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ORIGINAL ARTICLE

Expression of functional recombinant mosquito salivary apyrase: A potential therapeutic platelet aggregation inhibitor

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Abstract
Excessive platelet activation and accumulation can lead to vessel occlusion and thus present major therapeutic challenges in cardiovascular medicine. Apyrase, an ecto-enzyme with ADPase and ATPase activities, rapidly metabolizes ADP and ATP released from platelets and endothelial cells, thereby reducing platelet activation and recruitment. In the present study, we expressed a 68-kDa recombinant mosquito *(Aedes aegypti)* salivary apyrase using a baculovirus/insect cell expression system and purified it to homogeneity using anion-exchange chromatography on a large scale. A yield of 18 mg of purified recombinant apyrase was obtained from 1 litre of the medium. Kinetic analysis indicated that the recombinant apyrase had a K_m of 12.5 μM for ADP and a K_m of 15.0 μM for ATP. The recombinant apyrase inhibited ADP-, collagen- and thrombin-induced human platelet aggregation in a dose-dependent manner, indicating that the recombinant protein retained nucleotidase activity in a whole cell system, which suggests that it may serve as a therapeutic agent for inhibition of platelet-mediated thrombosis.

Keywords: *Platelet aggregation inhibitor, recombinant apyrase, baculovirus/insect cell expression system, adenosine diphosphate, mosquito, Aedes aegypti*

Abbreviations

ADP	adenosine diphosphate;
ATP	adenosine triphosphate;
K_m	Michaelis constant;
GP	glycoprotein;
cDNA	complimentary DNA;
MOI	multiplicity of infection;
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Introduction

Platelet activation is an essential component of normal hemostasis. However, excessive platelet activation is a contributing factor to several clinical disorders which include myocardial infarction, restenosis after angioplasty or bypass surgery, and stroke. After vessel injury, platelets adhere to exposed subendothelial structures, a process that is mediated by platelet glycoprotein GPIbα, GPIaIIa ($\alpha 2\beta 1$ integrin), and GPVI binding to subendothelial von Willebrand factor and collagen [1–4]. The platelets are concurrently activated by both the exposed collagen and thrombin generated by the coagulation cascade. Upon activation, platelets recruit additional platelets into the growing thrombus by two mechanisms. Firstly, thromboxane A_2 is synthesized and released; second, ADP is secreted from storage granules of activated platelets. Both thromboxane A_2 and ADP are platelet agonists that stimulate nearby platelets [5, 6].

Apyrase is an ATP diphosphohyrolase which inhibits platelet aggregation by metabolizing ADP released from injured cells or by activated platelets [7]. The secreted salivary apyrase of the mosquito, *Aedes aegypti*, shares a number of similarities with

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vertebrate pancreatic and endothelial apyrase, including approximate molecular weight, pH sensitivity, divalent cation dependence, and immunological cross-reactivity [8]. A cDNA encoding for apyrase has been cloned from an adult female *Aedes aegypti* salivary gland library [9, 10].

A baculovirus/insect cell system, which performs many of the post-translational modifications found in eukaryotic cells, has been used extensively for the production of large amounts of recombinant proteins with biological activity [11–15]. Moreover, the growth of insect cells in serum-free medium makes the purification of the recombinant protein easy by using simple laboratory techniques. By using this method, Faudry et al. produced and purified apyrase of *Triatoma infestans*, which is a 79-kDa 5′-nucleotidase with activity to hydrolyze ATP and ADP [16]. A recombinant apyrase, human CD 39, has an ecto-ADPase activity and strongly inhibits human platelet aggregation induced by ADP and collagen [17]. Herein we describe the expression, purification, characterization, enzyme kinetic parameters, as well as the effect of recombinant salivary apyrase of *Aedes aegypti* on human platelet aggregation.

Materials and methods

Construction of transfer vector

A 1.2-kb 3′-end cDNA clone, λSGG12, and a 0.47-kb 5′-end cDNA clone, λSGG12-5, encoding the *Aedes aegypti* salivary apyrase [9,10], were ligated to form the full-length cDNA and then cloned into baculovirus expression vector pBlueBacHis C as described previously [18]. The full-length apyrase cDNA (1.6 kb) was excised from pBluecriptSK-apyrase and inserted into pSE420 (Invitrogen, San Diego, CA) via restriction enzyme sites *Bam*HI and *Kpn*I. The cDNA was then excised by *Bam*HI and *Not*I and cloned into the baculovirus transfer plasmid, pVL1393 (Invitrogen). The structure of the insert was confirmed by DNA sequencing. The resultant pVL1393-apyrase was amplified using Top 10 cells (Invitrogen), purified by CsCl gradient centrifugation and used for transfection experiments.

Generation of recombinant baculovirus

The transfer vector, pVL1393-apyrase, and linearized AcMNPV viral DNA (Invitrogen) were co-transfected into *Spodoptera frugiperda* (Sf9) cells by the cationic liposome (Invitrogen) according to the manufacturer's instructions (Invitrogen). Briefly, Sf9 cells (2×10^6) were seeded onto 60-mm tissue culture plate and allowed to attach for 30 min. The transfection mix for each plate was prepared by mixing 1 μg of linearized AcMNPV viral DNA and 3 μg of purified pVL1393-apyrase with 20 μL of cationic liposome and incubated at room temperature for 15 min. After washing the cells with 2 ml of Grace's medium (Invitrogen) without supplements, 1 ml of transfection mix was added dropwise. Following a 4-h incubation at room temperature, 1 ml of complete TNM-FH media was added to each plate. The transfection media were harvested after the plates were incubated at 27°C for 48 h. The recombinant virus was purified by plaque assay and the occlusion negative plaques were verified by gene amplification. The amplification primers (Forward primer: 5′-TTTACTGTTTTCGTTAACAGTT TG-3′; Reverse primer: 5′-CAACAACGCACAG AATCTAG-3′) were provided by Invitrogen. Viral titers were increased by three rounds of amplification, and the titer of the final recombinant virus stock was determined to be $1–5 \times 10^8$ pfu/ml.

Optimization of recombinant protein expression

To determine the optimal condition for expression of recombinant apyrase by baculovirus-infected cells, High-Five cells were infected with pVL1393-apyrase at various MOIs and were harvested at various times post-infection. Cell lysates were evaluated by immunoblot analyses. Briefly, High-Five cells (5×10^5) were seeded into a 12-well tissue culture plate containing 3 ml of EX-CELL^{TM} 405 media (JRH Biosciences, Lenexa, KS, USA). Three wells each were infected with recombinant virus at an MOI of either 1, 5, or 50, and the contents of one well containing cells with virus at each MOI were harvested at 24, 48, and 72 h postinfection. As controls, one well was infected with wild-type baculovirus at an MOI of 10, and one well was uninfected. Control wells were harvested at 72 h postinfection. The harvested cells were lysed, and 10 μg of extract was subjected to Immunoblot analysis.

Expression and purification of the recombinant protein

In order to obtain a high yield of expressed protein, 200 ml of suspension culture of High-Five cells at 2×10^6 cells/ml cultured in EX-CELL^{TM} 405 media were infected with the pVL1393-apyrase recombinant baculovirus at an MOI of 5 in a 500-ml spinner flask. Suspension cultures were harvested after infection at the time when the maximum expression occurred and centrifuged at $3000 \times g$ for 20 min, and then re-centrifuged at $30000 \times g$ for 20 min. The recombinant apyrase was purified from the supernatant by anion exchange chromatography. The supernatant was concentrated to a small volume using an Amicon stirred cell equipped with a YM-30 Diaflo membrane, and then dialyzed against a large volume of buffer (10 mM Tris, pH 7.4). The concentrated, dialyzed supernatant

was mixed and incubated with DEAE Sephacel (Pharmacia) previously equilibrated with the dialysis buffer for 10 min at room temperature. The mixture was poured into a small column. Elution of proteins from the matrix was monitored at OD_{280} using a Uvicord S spectrophotometer (LKB), and was performed by first washing the column with dialysis buffer until the OD_{280} returned to the baseline, followed by elution with 0.5 M NaCl in 40 ml of dialysis buffer. The first absorbance peak in which the recombinant apyrase did not bind the DEAE Sephacel was analysed by immunoblot for the presence of the recombinant apyrase. Fractions containing the recombinant apyrase were pooled and desalted by dialysis against a large volume of buffer (10 mM Tris, pH 7.4). Finally, the products were concentrated with Microsep Centrifugal Concentrators (Filtron), and stored at −70°C.

SDS–PAGE and immunoblotting

Samples were run on a 10% SDS–PAGE under reducing conditions. Following their separation at 110 V for 90 min at 4°C, proteins were either transferred to nitrocellulose membranes using a Trans-Blot cell (BioRad) at 100 V for 90 min at 4°C, or stained using the BioRad Silver Stain kit. Non-specific binding to the nitrocellulose membranes was prevented by blocking overnight at 4°C with 3% BSA in PBST (0.02 M PBS supplemented with 0.05% Tween-20, pH 7.2). Membranes were subsequently incubated with rabbit anti-apyrase IgG (1:5000) in PBST containing 1% BSA for 1 h at room temperature. The rabbit anti-apyrase IgG was prepared using purified *Aedes aegypti* apyrase as described previously [9,10]. Dilution buffer without anti-apyrase antibody served as a negative control. The specific binding was detected by incubating the membrane with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:2000) (Jackson ImmunoResearch Lab. Inc., West Grove, PA, USA) in PBST containing 1% BSA for 1 h at room temperature, followed by the substrates BCIP (5-bromo-4-chloro-3-indoylphosphate *p*-toluidine salt) (BioRad) and NBT (*p*-nitroblue tetrazolium chloride) (BioRad).

Apyrase activity assays

Purified recombinant apyrase activity was measured by the liberation of inorganic phosphate using a highly sensitive malachite green colorimetric assay [19]. Incubations were carried out in the following medium: 50 mM Tris base, 50 mM imidazole, 8 mM $CaCl_2$, and 250 μM substrate in a final volume of 1.0 ml (pH 7.5). Reactions were started by adding 6.6 μg of protein at 37°C and stopped after 10 min with 250 μl of the malachite reagent. Rates of ATP and ADP dephosphorylation were derived from a standard curve constructed with KH_2PO_4. Rates of phosphate release were expressed in micromoles per minute per milligram of protein (mmol/mg/min). Protein concentration was estimated by the technique of Lowry's using bovine serum albumin as a standard. The apparent K_m for ATP and ADP were derived from Lineweaver–Burk plots, with substrate concentrations ranging between 2 and 200 μM. In the enzyme assay, less than 5% of the substrate was dephosphorylated [20].

Platelet aggregation

Human blood, obtained by venipuncture from healthy donors, was collected into citrate dextrose anticoagulant (3.8 mM citric acid, 75 mM trisodium citrate, 125 mM dextrose, pH 6.5; 1.9 ml anticoagulant per 8.1 ml whole blood). Platelet-rich plasma (PRP) was obtained following centrifugation of whole blood at 800×*g* for 5 min at 20°C. Washed platelets were prepared by centrifugation of PRP at 800×*g* for 10 min, subsequently, the pellet was washed with Hank's balanced salt solution (HBSS) modified by the addition of 4.45 mM dextrose, 3.35 mM $NaHCO_3$, 500 μM $MgCl_2$, and 0.1 mg/ml bovine serum albumin. Finally, the pellet was suspended at 2.5×10^8 platelets/ml in modified HBSS and stored at 37°C. Platelet suspensions (0.5 ml) containing 1 mM $CaCl_2$ were stirred in siliconized aggregometer tubes and aggregation was monitored photometrically using a Payton dual channel aggregometer (Payton Assoc., Scarborough, Ont.) at 37°C. Prior to the addition of ADP (30 μM), collagen (1.8 μg/ml) or thrombin (0.1 unit/ml), platelets were incubated for 3 min with varying concentrations of recombinant apyrase (0–2.4 μM), and the aggregation response recorded for a minimum of 5 min.

Results

Expression and purification of recombinant apyrase

The apyrase cDNA was subcloned at the 3′-end of the polyhedrin promoter of the baculovirus transfer vector pVL1393. The resultant recombinant baculovirus expression vector was cotransfected using the Lipofectin method with a linearized AcMNPV viral DNA into Sf9 cells. Four putative recombinant virus colonies were selected, plaque-purified, and their structure verified by gene amplification analysis. All colonies were found to contain the apyrase cDNA insert of the appropriate size (data not shown). A single, purified recombinant virus was prepared to optimize the level of recombinant apyrase expression in baculovirus-infected High-Five cells.

The results of optimizing the recombinant apyrase expression in baculovirus infected High-Five cells

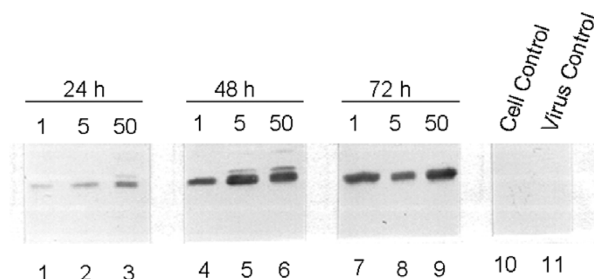

Figure 1. Optimization of the expression level of recombinant apyrase. High-Five cells infected with the recombinant virus at an MOI of 1, 5, or 50, respectively, were harvested at 24, 48, and 72 h post-infection and protein analyzed by immunoblotting with anti-apyrase antibody. Uninfected cells (lane 10) and cells infected with wild-type virus (lane 11) were used as negative controls.

are shown in Figure 1. Three wells each were infected with recombinant virus at an MOI of either 1, 5, or 50, and the contents of one well containing cells with the virus at each MOI were harvested at 24, 48, and 72 h postinfection. High-Five cell lysates were analysed by immunoblotting with anti-apyrase antibody. The maximum production of the recombinant protein was detected at 48 h post-infection with recombinant virus (MOI = 5). Uninfected cells and cells infected with wild-type virus did not express apyrase.

High-Five cells in spinner culture were used in the large-scale production of recombinant apyrase, which was purified from the expression medium supernatant by anion exchange with DEAE Sephacel. We found that recombinant apyrase was not adsorbed to the matrix and was included in the initial elution with dialysis buffer. Several other protein impurities were eluted as OD_{280} peaks following linear NaCl gradient elution (data not shown). The initial elution solution was pooled, desalted, lyophilized and reconstituted in a small volume of distilled water. Recombinant apyrase was shown to be relatively pure by SDS–PAGE through silver stain and binding with anti-apyrase antibody (Figure 2). The molecular size of the protein is 68 kDa. Approximately 18 mg of purified recombinant protein were recovered per liter of cell culture.

The kinetic parameters of the recombinant apyrase

To examine the activity of the recombinant apyrase, the ADPase and ATPase activities were measured by the liberation of inorganic phosphate using a sensitive malachite green colorimetric assay. The Lineweaver–Burk plots were used to estimate the apparent K_m for ADP and ATP (Figure 3). It indicated that the apparent K_m of the recombinant apyrase for ADP and ATP were 12.5 and 15 μM, respectively.

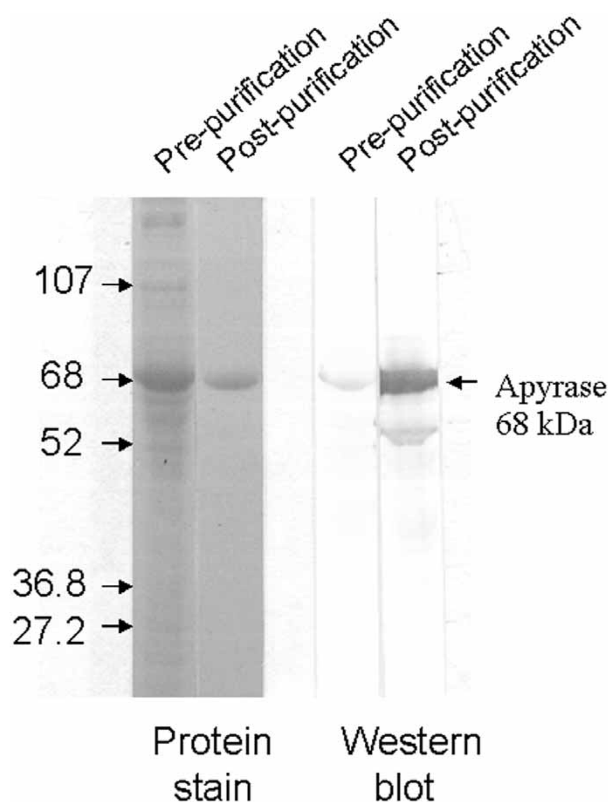

Figure 2. Purification of recombinant apyrase using DEAE Sephacel. The recombinant apyrase samples were applied to SDS–PAGE using 10% acrylamide under reducing conditions. Proteins were visualized by either silver protein staining using a BioRad kit (left panel) or transferred to a nitrocellulose membrane followed by immunoblotting (right panel). Pre-purification: unpurified, concentrated, dialyzed culture medium supernatant following expression; Post-purification: DEAE-purified recombinant apyrase.

Effect of the recombinant apyrase on platelet aggregation

Recombinant apyrase was able to inhibit ADP-induced platelet aggregation, as well as collagen- and thrombin-induced platelet aggregation. Platelet aggregation induced by 30 μM ADP, 0.1 u/ml thrombin and 1.8 μg/ml collagen were abruptly terminated by recombinant apyrase at 0.8, 2.4, and 1.6 μM, respectively, *in vitro* (Figure 4). Recombinant apyrase (0.4 μM) can inhibit 91, 20, and 68% of ADP-, thrombin- and collagen-induced platelet aggregation, respectively. The effect of recombinant apyrase on platelet aggregation was dose-dependent.

Discussion

Inappropriate platelet activation is a major contributing factor in the etiology of thrombotic conditions [21, 22]. Exposure of a thrombogenic surface, for example following the rupture of atherosclerotic plaque, triggers platelet activation. This process includes the exposure of fibrinogen-binding domains

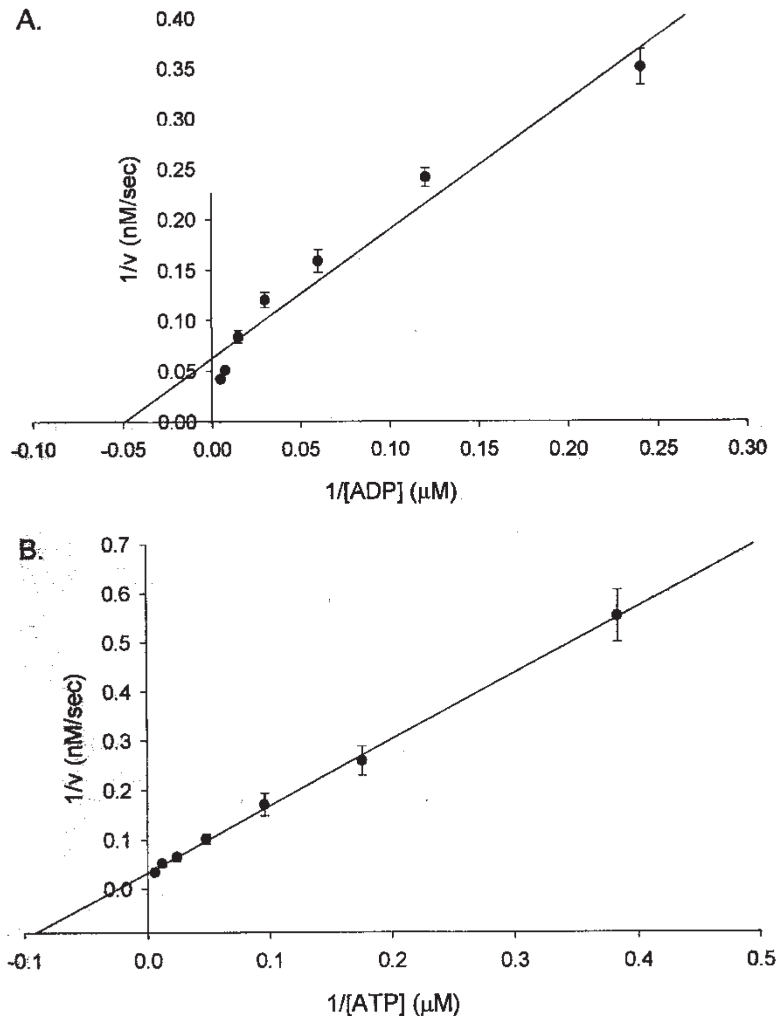

Figure 3. Lineweaver–Burk reciprocal plot. $1/v$ versus $1/[s]$ of the rates of metabolism at different concentrations of ADP or ATP using purified recombinant apyrase indicated that recombinant apyrase had a K_m of 12.5 μM for ADP and a K_m of 15.0 μM for ATP.

of the αIIb/β3 integrin on platelets, the cross-linking of which by fibrinogen consolidates platelet–platelet interaction, and also the release of inter-platelet mediators, notably thromboxane A_2 and ADP, which attract additional platelets to the lesion. Consequently, several anti-platelet drugs are routinely used in the management, and prevention, of several cardiovascular diseases [23–25]. Intravenously administered antagonists of the αIIb/β3 integrin, such as abciximab and tirofiban, attenuate platelet–platelet interaction during thrombus formation and have been shown to be effective in preventing ischemic complication in certain specific circumstances [26]; however, orally active αIIb/β3 antagonists have been disappointingly ineffective as thrombotic prophylactic agents [27].

Traditionally, aspirin has been considered the prophylactic drug of choice in individuals at risk of thrombosis [28]. Aspirin inhibits cyclooxygenase, thereby preventing thromboxane A_2 production and thus the subsequent accumulation of platelets. Although an effective anti-thrombotic, there has been an increasing appreciation of problems associated with aspirin resistance [29]. Thienopyridines, such as ticlopidine and clopidogrel, are pro-drugs whose active metabolites antagonize certain ADP receptors, prevent αIIb/β3 integrin expression, fibrinogen binding and platelet activation [30]. Both agents have been shown to be clinically useful in the attenuation of platelet function in specific clinical settings [23,25, 30, 31]. The importance of ADP in thrombotic disorders is highlighted by the presence on the endothelial cell luminal surface of CD39, an ecto-nucleotidase which metabolizes circulating ADP. CD39 null mice exhibit increased cerebral infarct volumes, and recombinant CD39 inhibits platelet aggregation *in vitro* [17], consistent with the ADP metabolic pathway serving as a potential target for anti-thrombotic agents.

CD39 exhibits a 48% amino acid homology with soluble apyrase, an enzyme widely used to inhibit platelet function, isolated from potato tubers [32]. Apyrase is also found ubiquitously in the saliva of hematophagous arthropods, such as blood-sucking insects and ticks [16, 33–35], where it acts as an inhibitor of platelet function to maintain vessel

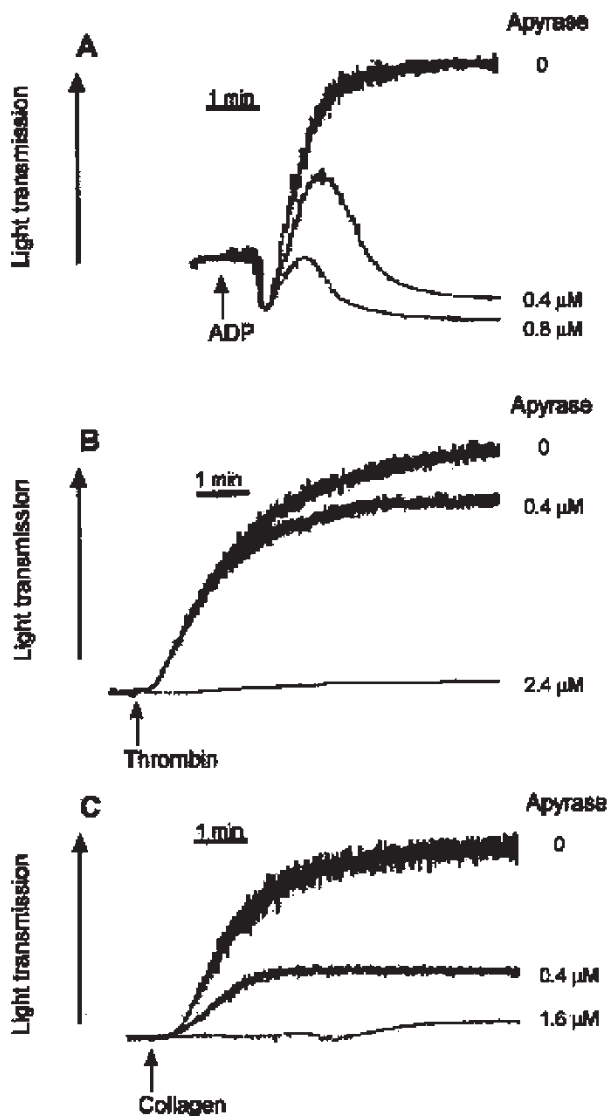

Figure 4. The effect of recombinant apyrase on inhibition of human platelet aggregation. Washed human platelets pre-incubated with various concentrations, 0, 0.4, 0.8, 1.6, and 2.4 µM, of recombinant apyrase before addition of (a) 30 µM of ADP, (b) 0.1 unit/ml of thrombin or (c) 1.8 µg/ml of collagen. Platelet aggregation was monitored continuously as described in the 'Methods'.

patency and allow successful feeding [35,36]. The process of purification of apyrase from potatoes or mosquito saliva is time consuming and labour intensive, and the yield is extremely low [9].

Recombinant DNA technologies may provide a solution to overcome the difficulty in obtaining large amounts of apyrase. A baculovirus/insect cell system has been used extensively for the production of recombinant proteins with biological activity [11–13]. This system has several advantages over prokaryotic expression systems. For example the growth of insect cells in a serum-free medium makes the purification of the recombinant protein relatively easy using simple laboratory techniques – most heterologous proteins are produced at amounts ranging from 1 to 100 mg per 10^9 cells (e.g., a 1-l culture) [14,15]. In the current study 18 mg of purified apyrase were obtained from 1 litre of cell culture which compares favourably to the low yield of CD39 expressed using CHO cells [37].

The purified recombinant apyrase inhibited platelet aggregation to ADP, collagen and thrombin. ADP-induced platelet aggregation was more sensitive to inhibition by the recombinant protein which demonstrates that the isolated ADPase activity, observed in the enzyme assay, is retained in the platelet aggregation system. Furthermore the effects of apyrase on collagen- and thrombin-induced platelet aggregation is consistent with a role for platelet dense granule-derived ADP in these responses.

Recent studies have highlighted the possibility that hematophagous arthropod-derived salivary apyrases have the potential as anti-thrombotic agents [39]. The high yield of the expression of functional recombinant mosquito salivary apyrase makes it an attractive alternative in studies addressing the potential of enzymes with ADPase activity as anti-platelet agents.

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