Secretion of biologically active human proapolipoprotein A-I in a baculovirus–insect cell system: protection from degradation by protease inhibitors

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Abstract: Studies of the structure and function of apolipoprotein A-I (apoA-I) often require its purification by delipidation of high density lipoprotein isolated from large quantities of human plasma and separation of apoA-I from other plasma apolipoproteins. To reduce the need for extensive purification procedures, we have developed an insect cell/baculovirus expression system for the production and secretion of human proapoA-I. The recombinant baculovirus containing full-length human apoA-I cDNA, when introduced into *Spodoptera frugiperda*, directs the synthesis of preproapoA-I, which is subsequently secreted into the growth medium as proapoA-I, indicating correct processing of the signal peptide during secretion. To prevent the extensive degradation of secreted proapoA-I, leupeptin and pepstatin A were added to the serum free cell culture medium. The protein was simply purified by filtration of the medium, which contained up to 80 mg/l proapoA-I, followed by chromatography on phenyl-sepharose CL-4B. The resultant proapoA-I was found to bind lipid and to activate lecithin:cholesterol acyltransferase as effectively as apoA-I from human plasma. The advantage of this expression system is the ease of purification of intact, biologically active apoA-I in high yield.—**Pyle, L. E., P. Barton, Y. Fujiwara, A. Mitchell, and N. Fidge.** Secretion of biologically active human proapolipoprotein A-I in a baculovirus–insect cell system: protection from degradation by protease inhibitors. *J. Lipid Res.* 1995. **36:** 2355–2361.

Supplementary key words expression system • lipid binding properties • LCAT activation • proteolytic degradation

Apolipoprotein A-I (apoA-I) is proposed to have a protective role against atherosclerosis. For example, the plasma level of apoA-I is as strong a predictor of risk of disease as that of high density lipoproteins (HDL) of which apoA-I is the principal protein moiety (1). ApoA-I is synthesized as a preproprotein by hepatic and intestinal cells. The preproprotein is cleaved intracellularly, releasing the proprotein into the circulation where the six amino acid residue propeptide is proteolytically cleaved to yield mature apoA-I (2). ApoA-I is the main activator of lecithin:cholesterol acyltransferase (LCAT), which, taken together with its ability to promote cholesterol efflux from cells (3), strongly supports a role for apoA-I in the process of reverse cholesterol transport.

To study the regulatory role of apoA-I, and to identify specific structural regions responsible for these actions, it is important to obtain apoA-I free of other apolipoprotein contaminants. In order to produce preparative amounts of apoA-I, we have utilized a baculovirus-mediated expression system that generates apoA-I free of the contaminating apolipoproteins present in plasma and in a form that facilitates purification without the need for harsh denaturing treatments. Although apoE had been expressed in and secreted from insect cells (4), a previous report on the expression of apoA-I in *Spodoptera frugiperda* described the production of intracellular apoA-I (possessing a modified amino-terminus) with a consequent requirement for more extensive purification (5). ApoA-I and proapoA-I possessing a modified amino-terminus have been expressed in *Escherichia coli* (6–8) which has led to complicated strategies for removal of the additional methionine (9). Several studies have described the expression of apoA-I as a fusion

Abbreviations: HDL, high density lipoprotein; apo, apolipoprotein; h-apoA-I, human apoA-I; Sf21, *Spodoptera frugiperda*; SF, serum free; MOI, multiplicity of infection; LCAT, lecithin:cholesterol acyltransferase; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonylfluoride; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PC, phosphatidylcholine; d, density.

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protein followed by cleavage to produce mature apoA-I. However, degradation during cleavage (10) and formation of an unintentionally truncated product has been a problem (11). Overall, a high yield of intact protein has been difficult to obtain. In the present report, the cDNA for preproapoA-I inserted into a recombinant baculovirus has been shown to direct the secretion of proapoA-I from infected Sf21 cells. The proapoA-I in the culture medium requires minimal purification, is structurally and functionally similar to apoA-I derived from plasma, and is produced at levels up to 80 mg/l.

MATERIALS AND METHODS

Materials

Egg yolk PC (Type IX), pepstatin A, leupeptin, aprotinin, PMSF, and benzamidine were obtained from Sigma. Sf-900 II medium, TC100 medium, and Lipofectin® reagent were from GibcoBRL. Pefabloc ®SC and α_2-macroglobulin were from Boehringer-Mannheim.

Construction of recombinant baculovirus

Recombinant baculovirus containing full-length human preproapoA-I cDNA downstream from the polyhedrin promoter of the baculovirus *Autographa californica* nuclear polyhedrosis virus was constructed as follows. A plasmid containing the complete coding sequence of human preproapoA-I in an 804 base pair (bp) Pst1 fragment in pUC18 was kindly provided by Dr. Shula Metzger, Hadassah Hospital, Jerusalem, Israel. The Pst1 fragment was subcloned into the non-fusion baculovirus polyhedrin promoter-based transfer vector pVL1392 (12) Due to a mutation in the start codon of the polyhedrin gene, this subcloning step directs the start of translation to the methionine residue of preproapoA-I. The resultant transfer vector, pVLhAI, was cotransfected into *Spodoptera frugiperda 21* (Sf21) cells with linearized *lacZ*-containing *Autographa californica* nuclear polyhedrosis virus DNA (13) using Lipofectin® reagent according to the method of King and Possee (14). Plaques were selected by X-gal screening and viral isolates were expanded by inoculating 1×10^6 Sf21 cells in 2 ml of medium per well in 6-well cluster plates (Costar). The medium for viral expansions and trial infections was TC100, containing 5% fetal calf serum (Cytosystems). Virus in the medium was harvested at 4–5 days and used to infect 2×10^6 Sf21 cells in 6-well plates. The medium was removed after 4–5 days and screened for the presence of apoA-I by Western blotting as described below. Three of the nine primary isolates screened were positive. One of these was taken through two further rounds of plaque purification and expanded further in monolayers in 75 cm^2 flasks or suspension cultures to provide a stock of inoculum.

Infection of cultured insect (Sf21) cells

Monolayers. To minimize contamination of expressed apoA-I with serum proteins, infections were carried out on Sf21 cells adapted to SF medium. Recombinant h-apoA-I baculovirus produced as described above in serum-containing medium was used to infect monolayers of Sf21 cells, at confluency in culture flasks (Costar), by replacing the medium with the viral inoculum for 1 h. Unless otherwise specified, the number of cells at infection was 5×10^6 in 25-cm^2 flasks. The multiplicity of infection (MOI) was approximately 10. The infected cell layer was then washed with a minimal volume of SF medium and fresh SF medium was added to the flask. Cultures were incubated at 27°C. Aliquots (0.4 ml) of medium were removed daily for analysis.

Suspension cultures. Cultures up to 300 ml were grown in stirred flasks or in 1-litre conical flasks on an orbital shaker set at 100 rpm. Baculovirus infections for the production of apoA-I in suspension culture were achieved by adding an aliquot of viral inoculum of up to 0.1 vol of the culture when the cell density, as monitored by haemocytometer, was in the range of $1–2 \times 10^6$ cells/ml. MOI was 5 or greater. A SF viral inoculum for suspension cultures was produced by first expanding the recombinant virus on SF medium-adapted cells in a 75-cm^2 culture flask. After inoculation, the cells were washed twice with SF medium to remove any serum proteins. The resultant virus was carried through one additional round of expansion in an SF suspension culture of Sf21 cells.

Detection of apoA-I

Immunoblotting was used to detect apoA-I in the medium of infected cells. Small aliquots of untreated medium were fractionated directly by SDS-PAGE (12%) in a Mini Protean II apparatus (Bio-Rad). Aliquots of > 20 µl were first concentrated by precipitation in a final concentration of 75% (v/v) ethanol and resuspension in sample buffer. Proteins were either stained with Coomassie Brilliant Blue or were transferred to nitrocellulose by electroblotting. The apparent presence of apoA-I in the stained gels was confirmed by Western analysis using a monospecific rabbit antiserum to h-apoA-I, then anti-rabbit IgG coupled to horseradish peroxidase (second antibody) to allow color development as described previously (15).

Isolation and purification of expressed apoA-I

Prior to chromatography, medium from cell cultures was prefiltered through AP15 (Millipore) filters and loaded onto a 3.5 × 1.5 cm (6 ml) phenyl-Sepharose

CL-4B column previously equilibrated with running buffer (0.05 M Tris-HCl, pH 6.6, 0.02% (v/v) sodium azide, 0.5 μg/ml leupeptin, and 0.7 μg/ml pepstatin A.) The column was washed with the same buffer until the absorbance reading of the effluent at 280 nm (Abs 280) approached zero. Desorption of the column (11) commenced with 30% (v/v) propylene glycol in running buffer; after the Abs 280 of the effluent approached zero, elution was continued with 70% (v/v) propylene glycol in running buffer. ApoA-I eluted in the 70% propylene glycol fraction, which was dialyzed against 0.01 M Tris-HCl, pH 8.0, and then concentrated 10- to 15-fold in an Amicon ultrafiltration cell equipped with a Diaflo YM10 membrane.

Purification of plasma apoA-I

Briefly, HDL_3 (d 1.125–1.210 g/ml) was isolated by ultracentrifugation (16), washed by re-centrifugation at the same density, and delipidated as per Herbert et al. (17) using chloroform–methanol–ether as extracting solvents. ApoA-I was purified by gel filtration and ion-exchange chromatography as described earlier (18).

Preparation of egg yolk phosphatidylcholine vesicles and formation of lipid–protein complexes

L-α-phosphatidylcholine (PC) prepared from egg yolk (Type IX-E, Sigma) and supplied in chloroform was dried under N_2 and reconstituted to a final concentration of approximately 1 mg/ml in 0.15 M NaCl, 0.05 M Tris-HCl and 1 mM EDTA, pH 7.4. The solution was kept at 0°C during sonication with a Branson Sonifier on setting 3 for 30–40 min under a stream of N_2. After sonication, the solution was centrifuged at 436,000 *g* for 1 h at 24°C to pellet titanium particles and to sediment large multilamellar vesicles. The supernatant containing the unilamellar vesicles was used to test the lipid binding properties of expressed apoA-I that was incubated with the PC at ratios of 1:4 or greater (w/w, protein:lipid) in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4, for 3 h at 37°C (19).

Analytical methods

Protein concentrations were measured according to the method of Lowry et al. (20) using BSA as standard.

Protein sequencing

After SDS-PAGE (12%), proteins were electroblotted to ProBlott membranes (Applied Biosystems), briefly stained with Coomassie Brilliant Blue, and appropriate bands were excised. N-terminal sequencing was performed directly on PVDF membrane using an Applied Biosystems Model 470A protein sequencer equipped with an on-line Model 120A PTH analyzer.

Quantitation of apoA-I

ApoA-I was quantitated by a competitive ELISA system using rabbit anti-human apoA-I polyclonal antiserum and purified human apoA-I as standard according to a previously reported procedure (21).

LCAT assay

LCAT was isolated from fresh human serum by a combination of ultracentrifugation, phenyl-Sepharose, ion-exchange (DEAE-Sephacel), and hydroxyl apatite chromatography as described previously (22). In order to assess the activity of apoA-I as a cofactor, the varying concentrations of apoprotein were preincubated with a constant concentration of substrate, comprising single bilayer vesicles of PC (25 μg), cholesterol (0.75 μg), and [^{14}C]cholesterol (0.5 μg, final activity 6×10^5 dpm/ml), prepared as described previously (23) for 1 h at 37°C. The reaction in a total volume of 0.25 ml was initiated by the addition of purified LCAT (0.1 μg) and, after 60 min at 37°C, the lipids were extracted and the cholesteryl ester fraction was obtained by TLC and quantitated by scraping and liquid scintillation counting (24).

Isoelectric focusing (IEF)

IEF was performed in a Mini Protean II electrophoresis chamber (Bio-Rad) on 8 cm × 7 cm 7.5% polyacrylamide slab gels (1.5 mm). Gel solutions contained 8 M deionized urea (Promega) and 5% (v/v) ampholine (pH 4–6.5: Pharmacia-LKB). Gels were prefocused for 30 min at 100 V; protein samples were loaded and focused at 200 V for 30 min then at 300 V for 2 h. The pH gradient was determined by cutting a small portion of blank gel into 3-mm slices, equilibrating each slice in 0.75 ml of deionized water for 30 min, and recording the pH of the resulting solution.

RESULTS

ProapoA-I expression by cultured insect cells

To determine optimum conditions for harvesting apoA-I secreted from cells, 5×10^6 infected cells were incubated as monolayers in 25-cm^2 flasks in SF-medium for up to 7 days. At various times, samples of medium were removed for analysis by SDS-PAGE and immunoblotting. As shown in **Fig. 1,** apoA-I was first detected 2 days after infection and continued to be secreted for several days. Immunoblot analysis established the protein to be apoA-I (data not shown) and N-terminal sequencing of the secreted protein confirmed its identity as proapoA-I, the prepeptide having been accurately cleaved during secretion.

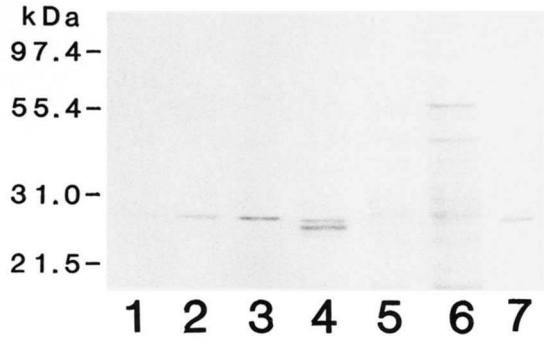

Fig. 1. Expression of recombinant proapoA-I by baculovirus-infected Sf21 cells. Proteins from aliquots (200 µl) of medium were detected by Coomassie Brilliant Blue staining after SDS-PAGE (12%). Lanes 1–6, samples of medium from days 1 to 6 post-infection, respectively. Lane 7, h-apoA-I from plasma.

As also demonstrated in Fig. 1, an apparent degradation product of proapoA-I, confirmed by immunoblot (data not shown), appeared within 2 days post-infection such that unchecked degradation caused the complete loss of proapoA-I protein within 5 days post-infection.

To investigate methods for preventing the degradation, we initially developed an assay for monitoring the extent of degradation. The assay consisted of plasma-derived human apoA-I as substrate, and day 7 conditioned medium from infected Sf21 cultures (in which expressed apoA-I was completely degraded) as a source of Sf21 proteases. Incubation for 16 h at 22°C resulted in plasma apoA-I being undetectable by immunoblot. It was found that a cocktail of inhibitors consisting of EDTA, PMSF, leupeptin, benzamidine, and pepstatin A, active against a range of proteases, added to the assay was effective in inhibiting degradation. By selective omission of each one of these inhibitors from the cocktail, it was deduced that the active components of the cocktail in suppressing the degradation of apoA-I were leupeptin and pepstatin A. Concentrations of inhibitors that appeared to allow normal growth of cells but were sufficient to prevent degradation of plasma apoA-I by conditioned medium from infected cells were determined to be 0.5 µg/ml for leupeptin and 0.7 µg/ml for pepstatin A (data not shown).

A comparison of the concentration of proapoA-I in the SF-medium of Sf21 cells grown with or without the combination of leupeptin plus pepstatin A (added at day 1 after infection with baculovirus and thereafter at 24-h intervals) is shown in **Fig. 2.** The extensive degradation of proapoA-I by day 5 in the absence of inhibitors is completely prevented by the presence of pepstatin A and leupeptin. Subjecting the medium from cultures with added inhibitors to SDS-PAGE (Fig. 2B) confirmed that the secreted proapoA-I remained intact for the 6-day post-infection period.

The intra- versus extracellular distribution of apoA-I was analyzed by ELISA in samples of sonicated Sf21 cells or the corresponding conditioned medium. The majority of proapoA-I (75–80%) was secreted into the growth medium (data not shown).

A variety of compounds was tested for the ability to increase the secretion of proapoA-I. Egg yolk PC (10 µg/ml), albumin-bound sodium oleate (1 mM), albumin-bound cholesterol (50 µg/ml) or butyric acid (5 mM), which stimulates apoA-I production in HepG2 cells by post-translational mechanisms, were added to Sf21 cell cultures. None of these treatments caused changes in the secretion of proapoA-I.

Optimization of proapoA-I production in monolayers

To determine the effect of cell density at infection on proapoA-I secretion, cultures in 25-cm^2 flasks were seeded with 1.25, 2.50, and 3.75 × 10^6 cells and incubated until the flask with the highest cell density was

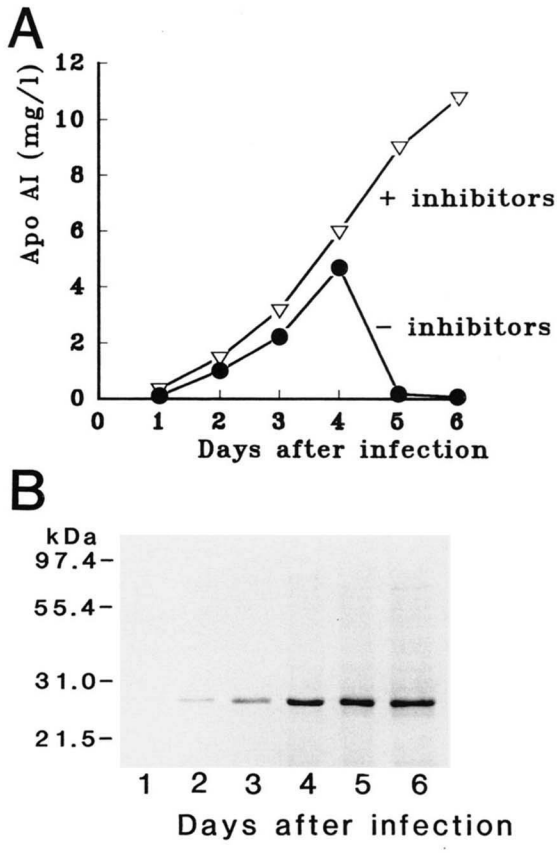

Fig. 2. Effect of cell culture in the presence of protease inhibitors on yield of proapoA-I. Sf21 cells infected with recombinant baculovirus were incubated with or without 0.5 µg/ml leupeptin and 0.7 µg/ml pepstatin A as described in Methods. A: Samples of medium were analyzed by ELISA for quantitation of apoA-I, (▽) culture plus inhibitors; (●) culture minus inhibitors. B: Aliquots (200 µl) of cell culture medium, which had been treated with inhibitors, from days 1 to 6 post-infection were subjected to SDS-PAGE (12%) and the proteins were detected by staining with Coomassie Brilliant Blue.

confluent and that with the lowest density was approximately 65% confluent. The cells were infected at an MOI greater than 10. Inhibitors were added as described above. ProapoA-I secretion varied markedly with cell density (data not shown), being approximately 10 times higher at 4 days after infection of the least dense monolayer compared with the higher cell densities.

Production of proapoA-I in suspension cultures

A trial to assess the yield of proapoA-I in suspension culture, rather than from Sf21 monolayers, was performed starting with 100 ml of Sf21 suspension culture in SF medium, infected at an MOI of 5. Protease inhibitors were added on days 2, 3, and 5 (i.e., at 31, 66, and 104 h) post-infection. The highest yield, of 30 mg/l, occurred between 4 and 5 days after infection, after which a small amount of degradation became apparent, suggesting that higher levels or more frequent application of the protease inhibitors may have been necessary to give protection to the secreted proapoA-I. In order to minimize degradation, possibly caused by the release of proteases due to cell death, incubation was held to 96 h post-infection in future experiments.

For producing the larger quantities of proapoA-I needed for physiological and physico-chemical studies, Sf21 cells adapted to SF medium were grown to a density of approximately 2.3×10^6 cells/ml in a volume of 300 ml in a 1-litre shake flask at 27°C, shaken at 100 rpm. The cultures were inoculated at a multiplicity of infection of 1.5 with recombinant virus grown in SF medium. Pepstatin A and leupeptin were added (to final concentrations of 0.7 μg/ml and 0.5 μg/ml, respectively) at 36, 61, 66, and 83 h post-infection. A final addition of the inhibitors was made on harvesting the medium at 96 h post-infection. While yields were somewhat variable, this procedure gave high quality proapoA-I with concentrations of up to 80 mg/l.

Characterization of secreted proapoA-I

The results of two different tests relating to the lipid-binding properties of the expressed proapoA-I are presented in **Fig. 3.** In the first, the question of whether proapoA-I was secreted in either a lipid-poor or lipoprotein form was examined by adjusting a sample of culture medium from baculovirus-infected Sf21 cells to d 1.25 g/ml and centrifuging at 436,000 *g* for 16 h at 4°C. The top and bottom fractions were separated, dialyzed against 0.01 M NH_4HCO_3, pH 8.0, and aliquots were subjected to SDS-PAGE for assay by immunoblotting. Lanes 2 and 3 (Fig. 3) show that most of the secreted proapoA-I was lipid-poor, being found in the d > 1.25 g/ml fraction.

The second experiment was designed to test whether the expressed proapoA-I met the essential criterion for its classification as an apolipoprotein, namely its ability to bind lipid. Samples of Sf21 culture medium were incubated with egg yolk PC unilamellar vesicles (see Methods) for 3 h at 37°C. The mixture was adjusted to d 1.25 g/ml and centrifuged as described above. As shown in Fig. 3, lanes 4 and 5, most of the proapoA-I was recovered in the top, lipid-containing fraction, demonstrating the lipid binding properties of the expressed apoA-I.

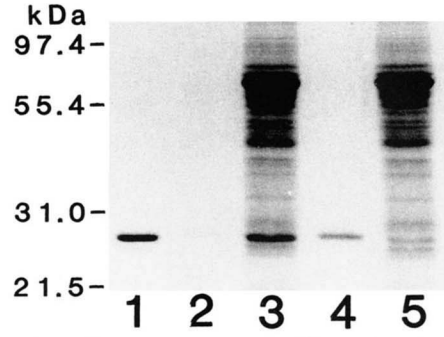

Fig. 3. Interaction of recombinant proapoA-I with lipid during and after synthesis and secretion. Cell culture medium from infected Sf21 cells expressing proapoA-I was adjusted to a density of 1.25 g/ml for separation of lipoprotein and non-lipoprotein fractions, either with or without prior incubation with PC vesicles as described in Methods. Samples were fractionated by SDS-PAGE (12%) and proteins were detected by Coomassie Brilliant Blue staining. Lane 1, plasma h-apoA-I; lane 2, the d < 1.25 g/ml fraction of culture medium; lane 3, the d > 1.25 g/ml fraction of culture medium; lanes 4 and 5, the same density fractions as lanes 2 and 3, respectively, obtained after incubation of culture medium with PC vesicles.

Another important function of apoA-I is its ability to activate LCAT. The purified recombinant proapoA-I was tested for this function by its addition to substrate mixtures containing PC and cholesterol (see Methods) followed by incubation with purified LCAT. Recombinant proapoA-I resulted in marked stimulation of LCAT activity, demonstrating that like plasma apoA-I, it possesses the physiological properties of the activator protein (**Fig. 4**).

As an additional indication of the authenticity of the expressed proapoA-I, its pI values were compared by IEF with those of the polymorphic forms of plasma h-apoA-I (25). Two bands of differing intensity were detected by Coomassie staining after IEF on the purified expressed apoA-I (result not shown). The minor band, proapoA-I, focussed at a pI consistent with it being two charge units more basic than the mature form. The major band, having a lower pI, equivalent to that of mature apoA-I, was presumably a deamidated product of the proform (25), as protein sequencing did not indicate the presence of the mature form of apoA-I.

DISCUSSION

Production of apoA-I free of other contaminating apolipoproteins is important for the investigation of the biochemical, physiological, and clinical properties of this apolipoprotein. The baculovirus expression system meets this requirement and, further, has the potential for producing mutated forms of the protein, enabling investigation of the candidate active regions of apoA-I. This system is one of several currently used to express mammalian proteins but has some potential advantages over the others, including intracellular processing similar to that which occurs in mammalian cells. In this case, a preproprotein was initially synthesized that followed a pathway of secretion similar to that in liver cells, with a proprotein product accumulating in the medium in concentrations of up to 80 mg/l. The signal peptide of preproapoA-I, in addition to being accurately cleaved, also directs the secretion of 75–80% of the expressed proapoA-I, suggesting that this peptide may be an interesting candidate for studies comparing the effects of different signal peptides on the secretion rate of expressed proteins (26).

To facilitate purification of the secreted proapoA-I and take advantage of the lack of other blood proteins, cells were adapted to grow in serum-free medium SF 900 II. However, in the absence of serum which contains naturally occurring inhibitors of proteolytic enzymes, degradation of expressed apoA-I was observed. This degradation was overcome by addition of the protease inhibitors pepstatin A and leupeptin to the cell culture.

The empirical approach, described here for determining the appropriate minimal combination of protease inhibitors and their effective non-toxic concentrations, may find useful application in the development of other systems for protein expression. For example, Yamada, Nakajima, and Natori (27) found considerable degradation of recombinant sarcotoxin IA expressed by *Bombyx mori* cells and found that addition of *p*-chloromercuribenzenesulphonic acid (PCMBS), a cysteine proteinase inhibitor, reduced this breakdown although the yield of protein remained low. When added to our test system alone, PCMBS had an effect similar to leupeptin. Combined with pepstatin A, PCMBS produced inhibition apparently equal to that of leupeptin and pepstatin A, suggesting that the approach of combining inhibitors may also be effective in *B. mori* systems.

In conclusion, the expressed and secreted proapoA-I showed the same immunochemical identity, electrophoretic migration, and biological activity (assessed by LCAT activation) as native apoA-I. Most of the proapoA-I secreted was in a lipid-poor form, as >95% was not buoyant at d 1.25 g/ml, a density at which most soluble lipoproteins float. It formed lipid–protein complexes when incubated with phospholipid vesicles that floated at a density similar to human HDL. These data, together with its hydrophobicity as evidenced by the elution pattern from phenyl-Sepharose, indicate that the expressed proapoA-I can be used for further studies of the structural and function of this important apoprotein.

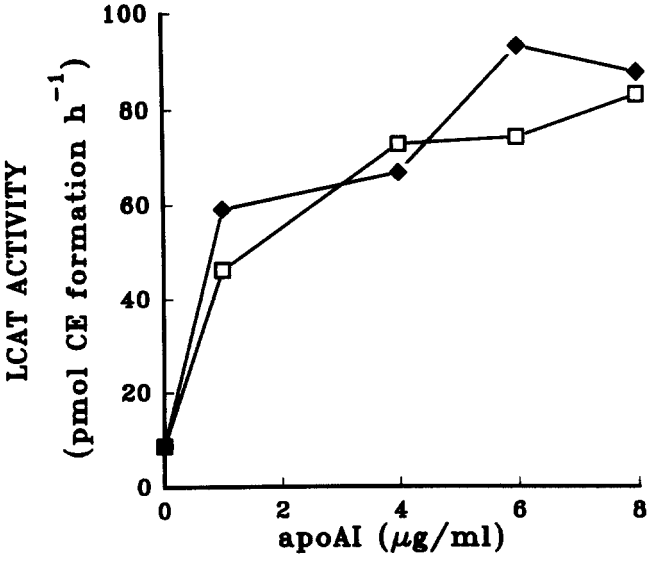

Fig. 4. Activation of LCAT by recombinant proapoA-I. The substrate was prepared as vesicles containing cholesterol (0.75 μg), [^{14}C]cholesterol (0.5 μg, final activity 6 × 10^5 dpm/ml) and egg PC (25 μg) per incubation and was equilibrated with recombinant (◆) or plasma (□) h-apoA-I at 37°C for 60 min before the addition of 0.1 μg/ml of purified LCAT in a total reaction mixture of 0.25 ml. After 60 min, lipids were extracted to determine [^{14}C]cholesteryl ester formation as described in Methods.

We gratefully acknowledge support from the Japan Foundation for Aging and Health and from the National Health and Medical Research Council for funds to undertake this work.

Manuscript received 24 April 1995 and in revised form 7 July 1995.

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