Li was purified, and 1 μ g of extracellular virus DNA was combined with 2 μ g of CsCl-purified pAcC4ADA in a cotransfection of Sf9 cells. Recombinant viruses that had undergone homologous recombination (designated AcMNPV strain Li/huADA) were identified by visual screening of plaque isolates, facilitated by the uptake of neutral red dye into uninfected viable cells (Fig. 2). Putative recombinant viral isolates were identified as nonstained cells containing no

polyhedrin protein occlusion bodies. Recombinant viruses were isolated and plaque-purified three times to obtain pure recombinant virus stocks.

Cell Culture and Larval Growth Conditions. Sf9 cells, grown as a monolayer in 25-cm² tissue culture flasks, were inoculated with the virus stock by incubation of virus and cells in culture medium (lacking fetal bovine serum) for 1 hr at 27°C (11). The inoculum was aspirated and replaced with TMN-FH medium supplemented with 10% fetal bovine serum, amphotericin B (0.3 μ g/ml), penicillin (60 μ g/ml), and streptomycin sulfate (270 μ g/ml). The cells were cultured at 27°C and collected for assays.

Insect larvae were produced by hatching *Trichoplusia ni* eggs. An egg sheet (containing about 20 eggs) was stapled to the lid of each of the Thompson cups containing room-temperature insect diet. The eggs hatched and about 75% grew to the appropriate size (3 cm in length, fourth instar) in 14 days at room temperature in the humid atmosphere of the

FIG. 2. Identification of recombinant baculovirus. Sf9 cells were cotransfected with AcMNPV and pAcC4ADA. An overlay of phenol red in semisolid medium was added to the cells and allowed to absorb overnight. A, area of cells containing potential recombinant virus; B, cells containing wild-type virus producing polyhedrin coat protein.

FIG. 3. Time course for the production of ADA enzyme activity in recombinant virus-infected cells. Sf9 cells (3×10^6) were seeded into 25-cm² flasks and infected with the recombinant virus. At the times indicated, cells were harvested and extracts were prepared and assayed for ADA activity. One unit of enzyme activity is defined as 1 μ mol of inosine produced per minute at 37°C.

closed cups. Larvae (fourth instar) were sedated by incubation on ice for 15 min and then injected near the proleg (forward along the body cavity) with 10 μ l of medium containing recombinant baculovirus. When optimum quantities of recombinant enzyme accumulated in the insects (4 days after injection), larvae were harvested and immediately frozen at -70°C .

ADA Assays. Cells were washed with phosphate-buffered saline, resuspended in 0.25 M potassium phosphate buffer, pH 7.4/1 mM 2-mercaptoethanol, and sonicated on ice (three 15-sec bursts). The sonicate was centrifuged (4°C) for 10 min in an Eppendorf centrifuge to clarify the supernatant solution. Larvae were added to extraction buffer (10 mM sodium acetate, pH 6.4/2 mM EGTA/8 mM benzamidine/10 mM 6-aminocaproic acid/1 mM phenylmethylsulfonyl fluoride) and homogenized on ice with a Polytron tissue disrupter (Brinkmann). The homogenate was centrifuged at $100,000 \times g$ for 1 hr. The radioassay for ADA activity, which employs [8-¹⁴C]adenosine as the substrate, was described previously (13). Protein was quantitated by the method of Lowry *et al.* (14) with bovine serum albumin as the standard.

Purification of Recombinant ADA. Frozen larvae (total, 7–8 g) were added to 20 ml of extraction buffer (see above) and homogenized (on ice) with two 15-sec bursts of a Polytron set at medium speed. The crude extract (25 ml) was centrifuged at $20,000 \times g$ for 1.5 hr. To remove viral DNA that would

inhibit binding of ADA to the DEAE ion-exchange matrix, the clarified extract was treated with 28 mg of protamine sulfate that had been neutralized with KOH. The clarified supernatant from this step was applied to a 100-ml DEAE-Sephadex column equilibrated in 10 mM sodium acetate (pH 6.4). The column was washed with 3 volumes of buffer and the ADA protein was eluted in the sodium acetate buffer containing 0.5 M NaCl. The eluate was brought to 70% saturation with ammonium sulfate and left at 4°C overnight. The ammonium sulfate precipitate was resuspended in 2.8 ml of phosphate-buffered saline (10 mM sodium phosphate/140 mM NaCl, pH 7.4) containing 1 mM EGTA and 0.02% sodium azide. The protein suspension was applied to a 50-ml adenosine-Sepharose (15) column and eluted with the same buffer at a flow rate of 0.2 ml/min. Fractions containing the bulk of the ADA activity were pooled and concentrated by using an Amicon ultrafiltration unit.

RESULTS

Isolation of Recombinant Baculovirus Encoding Catalytically Active Human ADA. The ADA transfer-vector construct contained the entire cDNA coding sequence directly downstream from the baculovirus polyhedrin protein promoter with no extra sequences 5' to the ATG start site (pAcC4ADA, Fig. 1). After coinfection of Sf9 cells with the transfer-vector construct and wild-type baculovirus, three independent recombinant isolates were identified by plaque morphology screening (Fig. 2). To confirm that these isolates contained ADA nucleic acid sequences, each culture was analyzed for the presence of ADA sequences by dot blot hybridization to a labeled ADA cDNA probe. Whereas uninfected wild-type Sf9 cell extracts did not exhibit hybridizable sequences, each of three isolates (8A, B, and C) did exhibit ADA sequences (data not shown).

Analysis of ADA enzymatic activity present in recombinant virus-infected cells is shown in Fig. 3. Enzyme activity was detectable at 24 hr postinfection and reached maximal levels at 60 hr. In contrast, cells infected with wild-type baculovirus contained virtually no ADA activity (<0.001 unit/mg) at 60 hr. Accumulation of ADA as a function of the amount of recombinant virus used in the infection was optimized. At the plateau optimum, infection of 3×10^6 cells yielded ADA with a specific activity of 71.5 units/mg of protein at 60 hr postinfection. This level of accumulation is a 70- to 350-fold enrichment in the enzyme specific activity over the two richest natural sources of human ADA (thymus, 1 unit/mg of protein; leukemic cells, 0.2 unit/mg of protein).

FIG. 4. SDS/PAGE and immunoblot analysis of recombinant human ADA present in Sf9 cells. Cell extracts were applied to 10% gels in SDS/PAGE. After electrophoretic separation, one gel was stained directly with Coomassie blue (*Left*). Lanes 1 and 8, molecular weight markers (top to bottom, M_r 97,400, 42,700, 31,500, 21,500, 14,400); lanes 2 and 7, 2.7 μ g of purified human thymus ADA; lanes 3 (21 μ g) and 5 (11 μ g), proteins present in AcMNPV Li/huADA-infected cells; lanes 4 (11 μ g) and 6 (11 μ g), proteins in wild-type Sf9 cells. Proteins in the other gel (*Right*) were subjected to electrophoretic transfer onto nitrocellulose and tested for reactivity with monospecific rabbit anti-human ADA. The antigen was detected by reaction with a peroxidase-coupled secondary antibody (Biogenex Labs, Dublin, CA). Lanes 9 and 18, 0.9 μ g of purified human ADA standard; lanes 10 (5.5 μ g), 12 (1.2 μ g), 14 (0.6 μ g), and 16 (0.1 μ g), uninfected cells; lanes 11 (5.5 μ g), 13 (1.1 μ g), 15 (0.6 μ g), and 17 (0.1 μ g), recombinant ADA virus-infected cells.

Table 1. Purification of recombinant human ADA from recombinant baculovirus-infected insect larvae

Fraction	Protein, mg	Volume, ml	Enzyme units	Specific activity, units/mg	Percent yield*
Crude extract supernatant	570	25	6450	11	100
Protamine sulfate supernatant	299	24	2651	8	44
DEAE eluate	109	51	5322	48	82
Adenosine-Sepharose eluate	9	23	3584	398	55

*Yield is based on recovery of enzyme activity. The high salt concentration inhibited ADA in step 2.

To ensure that the kinetic properties of the enzyme were maintained throughout the vector manipulations, we determined the K_m for the natural substrate, adenosine, as well as the K_i for a potent inhibitor, *erythro*-9-[3-(2-hydroxynonyl)]-adenine (EHNA). The K_m for adenosine was 50 μ M and the K_i for EHNA in the reactions was calculated to be 15 nM. These values were identical to those reported for naturally occurring human ADA (16).

Immunoreactive Human ADA in Virus-Infected Cells. SDS/PAGE analysis of lysates of Sf9 cells infected with recombinant virus revealed a Coomassie blue-stained protein identical in molecular weight to ADA isolated from human thymus (Fig. 4, lanes 3 and 5). The band was not observed in control uninfected wild-type Sf9 cells (lanes 4 and 6). From the Coomassie-stained gel, it appeared that $\approx 10\%$ of the protein in the crude extract represented human ADA. The protein band was identified as ADA on the basis of its crossreaction with a monospecific rabbit anti-human ADA antibody in an immunoblotting assay (lanes 11, 13, and 15). No immunoreactive material was present in uninfected Sf9 cells (lanes 10, 12, 14, and 16) or in Sf9 cells infected with wild-type baculovirus (data not shown).

Production of Recombinant Human ADA in Insect Larvae. Successful expression of human ADA in cultured insect cells prompted us to attempt virus infection of a natural host insect, which would then function as an inexpensive medium for accumulation of large quantities of the recombinant protein. For these experiments, we selected the larva of *Trichoplusia ni*, the common cabbage looper moth, as a

baculovirus host. Although potential problems with injection injury have been cited in the literature (19), we were able to inject up to 10 μ l of recombinant viral DNA stock (AcMNPV Li/huADA), with $<10\%$ injection-induced mortality. Under these conditions the life-span of virus-injected survivors was 5 days. Protease digestion of human ADA was not observed in the cultured insect cells (see Fig. 4). However, the whole-insect homogenates did appear to contain proteases as reflected in the SDS/PAGE profile (data not shown). Several strategies aimed at eliminating proteolysis were compared. Hemolymph, collected from each larva by bleeding, was assayed for ADA activity. However, the specific activity of ADA in this fluid was low in comparison to the whole homogenate and the extra effort required to collect the fluid was not warranted. Therefore, protease inhibitors (phenylmethylsulfonyl fluoride, benzamidine, 6-aminocaproic acid, and EGTA) were added to the solution used to homogenize frozen larvae. Under these conditions, intact ADA was readily detected in crude extracts (data not shown) and human ADA activity was not affected by the presence of these protease inhibitors. The specific activity of ADA ranged from 1.2 units/mg of protein on day 1 to 27 units/mg on day 4 (postinjection). Although $>80\%$ of the larvae were dead on day 5, they yielded ADA with a specific activity of 29 units/mg of protein. However, larvae were harvested routinely on day 4 following infection with the recombinant virus.

Purification of Recombinant Human ADA. The specific activity of human ADA in the larval homogenates was 10–300 times higher than the value obtained from human tissues that have been used traditionally to purify this enzyme. Therefore, we were able to simplify the purification protocol. A sample scheme is shown in Table 1. For this preparation, 22 frozen larvae were used. ADA obtained by this procedure exhibited a specific activity of ≈ 400 units/mg of protein [a value we have consistently observed for homogeneous preparations of the enzyme obtained by our standard method (8)] and was $>95\%$ pure on the basis of SDS/PAGE (Fig. 5, lanes 1–4). The Coomassie-stained protein bands comigrating with human ADA and at lower molecular weights crossreacted with anti-ADA antibody (data not shown).

DISCUSSION

This study has demonstrated that catalytically active human ADA can be expressed in recombinant baculovirus-infected insect cells to levels corresponding to 10–15% of the total cell protein. This level of ADA expression far exceeds that observed in any natural human tissue source. A number of other eukaryotic proteins have been successfully overproduced in insect cells by placing gene coding sequences under the control of the baculovirus polyhedrin promoter (17). However, production of large quantities of recombinant protein by this approach requires access to specialized large-scale tissue culture facilities as well as defined and expensive tissue culture media (17). To circumvent these requirements, we investigated the infectivity of the recombinant baculovi-

Fig. 5. SDS/PAGE analysis of purified recombinant human ADA produced in insect larvae. Samples were applied to 12.5% gels in SDS/PAGE. After electrophoretic separation, the gel was stained with Coomassie blue. Lanes 1–4, recombinant ADA (8, 32, 2.5, and 5.0 μ g, respectively); lane 5, molecular weight markers.

rus as well as the stability of the viral gene product in insect larvae. The activity of the polyhedrin promoter has been reported to be very high in larvae (18), but others have reported a high mortality rate in inoculated larvae and suggested that coinfection with recombinant and wild-type baculoviruses is necessary to produce encapsidated recombinant virus particles that can be used for infection *per os* (19). As demonstrated in this paper, injection of the recombinant baculovirus AcMNPV Li/huADA near the proleg caused negligible loss of larvae due to inoculation injury and resulted in expression of recombinant human ADA to 2–5% of the total insect protein. Maintenance and total manipulation of 40 larvae requires about 3 hr of labor per week. It was unnecessary to collect hemolymph from the insect, and the frozen larvae were homogenized whole. Purification of human ADA (9 mg) from this source (22 larvae) was accomplished in three stages within 2 days. The number of larvae used in each purification can be easily manipulated so that with ADA it should be feasible to produce about 50 mg homogeneous protein in 2 days. By contrast, employing standard tissue culture techniques would require sophisticated instrumentation for bulk processing of large quantities of insect cells (20) and would incur a substantial investment in media, labor, and training.

The use of the larvae as host for viral replication represents an easy and inexpensive method for production of recombinant proteins. We have tested this method for production of another recombinant human protein, terminal deoxynucleotidyltransferase, and found equally efficient expression and stability of that enzyme in the insect larvae (3900 units/mg of protein as compared with 10–15 units/mg of protein in calf thymus). It is our expectation that this procedure will be generally applicable for the production of a wide array of recombinant proteins.

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