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Recombinant human prorenin expression in baculovirus infected cells

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Abstract

Renin is an aspartyl proteinase implicated in the regulation of blood pressure and fluid balance. As many other secreted enzymes, it is synthesized as a precursor, prorenin, that is cleaved by a processing enzyme during its migration through the cell sorting and secretion pathways. The prosegment plays an important role in the folding, the secretion and the inhibition of renin. A prediction of the three-dimensional structure of this zymogen has been derived from those of other aspartyl proteinases but recent findings have raised some doubts about the validity of this model. The best solution would be to crystallize the human prorenin but problems have been found with bacterial, yeast and mammalian expression systems. This study reports the expression of human prorenin in baculovirus infected arthropod cells and the optimization of this system. Up to **8 mg/L** of recombinant human prorenin is produced by High-Five™ cells. Comparison with prorenin produced by GH_4 cells demonstrates that they have similar enzymatic activities and that they are recognized by the same antibody. However, the glycosylation of the recombinant prorenin produced in insect cells is different as it migrates at a lower molecular weight on a SDS-PAGE and it is not retained by an anion exchange column. By taking advantage of these different properties of the recombinant enzyme, a method for a large scale purification of prorenin has been designed.

Résumé

La rénine est une aspartyl protéinase qui joue un rôle essentiel dans le maintient de la pression artérielle et du volume sanguin. Tout comme un nombre important de protéines sécrétées, elle est synthétisée sous la forme d'un précurseur, la prorénine, qui est maturée lors de son passage dans la voie de sécrétion régulée de la cellule. Malgré tout, le prosegment joue un rôle très important dans le repliement, la sécrétion et l'inhibition de la prorénine. Les prédictions faites au sujet de sa structure tridimensionnelle ont été réalisées à partir des structures connues d'autres aspartyl protéinases mais des données récentes soulèvent des doutes quant à la validité de ce modèle. La cristallisation de la prorénine humaine permettrait de répondre à ces questions mais des problèmes sont survenus lors de tentatives d'expression dans des systèmes bactériens, mammifères ou chez la levure. Cette étude rapporte l'expression de la prorénine humaine dans des cellules d'insectes infectées à l'aide d'un baculovirus et l'optimisation de ce système en fonction de différentes conditions. Jusqu'à **8 mg/L** de prorénine recombinante ont été produite chez des cellules High-Five™ infectées avec le virus recombinant pPAPProR. La comparaison avec une prorénine recombinante produite par des cellules GH_4 démontre qu'elle a une activité catalytique similaire et qu'elle est reconnue par le même anticorps. Toutefois, la glycosylation de la prorénine recombinante produite chez les cellules d'insectes diffère de celle de l'enzyme mammifère puisqu'elle migre à un poids moléculaire moins élevé sur un SDS-PAGE et qu'elle n'est pas retenue lors de son passage sur colonne échangeuse d'anions. En prenant avantage des propriétés uniques de cette protéine, il nous a été possible de mettre sur pied une stratégie de purification efficace de notre prorénine humaine recombinante.

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1. Introduction

Hypertension is a pathology affecting near 20% of the adult population in North America where it is contributing to over 200 000 deaths annually and is a heavy burden on the budget of medical care[1]. Lethality often results from complications associated to sustained high blood pressures such as coronary heart disease, congestive heart failure and stroke. Also, prolonged hypertension causes hemorrhages in the kidneys leading to renal failure, uremia and ultimately death[2]. It is generally accepted that 20-40% of the variation in blood pressure is attributable to genetic heritability and around 50% to environmental influences[1]. Apart from three different cases where a single gene was demonstrated to be responsible for the elevated blood pressure, hypertension is considered a multifactorial trait[1]. Consequently, extensive research is in progress to determine the causes of hypertension and the molecular mechanisms implicated in blood pressure regulation. Findings in this field could potentially improve diagnosis, prevention and treatment of the disease.

1.1 The circulating Renin –Angiotensin System

1.1.1 Overview of the circulating Renin-Angiotensin System (RAS)

The Renin-Angiotensin System is of primordial importance to the modulation and long term regulation of blood pressure and salt balance[2]. It has been directly implicated in the development of hypertension in certain populations and renovascular hypertension. Also, it is suspected to be a major contributor to the pathogenesis of essential hypertension[1].

In the classical representation of the RAS (Figure 1)[3], renin is an enzyme released in the afferent arteriole by the juxtaglomerular cells of the kidney in response to a drop in the perfusion pressure detected by the renal baroreceptor, sympathetic stimulation or a decrease in the sodium concentration in the distal tubule[2]. This aspartyl protease cleaves the glycoprotein angiotensinogen (Aogen) in the circulation

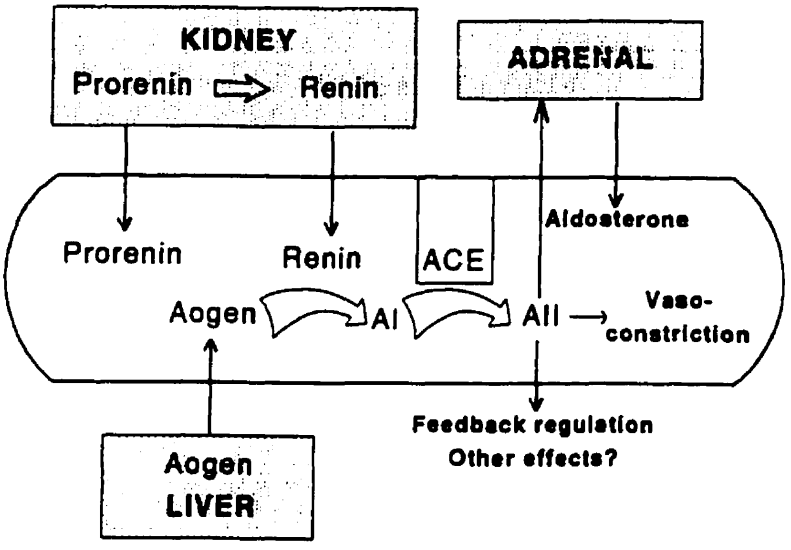


Figure 1: The classical view of the renin-angiotensin system (adapted from ref. 3 with permission).

Aogen: angiotensinogen **ACE**: angiotensin converting enzyme **AI**: angiotensin I **AII**: angiotensin II.

to generate angiotensin I (AI) that is rapidly processed to angiotensin II (AII) by the angiotensin converting enzyme (ACE) present on the surface of endothelial cells[3]. Angiotensin II then binds to the type I angiotensin receptor on target cells to promote smooth muscle contraction, increased secretion of aldosterone by the adrenal glands and feedback regulation on the expression of RAS components[3]. In turn, aldosterone increases sodium and water reabsorption by the proximal tubules in the kidney, thus elevating arterial blood pressure to normal levels. Also, AII acts directly on sodium reabsorption by the proximal tubules and constricts the efferent arteriole to increase the glomerular filtration rate[2]. In the end, AII is converted to angiotensin III and IV by the action of aminopeptidases. The physiological importance of these peptides is currently unknown but a new receptor that binds specifically AIV in the brain has been reported.

1.1.2 Angiotensinogen is the natural substrate of renin

Angiotensinogen is constitutively secreted by the hepatocytes and is produced to a lesser extent in other tissues such as the brain, the heart, the kidneys, the adrenal glands and the adipose tissue[4]. Nevertheless, circulating levels of Aogen reflect mainly liver synthesis and the plasma is considered as the main reservoir for this protein. The human angiotensinogen gene has been mapped to the long arm of chromosome 1, in proximity to the renin gene, and contains five exons and four introns that span over 13 kb. Its transcription is augmented in the liver by estrogens, glucocorticoids, thyroid hormones and possibly AII[4].

Angiotensinogen, a 452 amino acids globular protein, is the only naturally occuring substrate of renin in plasma and the release of the decapeptide angiotensin I from its N-terminus represents the rate-limiting step of the RAS in humans (Figure 2). Although, the circulating concentration of human Aogen is in the micromolar range[5] (~1.3 μM) - a thousand times the circulating renin concentration[4] - the K_m of human renin for Aogen is roughly equivalent (1.15 μM) to its circulating concentration[6]. Therefore, angiotensinogen is probably not in sufficient excess over renin and this reaction is dependent on the circulating levels of both proteins[4]. In contrast, the

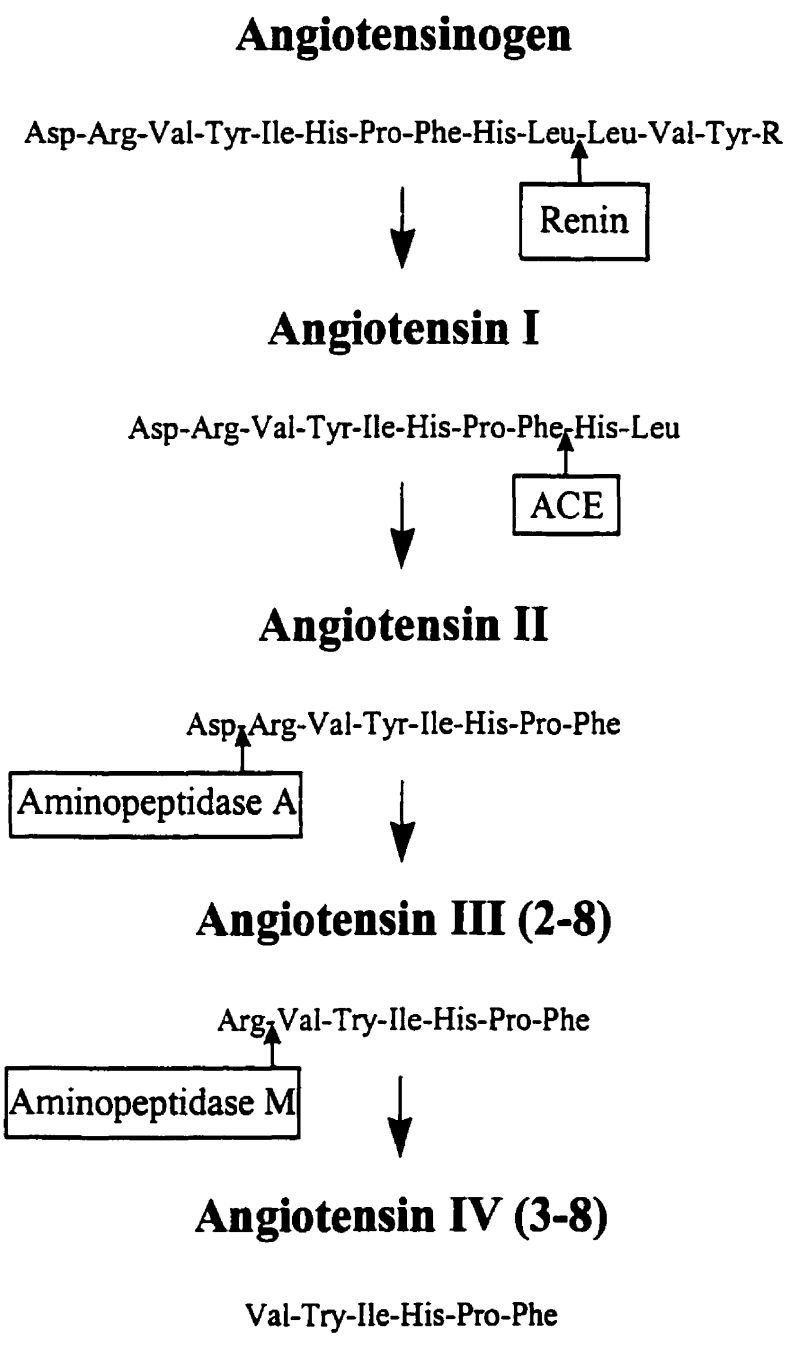


Figure 2: Cleavage site of angiotensinogen by renin and sequence of the angiotensin peptides. ACE: Angiotensin converting enzyme. Other aminopeptidases or carboxypeptidases have been implicated in the degradation of angiotensin II.

mouse circulating RAS is dependent on angiotensinogen concentration whereas in the rat, renin production and release is the limiting factor.

Taking advantage of the fact that renin and angiotensinogen show little cross-species reactivity[7], investigators have generated different transgenic models to test the physiological importance of the RAS in hypertension. Reports have shown that angiotensinogen knock-out mice are hypotensive[8] and that double transgenic mice expressing the human angiotensinogen and renin genes develop chronic hypertension that can be reduced by a human renin specific inhibitor[9]. Further, a molecular variant of angiotensinogen (M235T) has been identified from sibling genetic linkage studies[10] and is associated with an increased rate of gene transcription and angiotensinogen concentration in plasma. These results confirm the essential role of the RAS for blood pressure regulation and implicate angiotensinogen in the pathogenesis of essential hypertension in humans.

1.1.3 Active renin is produced in the JG cells of the kidney

Human[11], rat[12] and mouse[13] renin genes have all been cloned and characterized. In contrast with humans and rats that only have one copy of the renin gene expressed in the kidneys, inbred strains of mice carry the *Ren-1d* gene transcribed at high levels in the kidneys whereas the *Ren-2* gene is expressed in the submaxillary glands[3]. Nevertheless, all renin genes encode protein precursors of 400-406 amino acids that have a high sequence homology[3,9]. They contain a signal peptide driving their insertion in the rough endoplasmic reticulum (RER) and a pro region at their N-terminus. Upon insertion in the RER and removal of its signal peptide, prorenin is released and post-translationally modified. Two consensus sequences for N-glycosylation are present in human renin and three such sites can be found in rat and mouse renal renins[3]. During its migration through the RER and the Golgi apparatus, the sugar moieties of prorenin are modified to generate multiple species of prorenin and renin that can be separated by lectin affinity chromatography[14] or isoelectric focusing[15]. Further, partial phosphorylation of mannose residues on carbohydrate side chains of human renin creates a classic lysosomal targeting signal[16].

Circulating active renin is secreted by the juxtaglomerular cells of the kidney which are modified smooth muscle cells located between the afferent arteriole and the distal tubule, in proximity to the glomerulus[2]. They represent approximately 0.1% of the adult kidney cellular mass and are rich in dense core secretory granules, which is reminiscent of other neurendocrine cells[3]. Two striking characteristics distinguish them from other cell types : First of all, paracrystalline structures of rhombus shape are observed in membrane-bound structures in the cytoplasm or budding off from the TGN[3]. Second, the secretory granules of JG cells display properties that are similar to those of lysosomes. They are immunoreactive to antibodies against common lysosomal enzymes[17] and they contain multiple vesicular inclusions and membrane fragments, suggesting that they are capable of autophagy and micropinocytosis[18]. Taken together, these results have led investigators to suggest that JG cells have adapted lysosomes for the processing and regulated secretion of renin[18].

Three different mechanisms are responsible for the secretion of the products of the renin gene in JG cells (Figure 3). Primarily, prorenin has been shown to be released by the plasma membrane from low-density vesicles migrating from the TGN on microtubules, indicative of the constitutive secretory pathway[3]. Also, the paracrystalline structures that are sorted from the TGN, described as protogranules, are rich in prorenin and later join together to form immature (also referred as juvenile) secretory granules which can be seen by electron microscopy as low-density vesicles bound to membranes[3]. Sometimes, fusion with the plasma membrane will release prorenin, a process corresponding presumably to the basal secretion of prorenin[3]. Finally, the remaining immature granules undergo a condensation process that is associated with the maturation of prorenin to renin and a progressive increase in their density, as visualized under the electron microscope[3]. The most dense granules are referred to as mature granules and may contain inclusions. They are released from the JG cells as part of the regulated secretion pathway[3].

Human prorenin processing begins in the immature granule by enzymatic cleavage of a peptide bond following a pair of basic residues (Lys-Arg) near the N-

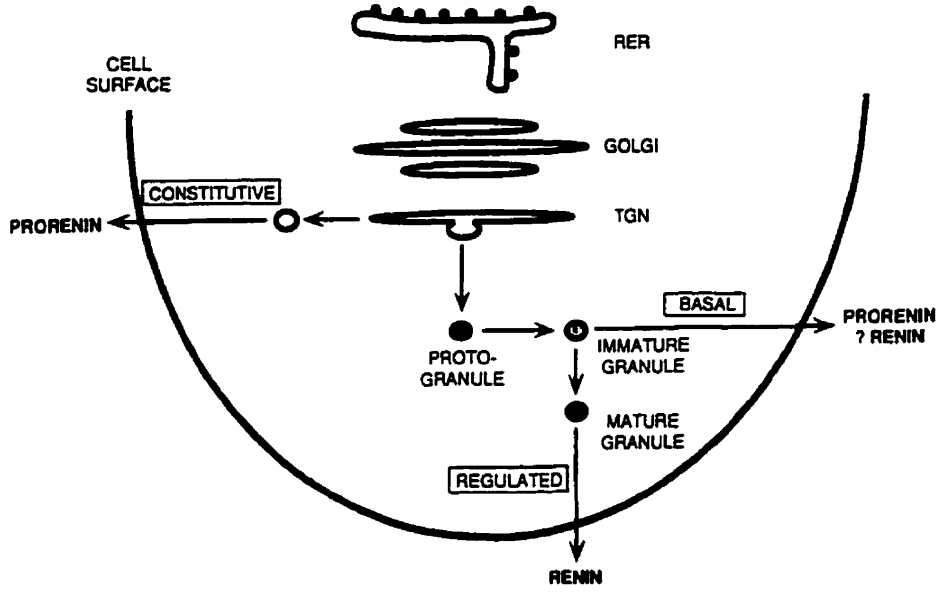


Figure 3: Representation of the proposed secretory pathways in juxtaglomerular cells. (Adapted from Ref. 3 with permission). RER: rough endoplamic reticulum. TGN: trans-Golgi network.

terminus of the protein and it results in the removal of the 43 amino acid prosegment[3]. To date, the maturation enzyme present in JG cells and responsible for the conversion of prorenin to renin has not been identified although candidates such as PC1[19], PC5[20] and cathepsin B[21] have been suggested.

1.1.4 The angiotensin converting enzyme (ACE)

ACE, also referred as kininase II, is a zinc metalloprotease that catalyzes the hydrolysis of the His-Leu dipeptide from the carboxy-terminus of angiotensin I (Figure 2)[3]. Early in the study of the RAS, the most important site of generation of AII was found to be the pulmonary circulation where ACE has been located by ultrastructural immunochemistry on the plasma membrane of the vascular endothelial cells[22]. Interestingly, the angiotensin converting enzyme has been detected in many other tissues like the kidney, the testis, the ovaries, the adrenal glands, and the brain. A soluble form can also be found in the circulation. As a result, this enzyme has been implicated in the inactivation of other oligopeptides such as bradykinin, susbtance P, LH-RH and the enkephalins[22]. A single human ACE gene has been found and it is translated to a 1306 amino acids protein with a molecular weight of 150 000 to 180 000 Da[22]. It contains a C-terminal hydrophobic region driving its insertion in the plasma membrane. Analysis of its structure revealed that this protein folds in two homologous domains, each presenting a zinc binding site and an active center[22]. However, further examination has shown that only the C-terminal active site is implicated in the hydrolysis process[22].

Numerous investigators have reported the beneficial effect of ACE inhibition in the treatment of hypertension[23]. Also, ACE inhibitors have been implicated in renal and cardiovascular protection[23] and their administration improves the survival of hypertensive patients, thus confirming the essential role of the renin-angiotensin system in the pathogenesis of these deseases.

1.1.5 Angiotensin receptors type I and II and their physiological role

Angiotensin II, the most physiologically important peptide of the RAS, has been reported to interact at the surface of target cells with two different 7 transmembrane domain receptors in humans: the AT_1 and AT_2 subtypes. Both receptors are distributed throughout the body but their localization varies within organs and between species. To date, all the biological functions of AII have been attributed to the type 1 angiotensin receptor[24]. These include vasoconstriction, increased aldosterone secretion from glomerulosa cells, stimulation of the release of antidiuretic hormone, increased cardiac contraction and stimulation of platelet aggregation. In addition, AII has been reported to stimulate proliferation and hypertrophy of vascular smooth muscle cells and it has been implicated in the long-term development of congestive heart failure[24]. The AT_1 receptor is linked to numerous signaling pathways in vascular smooth muscle cells[25]. It has been shown to be directly coupled via G_q-proteins to phospholipase C, the inactivation of adenylate cyclase and the release of calcium from intracellular stores [25]. Also, investigators have reported that phospholipase D and A_2 are also involved in signaling. Finally, the AT_1 receptor has been linked to tyrosine phosphorylation of numerous proteins and the activation of ras, JAK2, TYK2, NADH/NADPH oxidase, FAK and the MAP kinase pathway[25]. However, the intermediates involved in the transmission of the signal have not all been identified and further research is in progress to clarify this situation. In contrast, no direct signaling mechanism has been characterized for the AT_2 receptor but some reports have suggested a possible link with cGMP[25].

1.2 Tissue Renin-Angiotensin Systems

In recent years, a number of studies have suggested that local Renin-Angiotensin Systems could have autocrine or paracrine functions in several tissues such as the brain, the kidney, the adrenal and pituitary glands, the reproductive tissues, the heart and the vasculature[3,26]. Proteins, but mostly messenger RNAs, of all the required components have been co-localized in those tissues although sometimes in different cell types[26]. Nevertheless, prorenin maturation has not been demonstrated so far to be significant in any tissue RAS and this casts a doubt upon the functionality

of local systems. In humans and rats, the kidneys appear as the sole source of circulating active renin since its concentration drops to undetectable levels two days following nephrectomy[5,27]. Prorenin concentration also falls in humans but remains measurable whereas it is not detectable in rats. As expected, angiotensin levels are also suppressed in rats and no more than 7 percent of the normal levels are present in humans. Interestingly, isolated and perfused rat hind limbs are capable of generating angiotensin peptides[28]. However, nephrectomy prior to the experiment prevented this result, thus suggesting that active renin was taken up form the circulation[29]. Altogether, these results suggested that active renin is mainly, if not totally, produced in the kidneys but could be retained by certain tissues, presumably to play a local physiological role. In agreement with these observations, two binding sites for renin have been detected in purified membrane fractions of the rat mesenteric arteries and at lower levels in the aorta, lung and renal medulla[30]. Also, a high affinity and saturable binding site for human renin and prorenin was detected in the placenta (S.M.L. Chu : *Master's thesis*).

1.3 Analysis of the three-dimensional structure of renin

The first three-dimensional models of renin were based on the structure of other aspartic proteinases[31]. Indeed, they share a high degree of homology in their primary, secondary and tiertiary structures but differences exist in their catalytic properties, cellular localization and biological properties[32]. This computer model, used primarily to assess the interaction with inhibitors, predicted that the glycosylation sites were located on the surface of the protein and that the cleavage site of the pro region was easily accessible to the processing enzyme[31]. Later, crystallization of the recombinant human renin[33] and the mouse submaxilliary renin[34] confirmed the validity of the models. Human renin is composed of two similar domains separated by a long and deep cleft where the aspartic acid residues catalyzing the reaction and the substrate binding sites are located[8,33]. Further analysis has shown that the substrate specificity of renin could be attributable to secondary structure elements at the periphery of the binding cleft[34]. The highest homology with the models was found in the hydrophobic core, the ß-sheet and among residues in the

substrate binding sites whereas differences were noted in the surface loops and those surrounding the entrance to the active site[33].

1.4 Role of the prosegment of prorenin

A large number of proteins of eukaryotic origin and essentially all known extracellular bacterial proteases and aspartic proteinases are synthesized as zymogens and multiple functions have been associated to their prosegment[32,35]. In general, this region of the protein is found as an amino-terminal extension that follows the signal peptide and must be cleaved by another protease to release the active enzyme, but investigators have also described proteases with carboxy-terminal extensions or a combination of the two that have the same properties[35]. Their size is highly variable with reports of prosegments ranging form ~40 amino acids to ~60 kDa[35]. The N-terminal pro region of aspartic proteinases is usually 44 to 50 amino acids long[32]. Studies on different proteinases have demonstrated that the prosegment is required for proper folding of the protein where, unlike chaperones that prevent aggregation, it directly increases the rate of the forward folding reaction by lowering the high free-energy barrier leading to the native state[35]. The prosegment strongly interacts with the product of the folding reaction and probably limits the number of conformations that can be achieved. In addition, prosegments are involved in protein transport. An interesting example comes from experiments in the yeast *Yarrowia lipolytica* where secretion of the alkaline extracellular protease is inhibited by removal of its prosegment but can be restored if supplied in *trans*[36]. Finally, in all cases examined thus far, the pro regions are potent inhibitors of their respective enzyme[35].

In vitro, prorenin can be reversibly activated by acidification[37] or prolonged storage in the cold[38]. Suzuki *et al.*, using a model of the three-dimensional structure of rat prorenin based on the atomic co-ordinates of pig pepsinogen A and the homology to human renin, have suggested that lysine-43 in the prosegment is located within 3.0×10^{-1} nm of the catalytic residues Asp81 and Asp266[39]. Further, site directed mutagenesis of this lysine (Lys43Leu) increased the acid-activation speed of rat prorenin 3 fold compared to the wild-type and the mutant was more labile at 55°C

and neutral pH, suggesting that ionic interactions between Lys43 and the aspartic acids stabilizes the protein[39]. In addition, this group reported that truncation of the first 10 amino acids of the prosegment of rat prorenin increased its activity to 30% of the full renin activity whereas the wild-type precursor was inactive[40]. Thus, the pro region is also implicated in the inhibition of rat renin. Other investigators have confirmed these results and shown that the N-terminal fragment of pig pepsinogen A (1P-12P and 1P-16P), the pro region of cathepsin D and the N-terminal prosegment peptide (P10-P20) of prorenin inhibit their respective enzymes[32] (Here, P designates prosegment and the numbering begins after the signal peptide cleavage site). Analysis of the X-ray structure of rat pepsinogen A has shown that the N-terminal portion of the pro region is located within a ß-sheet and interacts with the S_3 binding site while the doublet Lys36P-Tyr37P and the conserved Tyr9P form hydrogen bonds with the aspartates of the active site and block the subsites S_1 and S_2[32]. On the other hand, Yamauchi *et al.* have shown that replacement in human prorenin of three arginines (Arg10P, Arg15P and Arg20P) almost completely activated the zymogen[41]. Finally, the prosegment of aspartic proteinases has been implicated in their sorting and folding. In particular, the interaction of procathepsin D with intracellular membranes has been reported and is independent of the mannose-6-phosphate (M6P) signal targeting the enzyme to lysosomes[32]. The presence and the maturation of the prosegment of human renin is also required for correct targeting of the protein to the secretory granules in AtT-20 cells[42] and it has been shown that mutations activating prorenin correlated with a decrease in the release of the protein in response to secretagogues[43]. A short peptide located at the N-terminus of the prosegment fused to the mouse IgG heavy chain directed this protein to the regulated secretory pathway[3]. Also, a small region of the prosegment from Arg-10P to Lys14P has been shown to be essential for the reversible refolding of the prosegment of human renin[44].

1.5 Why should we overproduce prorenin?

The activation of prorenin and other aspartic proteinases is initiated by a dramatic conformational rearrangement of the prosegment that is triggered at a low pH and permits the maturation of the zymogen[32]. This observation is supported by reports showing that replacement of basic residues in the prosegment of prorenin facilitates the activation of the protein[32,41]. Presumably, protonation of these acidic amino acids involved in the ionic interactions of the prosegment would lead to the conformational change. However, all of the reported interactions between the prosegment of prorenin and the catalytic site are derived from mutagenesis studies and models based on the tertiary structure of porcine pepsinogen A. Comparisons were made between different aspartic proteinases based on their primary sequence homology but predictions varied depending on their alignment[32]. Also, results that raise doubts about the validity of the current models have been obtained. Heinrikson *et al.* found that prosegment residues Arg-10P to Lys-14P are important for the folding and the inactivation of human prorenin[44]. This was latter confirmed in a study of site-directed mutagenesis of many amino acids of the prosegment of human renin. In that case, replacement of Leu13P by alanine activated the zymogen almost to its full potential (91.2%) whereas other mutations from 10P to 20P had a mild effect on activation[43]. Also, Cumin *et al.* have shown that among synthetic peptides corresponding to the pro region of human renin, only those that included the residues Phe-12P to Pro-17P were potent inhibitors of the enzyme[45]. No equivalent for the Lys36P interaction with one of the catalytic residue has been found in renin to date[44]. Consequently, this has led some investigators to propose that the prosegment folding and inactivation of prorenin might be quite different from that of pepsinogen[43,44]. Therefore, crystallization of human prorenin will be essential to fully characterize the interactions of the pro region, to determine which model is correct and to understand the conformational change that occurs upon activation of the protein. Renin inhibitor design would also benefit from this information. The establishment of a system for a large scale production of prorenin becomes a *sine qua non* condition. Large quantities of semi-purified human prorenin could also be used in competition studies or to raise new monoclonal or polyclonal antibodies. Once the system would be in

place and optimized, generation of mutant prorenins in biochemical quantities would be simplified.

1.6 Prorenin production in different expression systems

Many different expression systems have been developed over the years to over-express mammalian proteins and facilitate their purification. Each have their own advantages and flaws and the choice of a suitable system depends primarily on the properties of the protein of interest and the quantities required[46].

Large scale production of prorenin in different systems has already been tried but without success. Significant levels of the protein were obtained in bacterial expression systems or in the yeast *Sacharomices Cerevisiae* but prorenin aggregated within these cells and could not be renatured (T.L. Reudelhuber, *unpublished results*). On the other hand, prorenin was correctly folded and secreted by GH_4 cells but the yields obtained were too low (~100 µg/L; T.L. Reudelhuber, *unpublished results*). This result was also observed in stable transfections of dihydrofolate reductase (DHFR)-minus CHO cells which secreted up to 0.25 µg of prorenin/1 x 10^6 cells/day, but progressive selection with methotrexate of cell strains with an increased gene copy number augmented the production up to 4 µg of prorenin/1 x 10^6 cells/day in continuous suspension cultures with cell recycling[47]. However, analysis of the protein by SDS-PAGE gel electrophoresis showed two major bands corresponding to prorenin[47]. The source of the heterogeneity was predominantly located in the glycosyl moieties and a crystallization of prorenin was not possible. This situation is reminescent of the heterogeneity of prorenin in GH_4 supernatants where up to 5 different isoforms are separated by isoelectric focusing[15]. Moreover, investigators have reported that proteases secreted in the supernatant of CHO cultures were responsible for a progressive triming of the prosegment that generated a series of truncated and active prorenin species[44]. Purification of a truncated form of prorenin from the human chorion-decidua has also been described[48]. From these results, it can be concluded that the ideal system for a large scale production of human prorenin would combine the yields obtained from the bacterial or yeast expression systems to

the ability of targeting prorenin correctly to the constitutive secretory pathway. Further, the glycosylation of the protein would have to be limited and more homogenous and the presence of specific proteases with trypsin-like activities would have to be avoided. In our view, the baculovirus expression vector system was the most promising to fulfill all these requirements. **This study reports our attempt to establish a system where prorenin is secreted by High-Five™ insect cells infected with a recombinant baculovirus.**

1.7 Overview of the baculovirus expression vector system (BEVS)

Baculoviruses (*Baculoviridae*) belong to a family of large circular double-stranded DNA viruses that infect specifically different species of arthropods and that are inoffensive to vertebrates. Their genome varies from 80 to 200 kbp and is packaged into rod-shaped nucleocapsids[49,50,51]. Upon infection, virus particles enter susceptible insect cells by facilitated endocytosis or fusion and their DNA is uncoated in the host nucleus. Viral DNA replication occurs in the next 6 hours post-infection (PI). Afterwards, two different mechanisms are responsible for the release of virus particles : In the early phase of the infection cycle (10 to 24 hours), insect cells release extracellular virus particles (ECV) from their plasmatic membrane whereas latter in the infection (2 to 6 days), virus particles are assembled in the nucleus and embedded in a homogenous protein matrix to from occlusions[49]. These occlusions are then released when cell lysis occurs late in the infection cycle and they protect the virus from inactivation and degradation. In a natural environment, occlusions liberated from decomposing tissues will eventually be ingested by other insects and the occluded virus particles will be released in their midgut, permitting the horizontal transmission of the virus. After this initial infection, ECV particles are released in the insect hemolymph and propagate the infection to the entire organism[49,50,51].

Polyhedrin (29 kDa) is a major component of the homogenous protein matrix and is produced at very high levels by insect cells during the late phase of infection. Up to 1 mg/ml is synthesized per 1-2 x 10^6 infected cells, representing almost 30-50% of the total protein level[49]. Although this protein is one of the most abundant during

the infection, its role is limited and it is not essential for the baculovirus life cycle in cell cultures[49]. The most studied of the baculovirus strains is the *Autographa Californica* nuclear polyhedrosis virus (AcNPV) and its entire genome (127.8 kbp) has been mapped and partially sequenced. Apart from the polyhedrin protein, other genes from this strain like p10, p39 and the basic protein gene have been cloned and shown to be non-essential for virus replication and propagation[49].

The baculovirus expression vector system (BEVS) has been developed from these studies and is now commercially available[49,50,51]. Several strong promoters from non-essential genes of the AcNPV strain such as the polyhedrin promoter have been characterized and used in the construction of different baculovirus transfer plasmids[49,50,51]. All of these contain an *E. Coli* origin of replication and an Ampicilin resistance gene for amplification in bacteria using standard techniques. Also, two different sequences of the AcNPV virus enclose a strong baculovirus promoter that will drive the expression of the foreign gene of interest. Therefore, when the purified recombinant plasmid is transfected in a specific insect cell line along with the wild-type AcNPV DNA, homologous recombination will generate viruses where the gene of interest has replaced a specific baculovirus gene and is expressed by its strong promoter[49,50,51]. Replacement of the polyhedrin gene of the AcNPV virus results in the production of recombinant viruses that do not produce occlusions in the nucleus of infected cells and this specificity was exploited at first to isolate them from the wild-type strain. However, these viruses are the result of homologous recombination events taking place inside the cells and the efficiency of this process is low (0.1%)[52]. Furthermore, not all wild-type infected plaques show occlusions and this complicated the isolation of the recombinant virus[49]. Several manipulations of the wild-type AcNPV have been done to improve the efficiency of the recombination event and to facilitate the selection of the recombinant. Kitts *et al.* have demonstrated that linearization of the baculovirus improved homologous recombination with the transfer plasmid up to 25%[53]. Now, strategies like positive survival selection by rescuing a lethal mutation in the AcNPV virus[49], color selection by co-expression of the ß-galactosidase[50] gene or the green fluorescent protein[54], transposon-mediated

modification of the AcNPV and YAC-mediated recombination[52] have been developed by the industry to facilitate the purification of the recombinant virus.

1.8 Advantages of the BEVS in the production of eukaryotic proteins and prorenin

Several features of the baculovirus expression vector system have made it increasingly popular for large scale production of proteins and it is now a system of choice used for many applications[55]. First of all, foreign gene expression driven by very strong baculovirus promoters such as the polyhedrin promoter have yielded impressive amounts of proteins compared to higher eukaryotic expression systems and this would be ideal for a large scale production of prorenin. Concentrations up to 100 mg of protein per liter have been reported in the literature[55]. Nevertheless, many recombinant proteins are not produced at such high levels and it seems difficult to predict how the system would perform for prorenin. Since this system has been developed recently, extensive characterization of its properties has not been completed and definitive guidelines have not been defined to date.

Second, overexpression of eukaryotic genes in insect cells provides an environment where proteins are properly folded and most post-translational modifications are correctly processed[49,55]. Different reports have shown that disulfide bond formation, oligomerization, N- and O-linked glycosylation, phosphorylation, acylation, amidation, carboxymethylation, isoprenylation, signal peptide cleavage and proteolytic cleavage all occur in arthropod cell lines[49,55]. Therefore, the product most likely resemble the native protein both structurally and functionally. However, tissue or species-specific modifications are generally not present and a good knowledge of the protein of interest is required to achieve correct expression. In this study, cleavage of the pro fragment of renin was not expected since the specific protease thought to be found in the secretory granules of the JG cells was unlikely to be present in insect cells. Although a native insect protease could process the prosegment, it was suspected that prorenin would enter the constitutive secretory pathway as it has been observed in mammalian expression systems[3,45]. Moreover,

characterization of the glycosylation of foreign proteins expressed at high levels in insect cell lines has shown that some late modifications are not present and that lower levels of glycosylation and phosphorylation can be expected[49,50,55]. Since it has been reported that multiple glycosylated forms of prorenin were present in plasma[56], expression by the baculovirus system would be beneficial if a more homogenous pattern of glycosylation of prorenin could be found as it would facilitate the purification of the protein and its crystallization.

Third, many cell lines with different characteristics are now available on the market and can be tested to maximize the production of the protein of interest. The most frequently reported are *Trichoplusia ni* egg cells (High-Five™ cells) or the Sf21 and Sf9 lineages derived from *Spodoptera frugiperda* ovarian cells[50]. All have been used in serum-free conditions and adapted to suspension cultures.

Other advantages of the baculovirus expression system have been reported although they were not applicable to this study. The virus capsid structure of baculoviruses is expendable and permits the insertion of very large genes[49,55]. No known upper size limit has been reported to date. Furthermore, this system has the potential to express two or more genes simultaneously allowing the formation of complexes and their study[57]. Finally, arthropod cells can perform intron/exon splicing although certain patterns will not be observed if they require splicing factors not found in the cell lines that are used. Nevertheless, higher levels of expression have been achieved using cDNA inserts rather than genomic DNA as it was the case for this study[49].

1.9 Disadvantages of the baculovirus expression vector system

Expression of a foreign protein by the baculovirus system is relatively easy and reliable but is time-consuming. Virus purification can take as much as two to three weeks per cycle and optimization of the conditions for maximal protein expression by infected insect cells requires multiple experiments, adding to the time needed to set up the system[55]. In addition, insect cells normally have a doubling time averaging 24

hours and harvest of the product from infected cultures have been reported up to 7 days post-infection[58]. Compared to bacterial expression systems, this approach is several magnitudes longer and more costly. On the other hand, very high levels of expression have not always been obtained and reports have shown that some modifications to the proteins were necessary[55]. In some cases, the native signal peptide of the protein had to be replaced[55,59]. In spite of certain difficulties encountered, the BEVS can be very useful because it combines yields that are reminescent of bacterial or yeast expression systems and the presence of the machinery responsible for eukaryotic post-translational modifications.

1.10 Experimental outline of the steps involved in large scale production of prorenin by the baculovirus expression vector system

In order to develop a BEVS that will lead to the crystallization of prorenin, numerous steps were necessary to maximize the production of the protein and to develop a strategy for its purification. They are summarized below (Table 1).

Table 1: Experimental outline of the steps necessary to produce large amounts of pure prorenin in insect cells infected by a recombinant baculovirus.

1. Selection of the transfer vector.
2. Cloning of prorenin cDNA and amplification of the transfer vector in *E Coli*.
3. Establishment of insect cell cultures.
4. Co-transfection of the wild-type linearized AcNPV and the transfer vector in insect cells
5. Verification of the transfection efficiency.
6. Recombinant virus purification by the plaque assay (to be repeated if necessary).
7. Propagation of different virus clones and analysis of their efficiency to drive prorenin expression by insect cells.
8. Production of a large recombinant virus stock and determination of its titer.
9. Optimization of the production of prorenin by different insect cell lines in function of time, the multiplicity of infection, the cell density and the media.
10. Small scale production of prorenin.
11. Isolation of prorenin and testing of different purification steps.
12. Characterization of prorenin produced in insect cells.
13. Development of methods for a large scale production of prorenin

2. Material and Methods

2.1 Materials

All restriction and modifying enzymes used in the construction of the vectors were purchased from Pharmacia Biotechnologies, Inc.

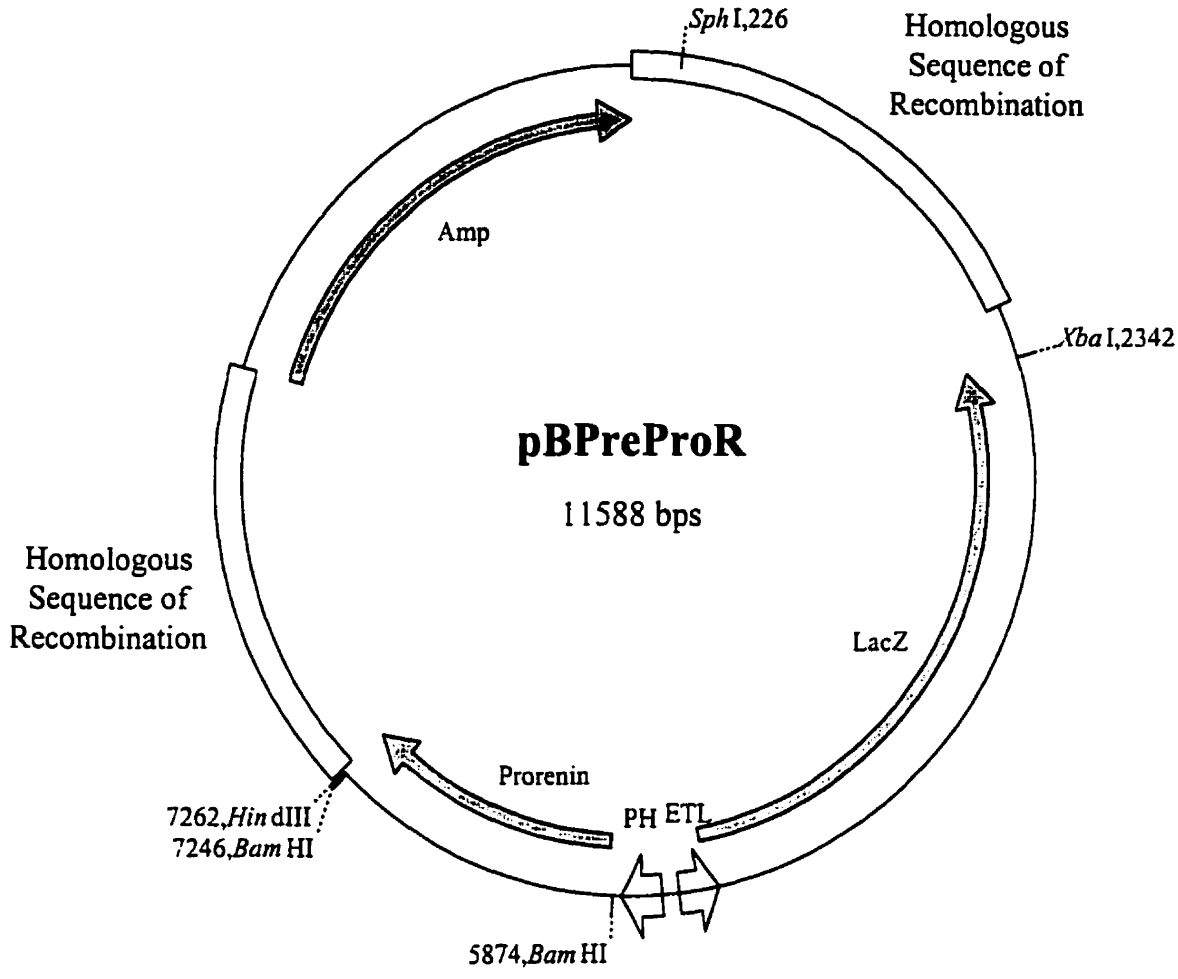
2.2 Vector construction

2.2.1 pBPreProR

In order to generate the vector pBPreProR, the vector pBbac (Invitrogen, Inc.) was digested using the *Bgl II* and *Nco I* restriction enzymes and the 5' overhangs were dephosphorylated by the bovine alkaline phosphatase overnight at 37°C. The pRHR1100 vector[60] containing the human preprorenin cDNA was digested by the *Hind III* and *Bam HI* restriction enzymes. Fragments from the two reactions were separated in a 0.8% agarose gel (ICN Biomedicals, Inc.) and both the vector pBbac and the cDNA were recovered on a NA45 DEAE-cellulose membrane and eluted in a high salt buffer according to the manufacturer's instructions (Schleicher & Schuell, Inc.). Following a brief phenol/chloroform extraction and ethanol precipitation, the purified fragments were incubated for half an hour in a solution containing one unit of the klenow fragment and 10 mM dNTPs in order to generate blunt ends. Finally, after an extraction and purification step, the insert and vector in a 2 to 1 ratio were incubated overnight at 16°C with the T4 DNA ligase in One for All buffer™. Correct insertion of the renin gene was easily verified by enzymatic digestion since the *Bam HI* site downstream of the renin gene was recovered in such a case (see figure 4). From one of the positive bacterial colonies, a large preparation of the plasmid was started and it was purified by a CsCl ultracentrifugation following standard procedures[61].

2.2.2 pPAPProR

The pPAPProR vector (Figure 6) was constructed in three steps because a restriction map of the parental vector pPbac[62] (Stratagen, Inc.) was not available at the



Feature	Start	End
Homologous region of recombination	*1*	*2098*
Beta-galactosidase gene	*5433*	*2344*
ETL promoter	*5476*	
Polyhedrin promoter	*5739*	
Preprorenin cDNA	*5892*	*7251*
Homologous region of recombination	*7273*	*9198*
pUC ori/Ampicilin resistance gene	*9199*	*11587*

Figure 4: The pBPreProR vector.

The preprorenin cDNA was cloned as described in the pBbac (Invitrogen, Inc.) commercial vector. Correct insertion of the fragment was verified by enzymatic digestion of the vector at the Bam HI restriction sites. PH: Polyhedrin promoter. ETL: Early-to-late promoter. LacZ: ß-galactosidase gene. Amp: Ampicilin resistance gene.

time and also because of difficulties encountered with direct cloning of the PCR fragment and the replication of the vector. In brief, the signal peptide of the prorenin cDNA was replaced by that of human Placental Alkaline Phosphatase (hPAP) in a two-step PCR reaction following standard procedures[63]. A schematic representation of the approach is presented below (Figure 5).

In the first reaction, the replacing signal peptide was amplified from 100 ng of the vector pPbac using a 5' oligonucleotide (309: ATC GGG CGT **GCT AGC**) that included the *Nhe I* restriction site and a 3' oligonucleotide (310: *GT CGG GAG* GCC CAG GGA GAG CTG) that created a fusion with the first amino acids of the renin prosegment (1). On the other hand, the prorenin cDNA from pRHR1100[60] was amplified with a 5' oligonucleotide (311: C CTG GGC *CTC CCG ACA GAC ACC*) complementary in part to 310 and a 3' oligonucleotide (27: *AAA GAA CAA TCA AGG GTC*) located downstream of the *Bam HI* restriction site (2). After a brief denaturation period of 3 minutes at 94°C, samples were incubated for 24 cycles (30 s; 94°C, 60 s; 50°C and 60 s; 72°C) with 2.5 units of Taq DNA polymerase (Bio/Can Scientific, Inc.) followed by a final step at 72°C for 10 minutes in a TwinBloc™ System PCR apparatus (Ericomp, Inc). The first reaction generated a single product of expected length (92 bp) that was separated from the pPbac vector in a low melt 3 % agarose gel and recovered using the MERmaid® DNA purification kit, whereas the second reaction created a fragment of approximately 1300 bp that was fractionated on a 1.0% agarose gel and recovered using the Geneclean® DNA purification kit (Bio/Can Scientific, Inc.). In a second PCR reaction with the same parameters, the hPAP signal peptide and the prorenin fragment served as templates (3) for an amplification using the oligonucleotides 27 and 309 that created the fusion product (4). After purification, the product was then ligated at 4°C to the linearized pGEM-T vector (5)(Promega, Inc.) designed to receive PCR fragments and a large preparation of the plasmid was realized from a single *E. Coli* colony and purified on a Tip-500 preparation column (Qiagen, Inc.). Finally, a large portion of the prorenin gene was replaced by the native sequence from pRHR1100 using the *Ava I* and *Bam HI* restriction sites to create a new vector (6) that was then digested with *Nhe I* and *Bam*

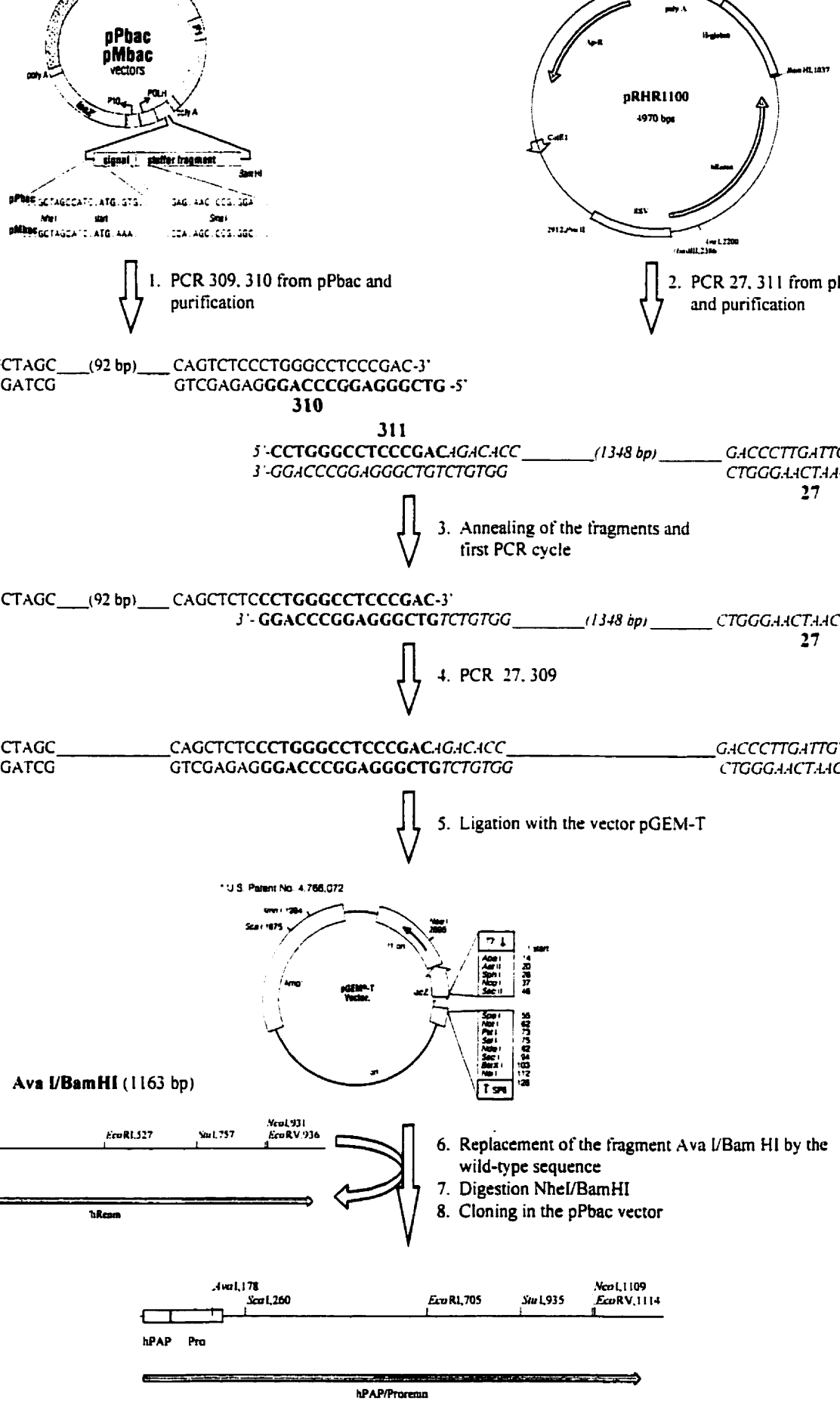


Figure 5: Construction of the pPAPProR vector.(Numbers refer to the description in the text)

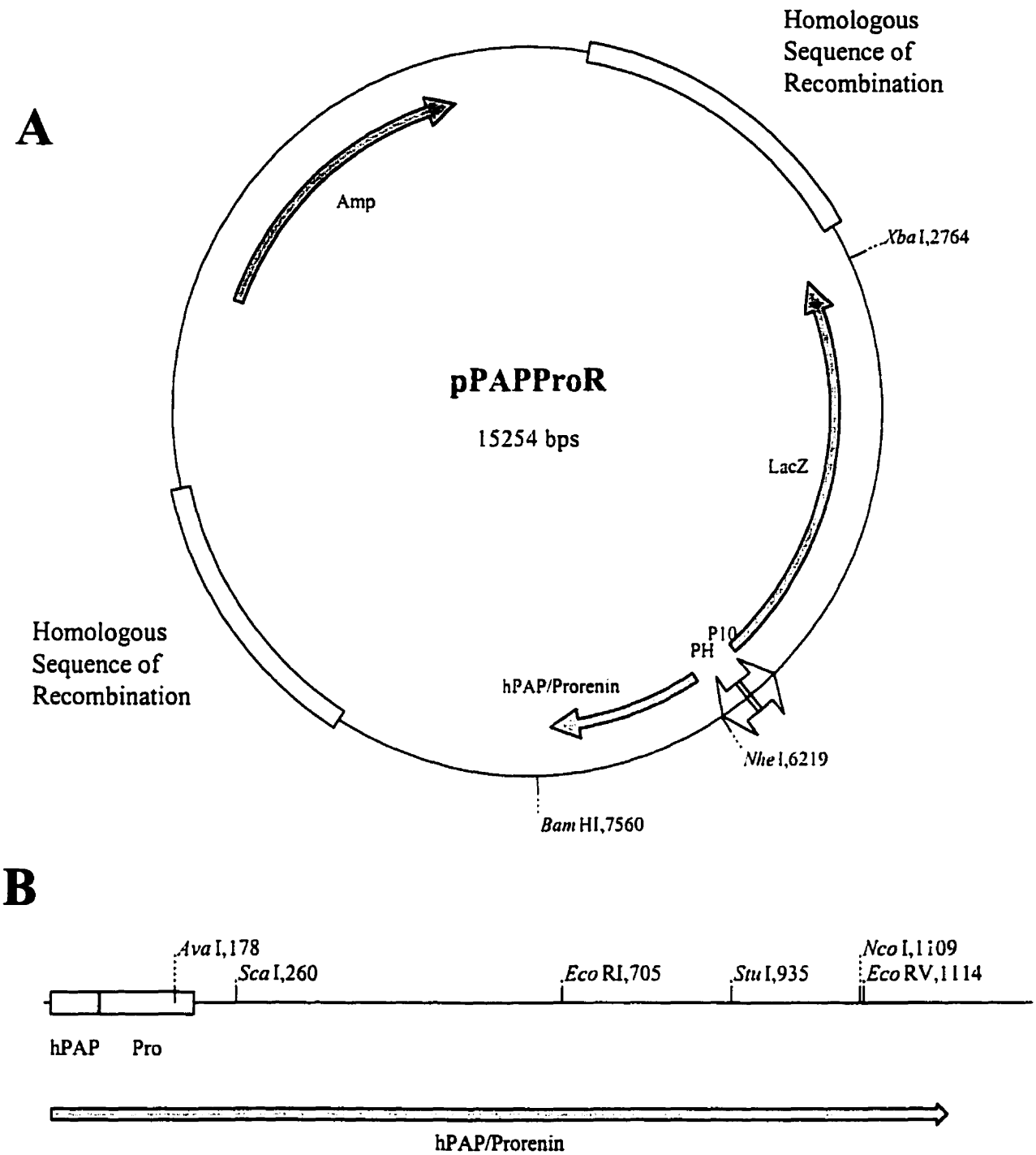


Figure 6: The pPAPProR vector.

The prorenin gene was cloned as described in the pPbac (Stratagene, Inc.) commercial vector between the *Nhe I* and *Bam HI* restriction sites. **A**: Schematic representation of the pPAPProR vector. **B**: The human prorenin cDNA fused to the human Placental Alkaline Phosphatase signal peptide (hPAP). The region from the *Ava I* to the *Bam HI* restriction site was replaced with the wild-type sequence. Pro: Prosegment

HI to release the insert (7) cloned in the commercial vector pPbac (8). Sequencing of the amplified fragment following the Maxam-Gilbert protocol[61] confirmed that no mutations were introduced in the construct and that fusion of the hPAP signal peptide was in frame with the prorenin gene.

2.3 Cell culture of arthropod cell lines

The Sf9 and High-Five™ (BTI-TN-5B1-4) cell lines were generously provided by the laboratory of Philippe Crine at the University of Montreal. Following common procedures[50] cells were placed in a humidified incubator at 27°C and they were subcultured two to three times a week in Grace's media supplemented with 7.5% fetal calf serum (TNM-FH), 10 mg/L of fungizone and 5 mg/L of gentamycin (Gibco/Life Technologies, Inc.). When required, cells were adapted over a one week period to a different environment by slowly increasing the proportion of the new media by increments of 25% every 48 hours when cells were subcultured. Adaptation of insect cells to suspension cultures were realized over a week and three cell passages were done prior to the infections. Pluronic acid (Sigma, Inc.) was also added to a concentration of 0.1% to decrease the shear stress of insect cells in such cultures.

Vials of cells were frozen and placed in a liquid nitrogen chamber in order to preserve a stock from which new cultures could be regenerated if desired. Briefly, cell cultures with good viability were resuspended in TNM-FH, counted with a hematocymeter and centrifuged at 1000 rpm for 15 minutes in an IEC Centra-8R centrifuge. The supernatant was removed and cells were resuspended in FCS 90% / DMSO 10% at a density of 1.0×10^7 cells/ml[50]. Vials of 1 ml were then placed at -20°C for one hour and then at -80°C overnight before being transferred to a liquid nitrogen chamber.

Insect cells are not immortal and their viability decreases with the number of cell passages. Over time, they loose their ability to adhere to the dishes, their infectivity decreases and they form aggregates in suspension. Therefore, new cultures

were regenerated every 4 to 6 months as recommended[50,51] and the number of cell passages was carefully followed. To start a new culture, one vial was removed from the liquid nitrogen chamber and quickly thawed in a 37°C bath before it was rinsed with ethanol and the cells were poured in 5 ml of cold fresh TNM-FH. After one hour at room temperature, the dish was placed in the incubator at 27°C and the medium was replaced quickly with fresh TNM-FH once cells had adhered to the dish. Then, the culture was inspected regularly to assess cell viability and the medium was replaced every 48 hours until cells were ready to be subcultured.

2.4 Transfection of insect cells

The wild-type prorenin recombinant baculovirus was produced using a commercial linear transfection kit (Invitrogen, Inc.) and by following the manufacturer's instructions[50]. A transfection mix comprising 3 μg of the vector, 10 μg of AcNPV DNA, 20 μl of Insectin™ liposomes (Invitrogen, Inc) and 1 ml of Grace's insect media was gently added over a layer of 2,5 x 10^6 Sf9 cells in a 60 mm Nunc™ dish (Gibco/Life Technologies, Inc.) and the culture was placed on a rotatory platform for an hour. Afterwards, the culture and a negative control from which the addition of the recombinant vector was omitted were placed in the incubator for a week. The recombinant virus was harvested two days PI but signs of infection only appeared after five days. Visual inspection of the infected cells showed a difference in morphology, a general increase in cell volume and more cell lysis compared to the negative control.

2.5 Recombinant virus purification

Purification of the recombinant baculovirus expressing prorenin was accomplished by the plaque assay technique[50,51]. Primarily, end-dilutions of the harvested supernatant were made in a range from 10^{-2} to 10^{-8} and Sf9 cells were plated at a density of 5.0 x 10^6 cells per 100 mm dish to generate a uniform layer of approximately 50% confluence. After a period of 3 to 4 hours on a rotatory platform to permit an even distribution of the cells, the supernatant of each culture was replaced by 2 ml of a given dilution and cells were incubated for one hour with mild

agitation in the presence of the virus. Finally, the supernatant of each culture was aspirated and an agarose solution was gently poured on the cells. This agarose solution was constituted by mixing 5 ml of TNM-FH containing 150 µg of X-gal/ml (Gibco/Life Technologies, Inc.), 2.5 ml of TNM-FH equilibrated at 46°C and 2.5 ml of autoclaved baculovirus agarose cooled down to 46°C (Invitrogen, Inc.). Then, after the agarose had solidified, the plates were placed in a humidified incubator at 27°C. Within 5 to 8 days, signs of infection were visible and viral plaques were easily identifiable. The recombinant viral plaques were blue whereas wild-type viral plaques where white, more opaque and showed extensive cell lysis. Moreover, viral occlusions were present in cells infected with the wild type baculovirus around the plaques. Nevertheless, total separation of the two viral types was uncertain at first and further cycles of purification were necessary. Recombinant viruses were recovered by puncturing the agarose layer with a sterile Pasteur pipette and resuspended overnight at 4°C in 2 ml of fresh TNM-FH each. From these solutions, cells were infected according to standard procedures[50] (see below) and the supernatants containing the viruses were recovered after 6 days. Prorenin production driven by each recombinant virus was evaluated by radioimmunoassay and only one was retained. New cycles of purification were then undertaken using the same procedure presented above to make certain the recombinant virus was completely isolated from wild-type contaminants.

2.6 Large scale virus production

Once the most productive recombinant baculovirus was purified, a high-titer stock of the virus was prepared according to standard procedures[50]. After the last cycle of purification, cells were infected to amplify the chosen virus and generate a primary stock of 10 ml (P1) which viral concentration was determined by the plaque assay[50]. Then, a larger viral stock (P2) was created by infecting Sf9 cells at a MOI of 5 and the virus concentration was determined again using the plaque assay. The P2 viral stock (60 ml) was then conserved at 4°C to be used in all subsequent experiments. Small aliquots were placed at -80°C for long term preservation.

2.7 Infection procedure

A simple protocol was used to infect arthropod cell lines and harvest prorenin[50]. Briefly, insect cells between passages 45 and 75 were resuspended from a log-phase growing culture, counted on a hematocymeter under an inverted light microscope and subcultured at a fixed density depending on the dish surface (Table 2). After a brief agitation period to ensure an even distribution on the dish surface, cells were incubated at 27°C for 3 to 5 hours to allow firm reattachment. Then, the supernatant was replaced by a solution containing the recombinant virus at a calculated multiplicity of infection (MOI) and the dishes were placed on a rotatory platform for one hour at room temperature. Finally, the supernatant was again replaced with a defined volume of the incubation medium and cells were transferred in a humidified incubator at 27°C until signs of infection were clearly visible. Supernatants were then recovered and centrifuged at 1000 rpm for 15 minutes in a IEC Centra-8 centrifuge before being transferred to a new tube and frozen at -20°C until further analysis. When necessary, the intracellular accumulation of prorenin was evaluated. First, cells were rinsed twice with a PBS volume equal to the supernatant fraction and incubated 5 minutes with an ABI solution (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0,1% SDS) corresponding to 90% of the supernatant fraction. 20% NP-40 was added to complete the volume to 100% and the cell lysate was passed 20 to 30 times through a 23G needle to breakdown DNA. The intracellular fraction was then recovered after 10 minutes of centrifugation at 13 000 rpm and stored at –20°C if necessary.

Table 2: Seeding densities of insect cells in different culture vessels

Type of vessel	*Number of cells plated*	*Minimal infection volume*	*Final incubation volume*
6-well plate	*0.6-3.6 x 10^6 cells*	*1 ml*	*2 ml*
60 mm dish (Nunc™)	*2.5 x 10^6 cells*	*1 ml*	*5 ml*
100 mm tissue culture dish (Sarsted Inc.)	*5.0 x 10^6 cells*	*2 ml*	*10 ml*
150 mm tissue culture dish (Sarsted Inc.)	*1.5 x 10^7 cells*	*5 ml*	*30 ml*
25 cm^2 T-Flask (Falcon™)	*2.5 x 10^6 cells*	*1 ml*	*5 ml*
150 cm^2 T-Flask (Falcon™)	*1.5 x 10^7 cells*	*5 ml*	*30 ml*

2.8 AI radioimmunoassays

An angiotensin I generating assay was performed with a commercial RIA kit (Dupont, Inc.) and it was used as a routine procedure to compare the production of prorenin from insect cells infected with different baculoviruses in the purification steps and also to determine the specific activity of renin. First, 40 µl samples were incubated at room temperature for one hour in trypsin activation buffer (5 µl of 10X TAB : 500 mM Tris pH 8.0, 1.0 M NaCl, 30 mM EDTA, 100 mM benzamidine-HCl and 5.0% BSA) with 5 µl of trypsin (3 mg/ml) to measure total renin activity or without to determine active renin concentration. Then, a mixture of converting enzyme and angiotensinase inhibitors (49 µl of maleate solution pH 6.0, 0.49 µl of 100 mM PMSF in isopropanol, 0.49 µl of dimercaprol and 0.49 µl of 8-hydroxyquinoline) was added to each sample along with the renin substrate (5 µl of semi-purified sheep angiotensinogen) and the reactions were placed in a 37°C bath for one hour. Afterwards, 50 µl of ^{125}I-AI (~360 cpm/µl) was added to each tube followed by 50 µl of AI rabbit antiserum and samples were incubated at room temperature for 90 minutes. This period allowed the establishment of a competition between the hot and cold AI for primary antibody binding. Finally, 250 µl of anti-rabbit serum was added and samples were incubated at room temperature for one hour. The antigen-antibody complexes that were formed were centrifuged at 3000 rpm for 10 minutes in a IEC Centra-8R centrifuge and supernatants were aspirated by a Pasteur pipette under the hood. Pellets were counted in an LKB-Wallac

Clinigamma 1272 automatic gamma counter and counts were compared to a standard AI curve to determine renin activity. In order to evaluate prorenin content, renin activity in non-treated samples was subtracted from total renin activity in samples incubated with trypsin and results were expressed as nanograms of AI produced per milliliter per hour (ng AI/ml/hr).

2.9 SDS polyacrylamide gel electrophoresis

All 10% polyacrylamide gels were polymerized in a mini-gel apparatus according to the manufacturer's instructions (Biorad, Inc.). Samples (4 to 15 µl) were fractionated by SDS- PAGE in non-reducing conditions at 200 V for 45 minutes. Coomassie brilliant blue (2,5 mg/L) staining of certain gels was performed according to standard procedures[61] in a fixing solution (45% dH_2O / 45% methanol / 10% acetic acid).

2.10 Western blot analysis

After proteins had been separated by electrophoresis, they were transferred onto a 0.2 µm nitrocellulose membrane (Biorad, Inc.) at 100 V for one hour following common procedures[61]. The blot was then washed rapidly in TBS-T (50 mM Tris pH 7.4, 150 mM NaCl and 0,5% Tween) at room temperature before it was incubated for 90 minutes in a TBS solution containing 5% Carnation™ instant skim milk powder and rotating in a hybridization oven at 25°C. Afterwards, the blot was rinsed twice with TBS-T and washed once for 15 minutes and four times for 5 minutes shaken on a rotatory platform at 50 rpm (the washing solution being replaced between each incubation). The blot was then incubated at 4°C overnight with a polyclonal anti-renin antibody (Babco 25) diluted 1 : 600 in TBS-T on a rotating apparatus. The next morning, the blot was transferred on a rotatory platform at 50 rpm and washed in fresh TBS-T for 15 minutes and four times 10 minutes. An anti-rabbit serum conjugated to horseradish peroxidase (made from donkey) was then added at a dilution of 1 :1200 in TBS-T and the membrane was placed in a hybridization oven at 25°C for one hour. Washes were repeated as previously before a solution consisting of equal volumes of luminol and stable peroxidase buffer (Pierce, Inc.) was poured on

the membrane. After a brief incubation of 45 seconds, the membrane was dried out, wrapped in cellophane and exposed for a period of 2 to 60 seconds depending on the intensity of the signal. Protein amounts in samples were evaluated by comparing the intensity of their signal to that of known quantities of purified recombinant human renin (a gift from Daniel Lamarre of Bio-mega) loaded on the same gel. The intensity of each signal was determined by spot densitometry of exposed X-ray films using a standard curve fitting program (Alpha Innotech corp. IS-1000 vers. 3.21)

2.11 Purification of prorenin secreted in the supernatant of infected HF cells

2.11.1 Ammonium sulfate precipitation of prorenin

Ammonium sulfate precipitation of prorenin was accomplished according to a standard protocol[65]. First, supernatants of HF cell cultures harvested 7 days post-infection were pooled and agitated continuously. Ammonium sulfate was added gradually to the medium over a period of 15 to 30 minutes at room temperature to achieve a final concentration of 30, 40 or 45% w/v (Table 3). Fractions were then spinned for 45 minutes at 10 500 rpm in a refrigerated (4°C) Beckman centrifuge using a Sorvall SS-34 rotor (Dupont, Inc.). Following this first precipitation, supernatants were again collected and ammonium sulfate was added slowly to raise its concentration up to 70, 75 or 80% w/v (Table 3). These solutions were then placed in a cold chamber for a two hours incubation with agitation. Finally, a second centrifugation was accomplished under the same conditions and the pellets were recovered. All pellets were resuspended in 3 ml of the dialysis solution (Tris 10 mM, EDTA 5 mM) and 1 ml samples of the two supernatants were also collected for analysis.

2.11.2 Dialysis of the precipitate and supernatant factions

All fractions were transferred in 12 000-14 000 mw dialysis membranes and incubated for 36 hours in a cold chamber with mild agitation. Samples were dialysed against a volume of 4 liters of a solution (Tris 10 mM, EDTA 5 mM) that was changed twice during the incubation (for a total volume of 12 liters). In the end, samples were recovered after a brief centrifugation at 10 500 rpm to remove any

debris and their volume was measured. In addition, the protein concentration in each fraction was determined by the Bradford quantification method[61] and samples were used for Western blot analysis.

2.11.3 DEAE-Sephacel gel chromatography

To begin, DEAE-Sephacel columns were prepared in a cold chamber by following standard protocols and washed with a solution of Tris 25 mM, EDTA 1 mM. The column total volume was determined in function of the volume of the sample and its protein content. No more than 10% of the column maximal capacity (around 30 mg of proteins per ml) was loaded at any time. After the initial sample was deposited and began to flow through, the column was rinsed with a solution of Tris 25 mM, EDTA 1 mM equal to three times its volume. Then, proteins retained on the column were eluted progressively with three solutions of Tris 25 mM, EDTA 1 mM with increasing concentrations of NaCl (50 mM, 100 mM and 200 mM). Again, each elution step was performed with three times the column volume. Fractions were collected from the beginning and tested for prorenin content by RIA and Western blot.

3. Results

3.1 Construction of the pBPreProR vector and purification of the derived recombinant virus

In order to generate a recombinant baculovirus, the first step is to introduce the gene of interest in an appropriate transfer vector. Therefore, the complete cDNA of the human preprorenin gene was cut from the pRHR1100 vector[60] and was inserted in the commercial pBbac vector downstream of the polyhedrin promoter to obtain the vector pBPreProR shown in figure 4 (see Materials and Methods). This vector also expressed the ß-galactosidase gene under the control of the ETL viral promoter to simplify virus purification and an ampicilin resistance gene for bacterial selection. Further, two regions of the vector enclosing the prorenin and ß-galactosidase genes are homologous to the wild-type virus and favor the recombination event that will lead to the deletion of the polyhedrin gene. Following the manufacturer's instructions[50] (Invitrogen, Inc.), Sf9 cells were transfected with the pBPreProR vector along with the wild-type virus AcNPV and supernatants were harvested after 53 hours. Signs of infection appeared only five days later as seen by an increase in cell volume, regions of cell lysis and the presence of viral occlusions in the nucleus of insect cells when compared to a negative control. In parallel, purification of the virus was undertaken using dilutions of the supernatants and according to the plaque assay procedure[50]. Visual inspection of the cultures after a week showed that approximately half of the viral plaques were blue (20/38) in agreement with the expected results[50]. Out of the recombinant viruses, six were retained for analysis. Prorenin accumulation in the supernatant of infected Sf9 cultures was determined for each virus by radioimmunoassay before a second purification was accomplished (data not shown). Again two viruses from three different sources were selected and analyzed (data not shown). A P1 viral stock was generated for each one and production of prorenin by Sf9 cells was measured over a period of 4 days. Finally, the most productive virus was chosen to generate a P2 viral stock (1.2×10^8 pfu/ml) which was used in further experiments.

3.2 Prorenin production by Sf9 cells using the pBPreProR recombinant virus

Sf9 insect cells were infected by the purified pBPreProR recombinant virus and prorenin accumulation in their supernatant as a function of time and the MOI was measured by radioimmunoassay. Results suggested that a multiplicity of infection of 4 yielded the maximal production after an incubation of 3 days at 27°C (data not shown). However, these cell cultures were deteriorating rapidly and the incubation period could not be prolonged without risking an important increase in cell lysis and the release of intracellular proteases and misfolded products. Compared to non-infected controls, infected cells showed an increase in volume and their morphology was altered, but no viral occlusions indicating the presence of the wild-type virus were discernable. Also, the proportion of floating cells was increased in the supernatant.

Unfortunately, prorenin concentration measured in the supernatant was low and not suitable for a large scale production. One possible explanation was that prorenin is not properly secreted by insect cells or had a tendency to form intracellular aggregates in the constitutive secretory pathway. In order to resolve this question and to verify that the secreted prorenin had the expected molecular weight, both the intracellular fractions and supernatants of Sf9 infected cultures were collected over 72 hours and samples (10 µl) were analyzed by non-reducing SDS-PAGE and Western blot (see Materials and Methods). As shown in figure 7, the proportion of prorenin secreted in the medium is extremely low compared to the accumulation of its precursor in insect cells. In the intracellular fractions, two forms reacted specifically with the first antibody. According to their molecular weight and their relative position to prorenin (~49000 Da), the first band would correspond to preprorenin (signal peptide attached) whereas the second band could be renin or a degradation product of preprorenin. Bands of higher molecular weight probably correspond to hetero or homodimers of the mentioned renin forms since no reducing agent was added to the gel. This result clearly confirmed that prorenin was not properly secreted by Sf9 cells but also suggested that optimization of the processing of the signal peptide could result in an important increase in the productivity of the system. Presumably, the

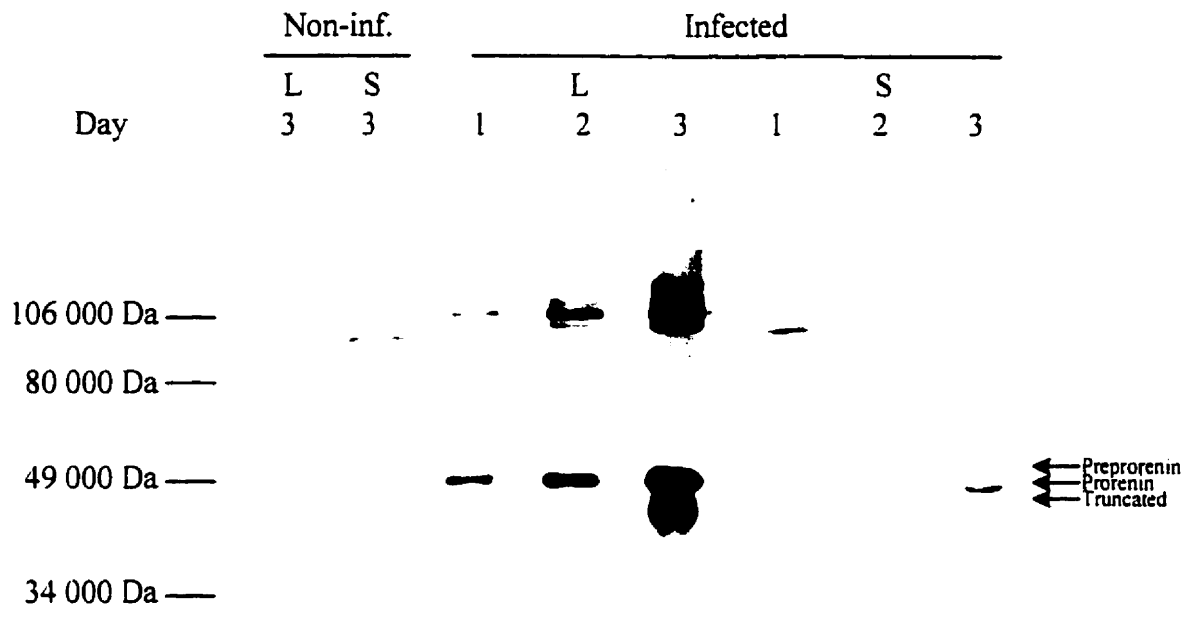


Figure 7: Prorenin production in Sf9 cells infected with the pBPreProR baculovirus.

Insect cells cultured in TNM-FH were infected according to standard procedures. Intracellular and supernatant fractions were harvested each day until cell lysis. L: cell lysate. S: supernatant.

accumulation of preprorenin within the cell could explain the lethality of the recombinant virus if it would interfere with the RER translocation machinery and prevent the insertion of other proteins. However, this hypothesis was not tested and remains to be proven.

Since problems with signal peptide cleavage have been reported by other investigators[55] but no typical requirements are known for their correct processing in insect cells, a new construction was generated and the signal peptide of prorenin was replaced by that of human Placental Alkaline Phosphatase (hPAP) for a trial.

3.3 Construction of the pPAPProR vector and purification of the derived recombinant virus

Previous reports had shown that the human Placental Alkaline Phosphatase signal peptide was correctly processed in insect cells and therefore suggested that it would be an appropriate replacement for that of prorenin[64]. The pPAPProR[62] vector (figure 6) was constructed in three steps to overcome technical difficulties but also to facilitate the replacement of a major portion of the amplification product by the wild-type prorenin sequence. Since both the pBbac (Invitrogen, Inc.) and the pPbac (Stratagen, Inc.) vectors are derived from the same precursor, their structure is very similar. Apart from the insert, the only significant difference in the pPAPProR vector is the promoter driving the expression of the ß-galactosidase gene that has been substituted by the p10 promoter (a late promoter in the virus cycle).

Transfection of Sf9 cells was accomplished as previously described but for the Insectin™ liposomes that were replaced by 50 µl of lipofectin™ (Gibco/Life technologies, Inc.) without interfering with the recombination event. Again, supernatants were recovered 48 hours afterwards and serial dilutions from 10^{-2} to 10^{-6} were made to begin a first cycle of purification. Six days latter, four blue plaques were punctured in the agarose layer and viruses were each resuspended in 1 ml of fresh TNM-FH media overnight at 4°C before they were amplified in Sf9 cells (see Material and Methods). Supernatants were collected 6 days post-infection and were

tested for prorenin content by RIA. Thereafter, two distinct viruses were selected for a second round of purification. Again, supernatants were tested by radioimmunoassay and Western blot (data not shown). Overall, only one virus was retained from the first purification in order to generate a large P2 stock of 60 ml (1.0×10^8 pfu/ml) used in all further experiments. Visual inspection of cultures infected with this solution did not show any contamination by the wild-type AcNPV as no occlusions were discernible. Furthermore, cellular toxicity was not as pronounced compared to a wild-type infection since cultures could be maintained for up to a week in fresh media without extensive cell lysis.

3.4 Prorenin production in insect cells using the pPAPProR recombinant virus

During the purification process, preliminary results suggested that the proportion of prorenin that was released in the supernatants of infected Sf9 cells was increased compared to the previous virus infections. To confirm these observations, both Sf9 and newly obtained High-Five™ cells were subcultured in 6-well dishes at a density of 5×10^5 cells/well and were infected with the P2 virus at a MOI of 2. Supernatants were then collected every 24 hours until extensive cell lysis occurred, whereas two intracellular fractions were obtained three days post-infection. First, prorenin accumulation in the supernatant as a function of time was measured by RIA to compare the two different cell lines (Figure 8A). Looking at these results, HF cells appeared to release prorenin over a longer period, leading to a greater accumulation of the protein after 3 days of incubation, but no significant differences were distinguishable between the two cell lines in the early phase of the infection. However, cultures were maintained for a prolonged period without extensive cell lysis when compared to the cultures infected with the pBPreProR recombinant virus, suggesting that the use of the hPAP signal peptide reduced the cellular toxicity seen with the previous construction. To study the secretion efficiency of prorenin, samples were loaded on a SDS-10% polyacrylamide gel, electrophoresed and transferred onto a nitrocellulose membrane for Western blot analysis. As shown in figure 8B, no accumulation of the intracellular precursor was detectable after 3 days of incubation

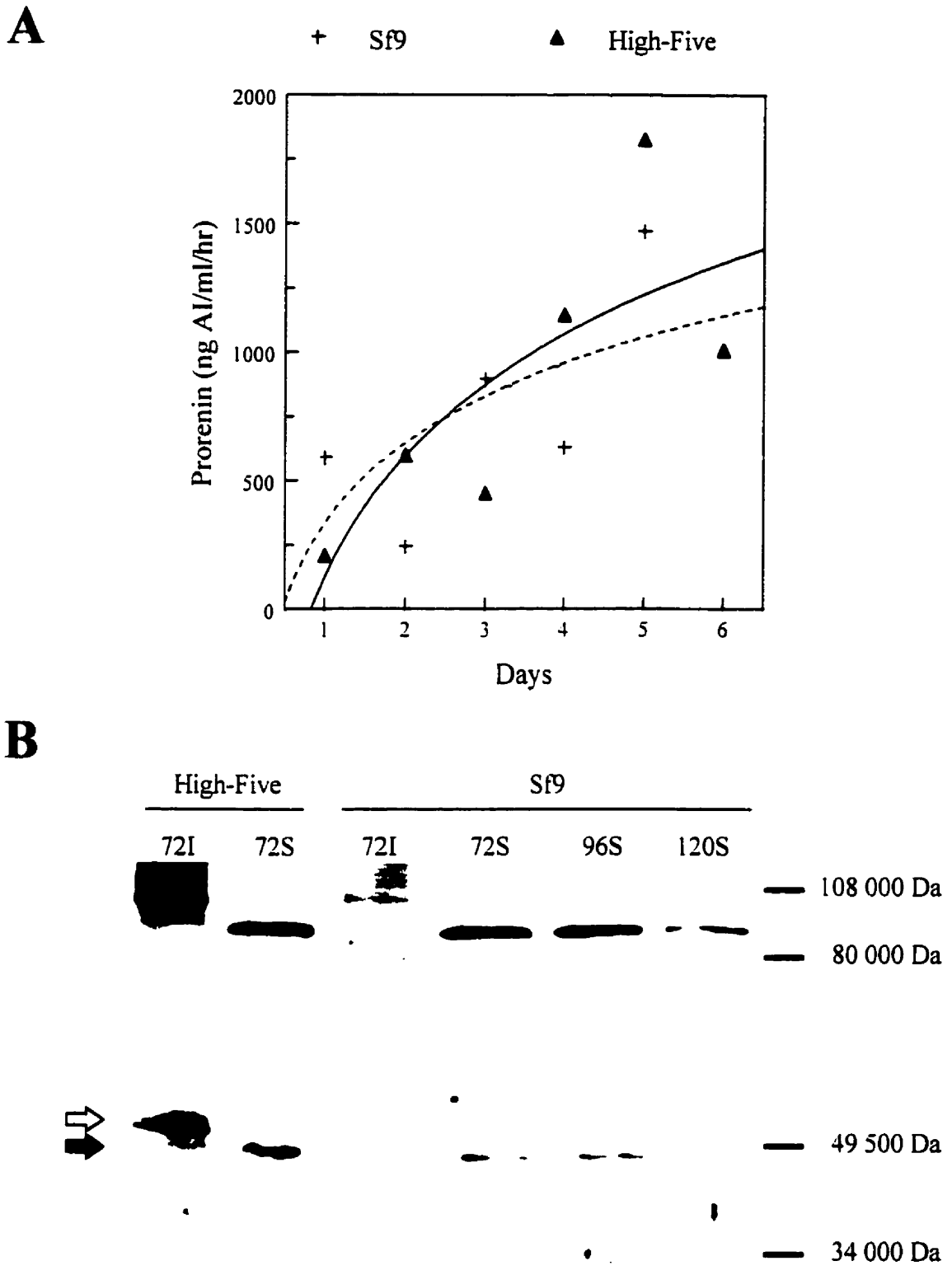


Figure 8: Comparison of the productivity of High-Five™ and Sf9 cells.

Insect cells plated at a density of 5 x 10^5 cells per well in 6-well dishes were infected with the recombinant virus at a MOI of 2 and samples from the supernatant were collected every day. Two intracellular fractions were harvested after 72 hours. **A**: Graphic representation of prorenin accumulation in the supernatant of HF and Sf9 cultures as determined by RIA. **B**: Western blot of the intracellular (I) and supernatant fractions (S) at different times and for the two cell lines. Numbers represent hours PI. Black arrow: prorenin. Open arrow: Preprorenin.

in Sf9 cells and the prorenin secreted in the media had the expected molecular weight of 49 000 Da, suggesting that the hPAP signal peptide was indeed processed correctly in insect cells and at the proper cleavage site. However, results in HF cells suggested that the new signal peptide was not completely removed after 72 hours since there was a significant accumulation of the precursor in the intracellular fraction. The level of prorenin secretion in the supernatant of HF cells was higher, in agreement with our previous results. In addition, the intensity of the precursor band suggested that optimization of the conditions of infection could improve the yields obtained with this cell line. Considering that High-Five™ cells already produced more prorenin than the Sf9 cells and that higher levels of expression could still be achieved, they were selected for further experiments.

After optimization of the production of prorenin by HF cells, the supernatants and the intracellular fractions harvested from 4 to 7 days were analyzed by Western blotting (Figure 9). Overall, no accumulation of the precursor was detectable in any of the intracellular fractions whereas accumulation of the product increased progressively in the supernatants, as expected. Clearly, this experiment shows that the hPAP signal peptide is also correctly processed in HF cells.

3.5 Prorenin production by HF cells infected with the pPAPProR virus as a function of cell density

Since optimization of the system is one of the key factor in the efficient production of a protein by the recombinant baculovirus, prorenin accumulation in the supernatant of insect cell cultures was determined by Western blot analysis as a function of the cell density. High-Five™ cells were plated at three different densities in 6-well dishes and supernatants were collected a week after they were infected at a MOI of 5 (figure 10). The cell densities that were chosen represented approximately 90% (3.6 x 10^6 cells/well), 40% (1.2 x 10^6 cells/well) and 20% (6 x 10^5 cells/well) of confluency. Results have shown that no significant increase in prorenin production was observable if cells were plated at a density superior to 1.2 x 10^6 cells/well. Presumably, results were not higher when the cell censity was increased because the

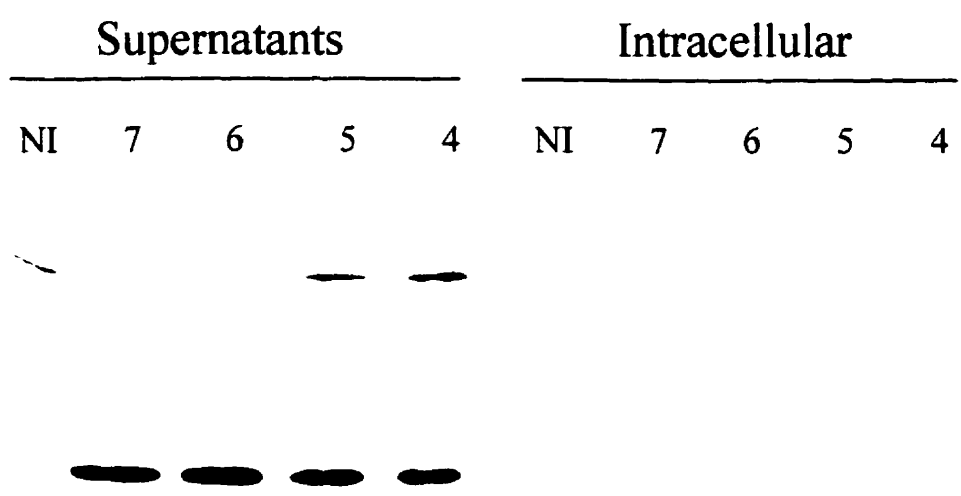
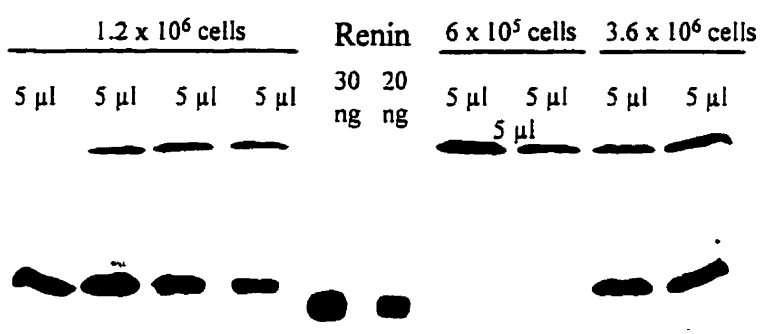


Figure 9: Prorenin expression in High-Five™ cells in optimal conditions.

High-Five™ cells were subcultured in TNM-FH at a density of 3.6 x10^6 cells/well and were infected by the recombinant virus 4 at a MOI of 6 according to standard procedures. Supernatants and intracellular fractions were recovered for a week and samples were analyzed by Western blot. **NI**: Non-infected cells (Day 7). Numbers represent days.

A



B

Prorenin production as a function of the cell density			
Cells/well	*6.00E+05*	*1.20E+06*	*3.60E+06*
Prorenin (mg/L)	0.56	6.53	6.65
	0.12	7.38	5.65
		5.88	6.41
		3.50	5.06
Mean (mg/L)	**0.34**	**5.82**	**5.94**
Standard deviation		1.66	0.73
n	2	4	4

C

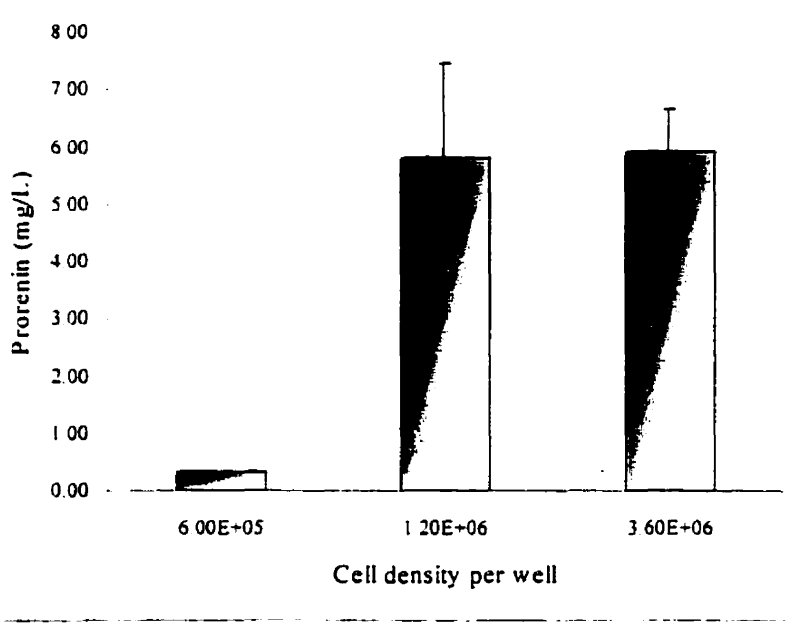


Figure 10: Prorenin production in infected HF cells as a function of cell density.

High-Five™ cells were subcultured in 6-well dishes at the indicated density and were infected with the recombinant pPAPProR baculovirus at a MOI of 5. **A**: Western blot of prorenin **B**: Prorenin concentration in 7 days supernatants as determined by Western blot analysis. **C**: Graphic representation of the results.

efficiency of the infection was decreased. Since cells are more tightly packed at a higher density, one can hypothesize that the membrane area exposed to the supernatant and the virus is lower in these cultures. Consequently, during the one hour infection period, the possibility that a given cell would be infected would be reduced in that case. On the other hand, the great difference in productivity at a lower cell density can be explained by two factors. First, the reduced number of cells to be infected would result in less prorenin produced, as expected. Second, High-Five™ cells plated at a low cell density tend to multiply more slowly, suggesting that cellular contacts are important for growth. Thus, the culture conditions are not maximal in that case, a factor known to reduce the productivity of the BEVS.

3.6 Time course of prorenin release in the supernatant of insect cell cultures infected with the pPAPProR recombinant virus

Another factor important to consider is the time frame of maximal accumulation of the product in the supernatant. Over time, insect cells secrete in the media the product of interest but cellular viability also decreases. If one should collect the supernatant of the culture too soon, yields of the product per liter would not be maximal and result in an increase in expense for large scale production of the protein. On the other hand, prolonged incubation of the culture would result in a depletion of the nutrients in the media and cell lysis due to the infection, both resulting in cell death and the release of intracellular proteases that will eventually degrade the product of interest. Therefore, for a given set of conditions, an equilibrium must be obtained.

To determine the most efficient time frame for harvesting the supernatants of monolayer cell cultures, High-Five™ cells were subcultured at a density of 3.6×10^6 cells per well in 6-well dishes and they were infected by the virus P2 at a MOI of 5-6. Samples of 10 µl were then collected every 24 hours until signs of infection were evident compared to a negative control and floating cells were beginning to appear in the media. Western blot analysis (Figure 11) has shown that maximal accumulation of prorenin was between 6 and 7 days. Longer incubation periods were not tested

A

Day	Prorenin (mg/L)			Mean (mg/L)	Stdev (mg/L)
4	N.D	1.77	2.85	**2.31**	N.D
5	2.37	3.30	4.03	**3.23**	0.83
6	5.38	7.98	9.04	**7.47**	1.88
7	5.06	6.28	6.41	**5.92**	0.74

B

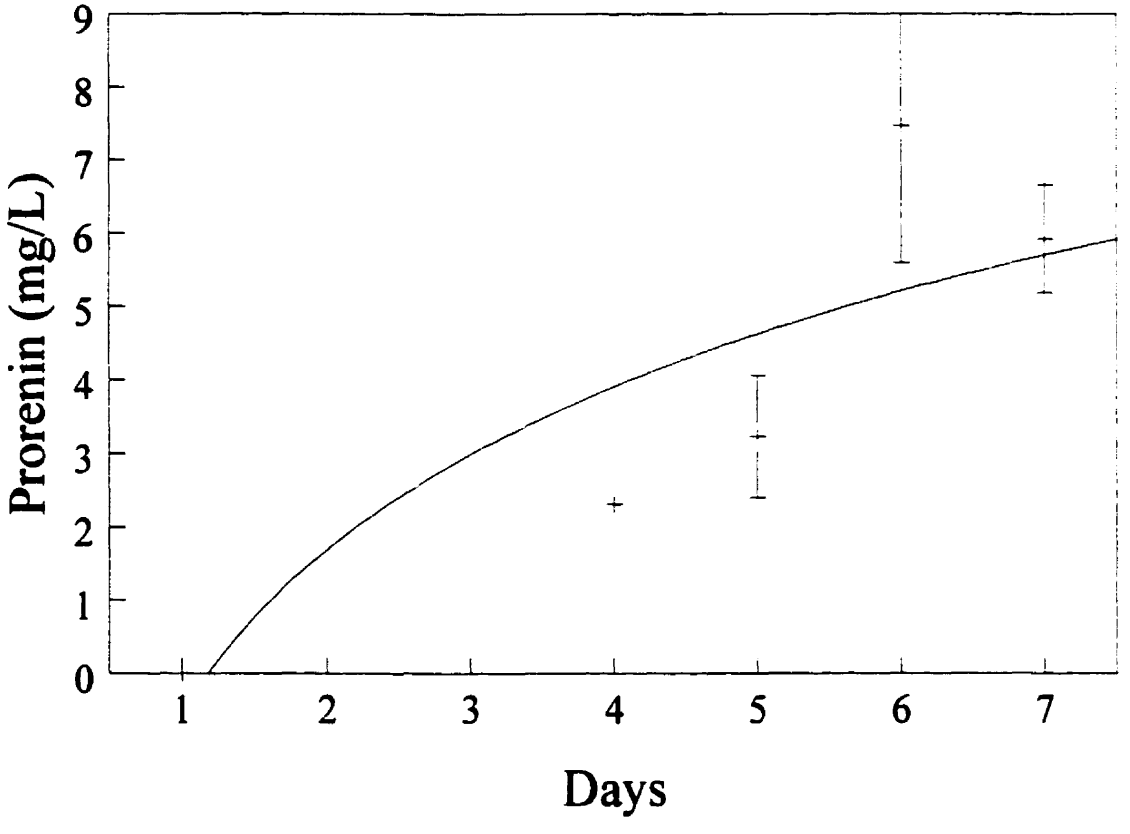


Figure 11: Time course of prorenin secretion by High-Five™ cells (3.6 x 10^6 cells/well) infected with the recombinant baculovirus pPAPProR at a MOI of 5-6.

A: Prorenin concentration in supernatants as determined by Western blot analysis.

B: Graphic representation of the results.

because cell lysis was too high after a week, probably due to the virus infection or the lack of nutrients in the media. Although this time frame is relatively high compared to the expression of polyhedrin in a wild-type infection, other investigators have reported similar results[58].

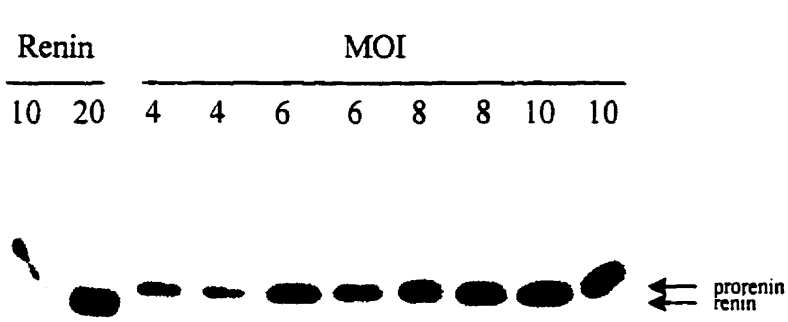
3.7 Prorenin production as a function of the multiplicity of infection

In another set of experiments, the multiplicity of infection used was considered as a function of time and prorenin secretion in the supernatant of HF cell cultures was evaluated by Western bolt analysis at 6 and 7 days (Figure 12). Results from the infections of 3.6×10^6 cells per well in 6-well dishes suggested that the most effective MOI would be 5 or 6. Indeed, at a lower MOI, prorenin accumulation was not as high, presumably because the number of cells infected over the one hour incubation period was reduced compared to infections with a greater number of virus particles. In such a case, the non-infected cells probably continued to divide and were only infected once other ECV particles were released from dying cells. The secretion of prorenin was probably spread over a longer period of time. This appraoch was not ideal for maximal production of any protein because cell lysis was not synchronized and the release of intracellular proteases could decrease the amount of prorenin released. Also, prolonged incubation was not possible since the nutrients in the media were probably very low after a week of incubation. On the other hand, infections with a greater MOI did not increase significantly the production of prorenin. In that case, the virus was in excess and no further improvement in the percentage of cells infected was possible. Clearly, a plateau in prorenin secretion by HF cells was achieved 7 days following the infection at a MOI of 6. This MOI was selected because it permitted a maximal accumulation of prorenin for the lowest quantity of virus particles spent. Moreover, cell lysis was synchronized using these parameters.

3.8 Prorenin production in different media

Once the parameters for maximal production of prorenin by High-Five™ cells were determined in TNM-FH, assays in serum-free media were performed to evaluate the yields that could be obtained in these conditions (Figure 13). Prorenin

A



B

	Production (6 days)			Production (7 days)		
MOI			*Mean*			*Mean*
2	1.71	2.00	1.85	2.42	2.58	2.50
4	2.09	1.61	1.85	4.11	4.83	4.47
6	3.06	3.38	3.22	5.23	5.63	5.43
8	4.14	4.88	4.51	5.08	6.11	5.60
10	5.32	5.62	5.47	5.63	6.47	6.05

C

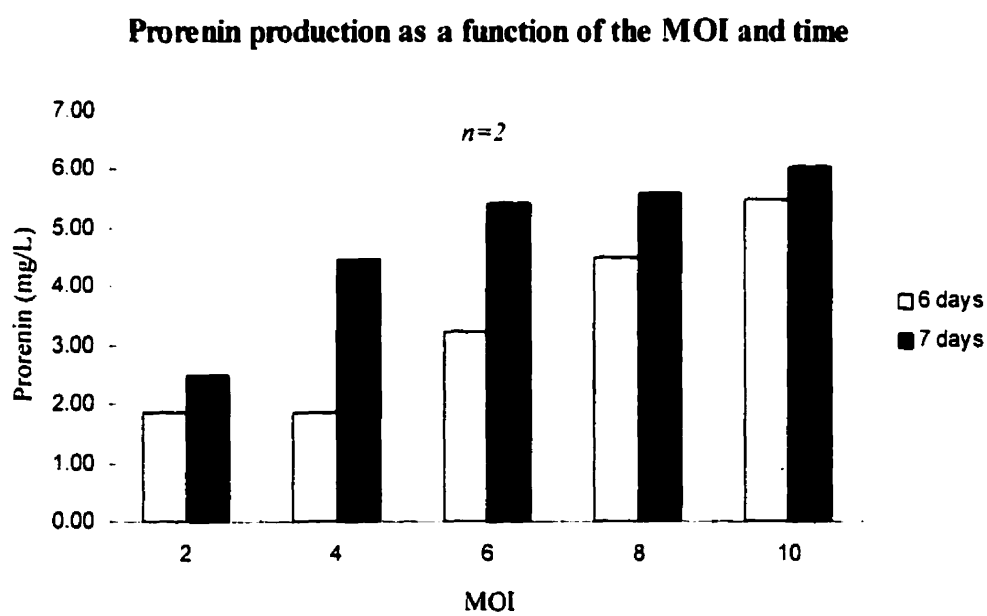


Figure 12: Prorenin accumulation in the supernatant of infected cultures as a function of the muliplicity of infection and time.

High-Five™ cells were subcultured in TNM-FH at a density of 3.6 x 10^6 cells/well in 6-well dishes and supernatants were collected after 6 and 7 days. **A**: Western blot to determine prorenin concentration by spot densitometry using fixed renin standards (6 days). **B**: Concentrations measured after 6 and 7 days of incubation in function of the MOI. **C**: Graphic representation of the results.

A

	SFM-II					Baculogold				
Days	*Prorenin (mg/L)*			*Mean*	*Stdev*	*Prorenin (mg/L)*			*Mean*	*Stdev*
4	3.03	1.78	3.18	**2.66**	0.77	3.75	3.81	4.93	**4.16**	0.66
5	2.30	2.58	3.38	**2.75**	0.56	4.32	5.14	6.40	**5.29**	1.05
6	1.99	1.99	4.20	**2.72**	1.28	5.01	6.41	7.66	**6.36**	1.33
7	3.98	3.72	3.98	**3.89**	0.15	4.83	4,57	4.98	**4.79**	0.21

B

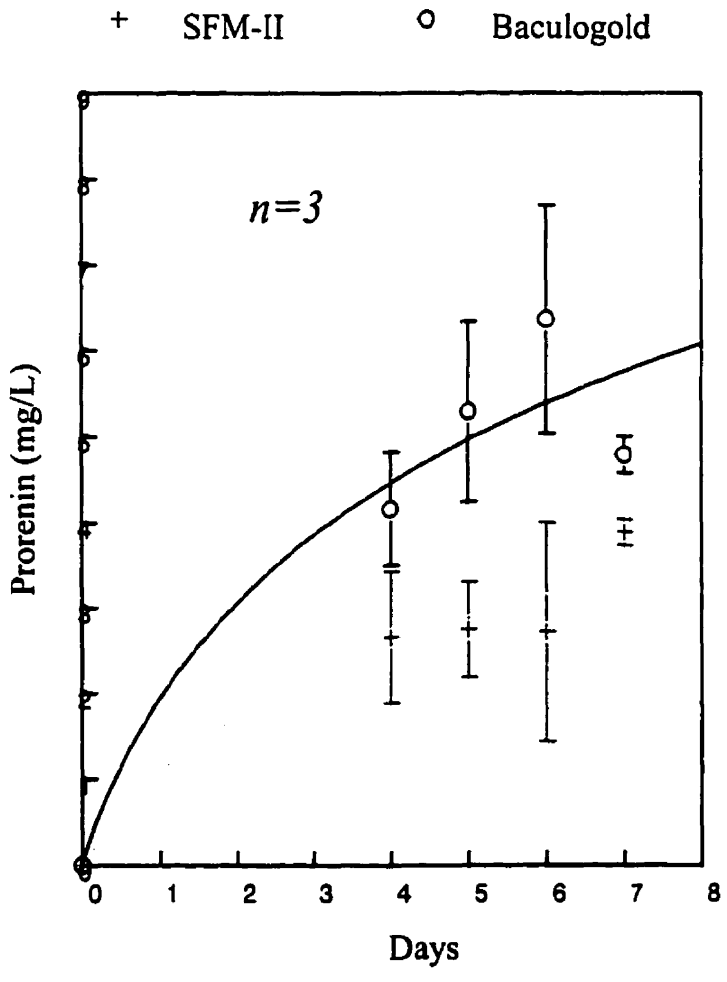


Figure 13: Prorenin secretion by High-Five™ cells in different serum-free media.

HF cells were subcultured in 6-well dishes at a density of 3.6 x10^6 cells /well and infected by the recombinant pPAPProR baculovirus at a MOI of 6. **A**: Prorenin concentration as determined by Western blot analysis in the SFM-II™ and Baculogold™ media. **B**: Graphic representation of the results.

purification would be greatly facilitated by the absence of serum in the supernatant.

Again, HF cells (3.6 x 10^6 cells per well in 6-well dishes) that were adapted progressively to the new medium were infected by the virus P2 at a MOI of 6 and samples were harvested every day for Western blot analysis. As presented in Figure 13, prorenin concentration increased over time and maximal accumulation was obtained after a week as expected from the previous results. This suggested that cells were not deprived from nutrients over the incubation period. Further, cell lysis was limited and no greater extent than in other infections. Nevertheless, maximal accumulation of prorenin was not as high as previously measured, particularly in SFM-II™ medium (Gibco/Life Technologies, Inc.). This suggested that although cells were able to survive over a week in these media, some essential nutrients were probably missing in the serum-free media. Indeed, the fact that a better expression was achieved in Baculogold™ (Pharmingen, Inc.) indicates that the composition of the medium greatly influences the yields that can be obtained from the infections. In addition, preliminary experiments with the HyQ medium (HyClone, Inc.) were realized and did not show any improvement over these results (data not shown).

3.9 Prorenin production in a suspension culture

Prorenin accumulation in the supernatant of High-Five™ infected cells was evaluated by Western blot analysis in a suspension culture and it was compared to monolayer cultures plated at the same density (Figure 14). Cells were adapted over a week to this new condition and were subcultured three times before they were gently centrifuged at 1000 rpm in a IEC Centra-8R centrifuge and resuspended in 10 ml of fresh TNM-FH/7.5%FCS. To this volume, 10 ml of the virus P2 was added (MOI of 6) and cells were incubated for one hour on a rotatory platform before being transferred to the incubation chamber. The volume was then adjusted to 130 ml to achieve a cell density of 1.0 x 10^6 cells/ml. In parallel, three different monolayer cultures plated the same day at 1.0 x 10^6 cells/ml were infected normally. Afterwards, samples were harvested every day and prorenin content in the supernatants was evaluated by Western blot analysis. However, 120 ml of supernatant had to be added

A

Prorenin concentration in the supernatants		
Day	*High-Five cells in a suspension culture*	*High-Five cells in monolayer cultures*
6	2.79	7.99
7	2.99	7.08
n	1	3

B

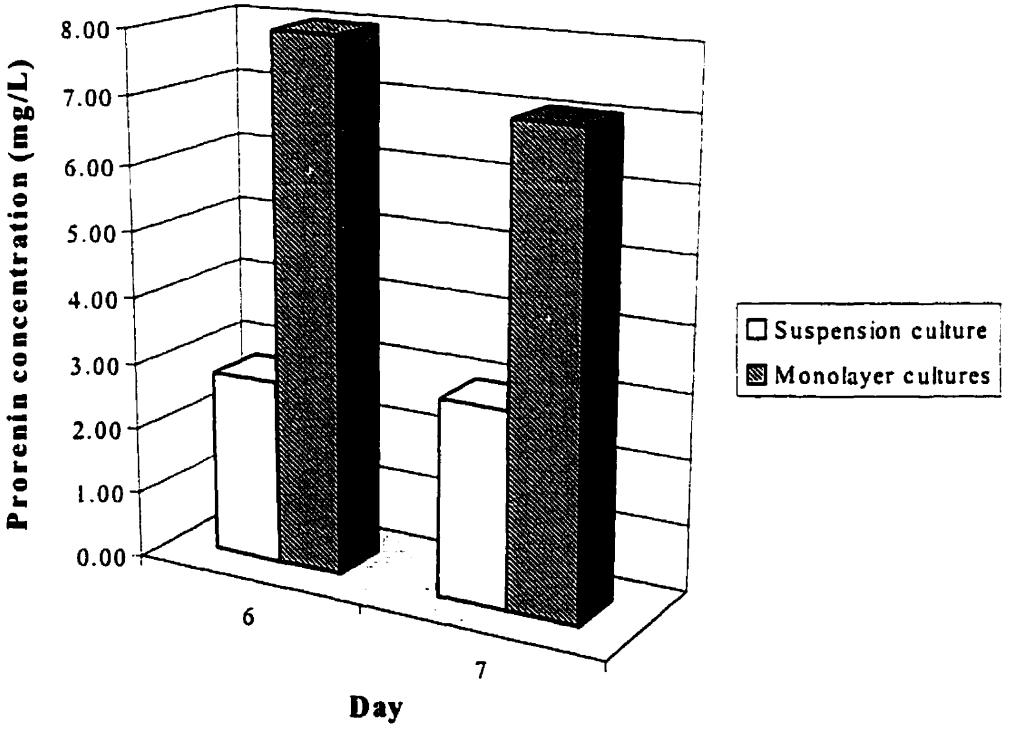


Figure 14: Prorenin accumulation in the supernatant of infected High-Five™ cells in a suspension culture and comparison with monolayer cultures.

Cells were infected at a MOI of 6 and samples were collected after 6 and 7 days. **A**: Results from a Western blot analysis expressed as the mean of mg of prorenin per liter of culture. **B**: Graphic representation of the results.

to the suspension culture over the 7 day incubation to prevent the aggregation of the insect cells as they were inspected regularly. As shown in figure 14, total accumulation of prorenin in the monolayer cultures were approximately 2 to 3 fold superior to the final concentration in the suspension culture. Considering that the final volume of the suspension culture was in fact twice the initial volume, the total production of prorenin as a function of the cell density at the moment of infection was similar in both conditions. Nevertheless, purification of prorenin produced in suspension cultures would be more difficult because it was diluted and this approach had to be rejected due to problems associated with it. Indeed, large suspension cultures are more easily contaminated than monolayer cultures and insect cells seem particularly susceptible to form aggregates in these conditions.

3.10 Development of a strategy for prorenin purification

Preliminary experiments were undertaken in order to define the steps that could be used in prorenin purification from TNM-FH supernatants of infected High-Five™ cells and to evaluate if the production in serum-free medium would be more advisable.

3.10.1 Ammonium sulfate precipitation of prorenin

Ammonium sulfate precipitation of proteins that are present in the supernatant of infected insect cells was tested as a first purification step and a concentration procedure to permit the loading of a column latter in the process. According to earlier reports[65], prorenin precipitates from solutions of ammonium sulfate in the range of 40% to 70% weight per volume. Since the glycosylation state of prorenin produced in HF cells was not known and might affect solubility, different concentrations for the precipitation steps were first tested using 30 ml supernatants of infected cells. After dialysis of the fractions, their prorenin content was evaluated by Western blot analysis in order to determine the efficiency of the purification and the best conditions. Results are summarized in table 3. The percentage of prorenin that precipitated in the first step was lower than that of the other proteins no significant losses were observed. As expected, the amount of protein that precipitated in the first step was proportionnal

Table 3: Prorenin precipitation from TNM-FH supernatants at different ammonium sulfate concentrations.

Ammonium sulfate concentrations[1]		***First precipitation pellet***[2]				***Second precipitation pellet***[3]				
First precipitation	Second Precipitation	Prorenin (µg)[4]	Proteins (mg)[5]	Prorenin lost[6]	Proteins lost[7]	Prorenin (µg)	Proteins (mg)	Prorenin Yields	Proteins Yields	Fold of purification[8]
30%	*70%*	*1.86*	*1.97*	*0.7%*	*1.3%*	*62.9*	*52.3*	*51.9%*	*78.1%*	*0.66*
30%	*80%*	*1.86*	*1.97*	*0.7%*	*1.3%*	*152.2*	*67.4*	*125.6%*	*100.7%*	*1.25*
40%	*70%*	*6.83*	*26.01*	*2.4%*	*16.6%*	*30.2*	*43.7*	*25.0%*	*65.3%*	*0.38*
40%	*80%*	*6.83*	*26.01*	*2.4%*	*16.6%*	*81.9*	*38.3*	*67.6%*	*57.2%*	*1.18*
45%	*75%*	*7.09*	*50.62*	*2.5%*	*32.4%*	*103.9*	*42.2*	*85.7%*	*63.0%*	*1.36*
Content in the initial volume		282.8	156.1			121.2	66.9			

1. The ammonium sulfate concentrations for the two precipitation steps are in tandem on the same line
2. The first pellet was resuspended in 1ml, dialyzed and protein and prorenin content were measured.
3. The second pellet was resuspended in 1ml, dialyzed and protein and prorenin content were measured.
4. Prorenin concentration was determined by Western blot analysis and multiplied by the dialysis final volume to obtain total prorenin.
5. Protein concentration was determined by the Bradford method and multiplied by the dialysis final volume to obtain total proteins.
6. Prorenin percentage that was lost or present in the final pellet (yields) was determined by dividing the total amount found by the content in the initial volume for this purification step.
7. Protein percentage that was lost or present in the final pellet (yields) was determined by dividing the total amount found by the content in the initial volume for this purification step.
8. The fold of purification obtained was determined by dividing the prorenin yields by the proteins yields.

to the percentage of $(NH_4)_2SO_4$. From these results, it was concluded that ammonium sulfate precipitation would be a good approach to concentrate prorenin in order to load a separation column but it does not seem to allow a differential precipitation of other proteins since the yields that were obtained in the second precipitation were similar in all cases. Prorenin was only 1.36 fold more pure in the best assay (Table 3). Somehow, important quantities of prorenin were lost between the precipitations or during the dialysis.

3.10.2 DEAE-Sephacel column chromatography

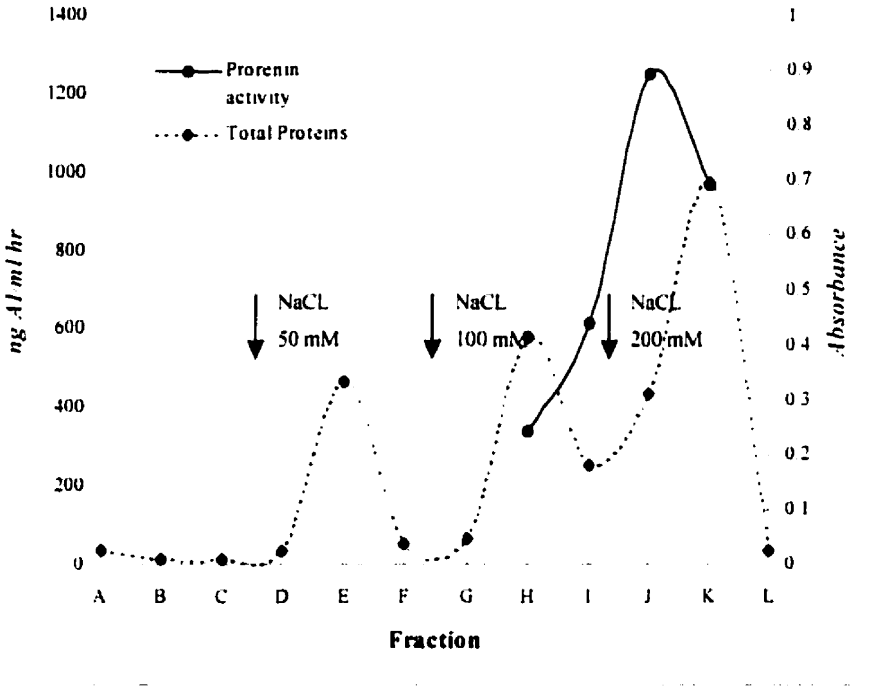
As a second step in the purification process, a sample of the dialyzed pellet from the second ammonium sulfate precipitation was loaded on a DEAE-Sephacel column prepared as described (see Materials and Methods). In parallel, prorenin expressed by mammalian cells was precipitated by the same procedure from a supernatant of GH_4 transfected cells and another sample was loaded on an equivalent column. This was done to compare the properties of both prorenins. Three fractions per elution step were collected as the column was eluted with each buffer, yielding a total of 12 different fractions that were subsequently analyzed. Protein content in each fraction was determined by measuring its absorbance at 260 nm and visualized on a SDS-PAGE stained with Coomassie brillant blue. In parallel, prorenin concentration was determined by radioimmunoassay and confirmed by Western blot analysis.

Results for the purification of prorenin produced by transfected mammalian cells and the infected insect cells are presented in figure 15 and 16, respectively. As expected, the prorenin that was produced by mammalian cells eluted from the column in the last fractions when the NaCl concentration in the buffer was raised from 100 mM to 200 mM (15B). This was in agreement with previous reports[78]. The elution profile representing the activity of the protein after trypsin activation was determined by AI radioimmunoassay and the presence of prorenin in the fractions I, J, K and L was confirmed by Western blot analysis (15D). Although it could not be seen on the gel, renin was also detected in these fractions by RIA. This was not the result of any

A

Fraction	Total Proteins *Absorbance*	Prorenin activity *ng Al/ml/hr*
A	0.025	
B	0.008	
C	0.008	
D	0.025	
E	0.333	
F	0.038	
G	0.048	
H	0.415	340
I	0.181	616
J	0.312	1251
K	0.696	967
L	0.025	

B



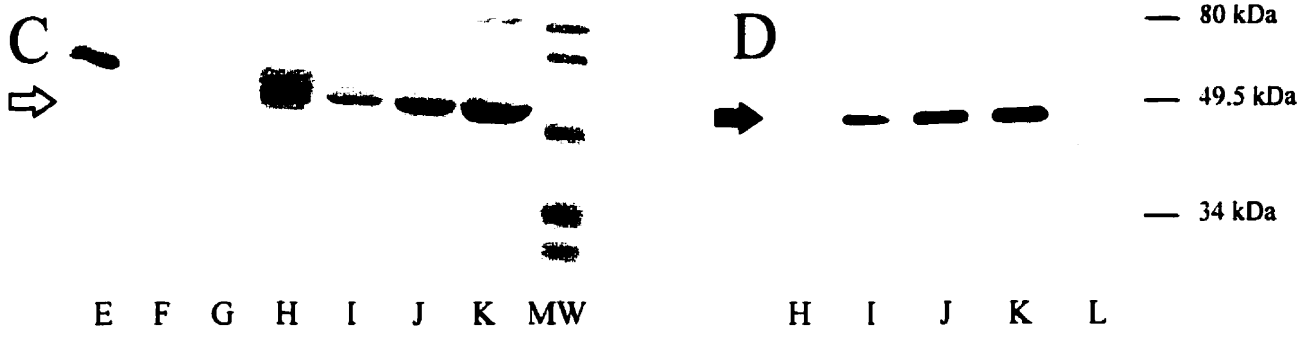


Figure 15: Purification of prorenin produced by mammalian cells on a DEAE-Sephacel column and analysis of the fractions obtained.

A: Measurement of the absorbance of each fraction at 260 nm and prorenin activity determined by RIA. **B**: Graphic representation of the results presented in A. **C**: Coomassie brillant blue staining of a SDS-PAGE gel to visualize total proteins. **D**: Western blot of a SDS-PAGE of selected fractions to visualize prorenin. Black arrow: Prorenin (~49 kDa) White arrow: Albumin (~55 kDa).

maturation of prorenin during the purification process but of the secretion by GH_4 cells of renin, since it was detected in the original supernatant. Most of the other proteins also eluted at this salt concentration and a good separation was not achieved (15B). As seen on the Coomassie brillant blue stained gel (15C), the majority of the proteins present in the supernatant came from the serum and they were concentrated in two bands, one of which is probably albumin (55 000 Da). In contrast, prorenin produced by insect cells was not retained by the column and was found in the first fractions that were collected (16B). Its presence in fractions 2, 3 and 5 was detected by AI radioimmunoassay and confirmed by Western blot analysis (16D). The presence of another peak around fraction 5 could suggest that two different isoforms of prorenin were secreted by HF cells but remains to be confirmed. However, most of the other proteins eluted in the last fractions at a 200 mM NaCl concentration (16B). This result, although surprising at first, can be explained by the structure of the glycosyl residues found on the proteins secreted by insect cells. The fact that prorenin was not retained by a positively charged column suggests that its net charge is almost neutral or positive. This idea is supported by experiments demonstrating the absence of phosphorylation and complex maturation of the mannose residues found on insect proteins[69]. A good separation was attained using this method and it could be used as a first step during the purification process. These results were confirmed in a second experiment where 100 ml of supernatant from infected HF cells were first concentrated by ammonium sulfate precipitation, dialysed and loaded on a DEAE-Sephacel column. Proteins were eluted as before but more fractions were collected instead. As previously, prorenin was found in the first fractions whereas the other proteins eluted at higher salt concentrations (Figure 17).

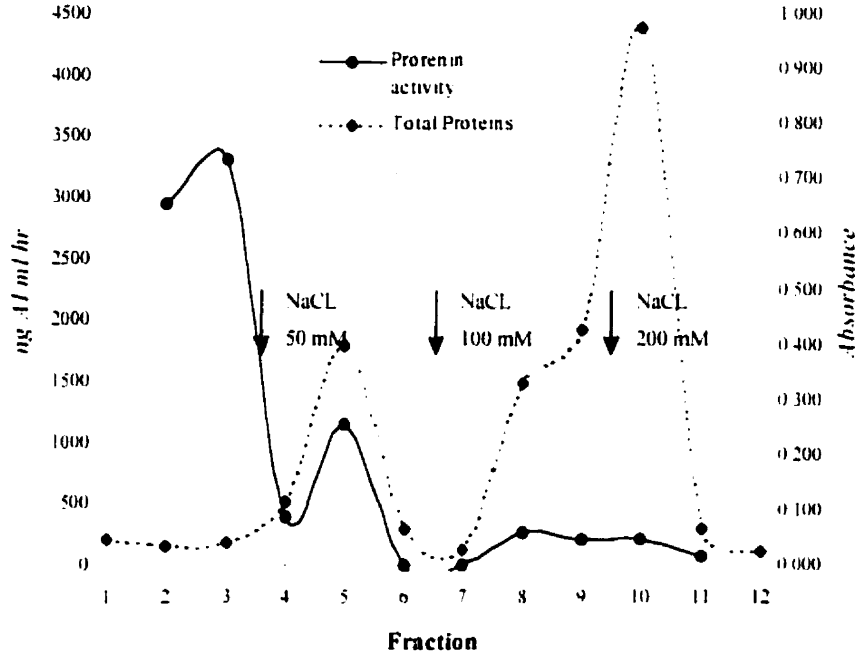
3.11 Comparison of the activity of the recombinant prorenins that were produced in HF cells and GH_4 cells

To determine if the recombinant prorenin produced in High-Five™ cells had similar enzymatic properties to the one that was secreted by GH_4 cells, similar quantities of both mature forms (as determined by Western blot analysis) were tested by RIA at a same dilution factor (Figure 18). Results have shown that both prorenins

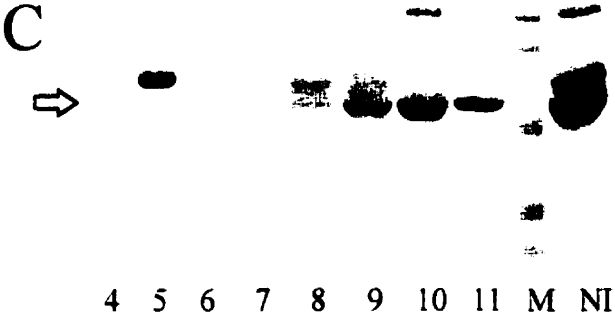
A

Fraction	Total Proteins *Absorbance*	Prorenin activity *ng Al/ml/hr*
1	0.048	
2	0.035	2944
3	0.041	3312
4	0.114	389
5	0.398	1145
6	0.064	0
7	0.028	0
8	0.330	265
9	0.426	204
10	0.975	205
11	0.064	69
12	0.024	

B



C



D

Figure 16: Purification of prorenin produced by High-Five™ cells on a DEAE-Sephacel column and analysis of the fractions obtained.

A: Measurement of the absorbance of each fraction at 260 nm and prorenin activity determined by RIA. **B**: Graphic representation of the results presented in A. **C**: Coomassie brillant blue staining of a SDS-PAGE gel to visualize total proteins and albumin (White arrow). **D**: Western blot of a SDS-PAGE gel of selected fractions to visualize prorenin (Black arrow). NI: Non-infected supernatant.

A

Fraction	Total Proteins *Absorbance*	Prorenin activity *ng Al/ml/hr*
Blanc	0.000	0
2	0.001	0
3	0.008	0
4	0.083	0
5	0.237	0
6	0.175	46
7	0.244	1159
8	0.440	1161
9	0.162	6
10	0.088	20
11	0.290	35
12	0.991	128
13	0.623	83
14	0.231	
15	0.086	
16	0.045	
17	0.157	
18	1.195	
19	1.186	
20	0.681	
21	0.332	
22	0.202	
23	0.548	
24	1.291	
25	0.913	
26	0.583	

B

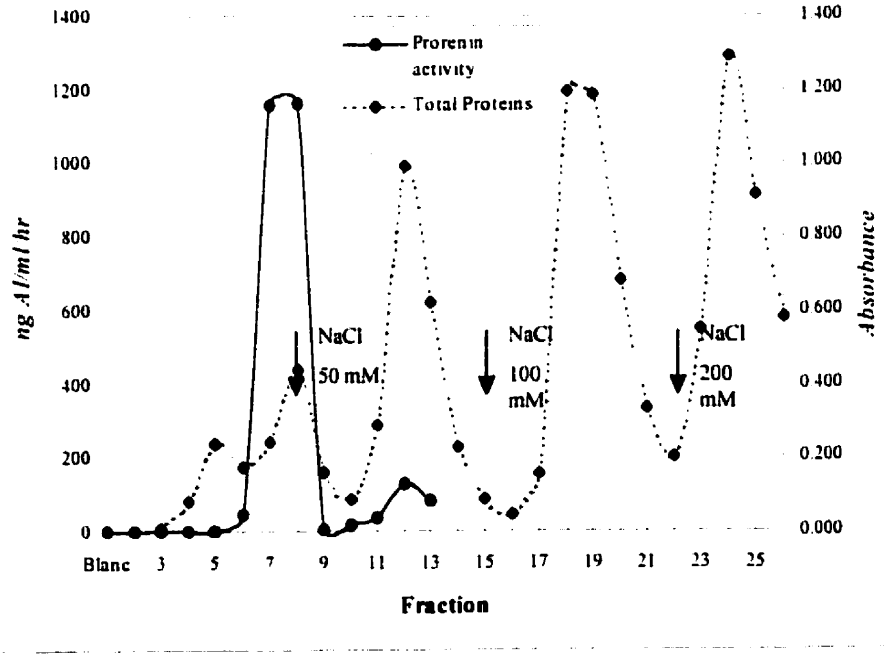
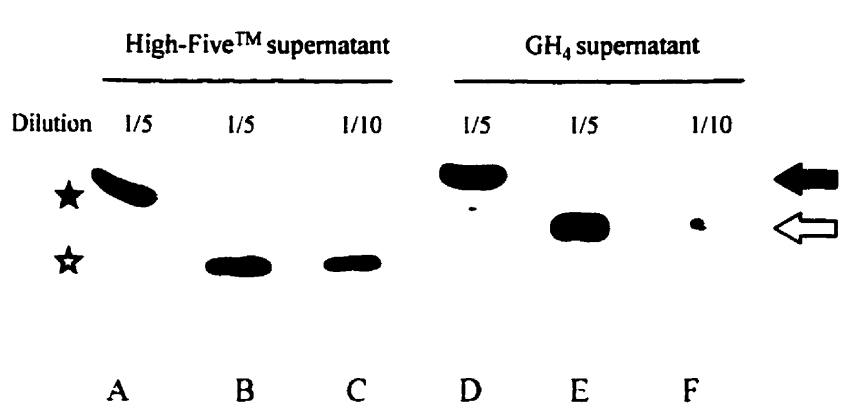


Figure 17: Purification of prorenin produced in High-Five™ cells on a DEAE-Sephacel column and analysis of the fractions obtained (second trial).

A: Measurement of the absorbance of each fraction at 260 nm and prorenin activity determined by RIA. No prorenin activity was measured by RIA for the fractions 14 to 26 in a separate assay. **B**: Graphic representation of the results.

A



B

	High-Five supernatant	GH_4 supernatant	Dilution
Renin (ng)	88.0	104.5	
Prorenin activity (ng AI/ml/hr)	256.4	287.9	1/200 000
Specific activity (ng AI/ml/hr/ng of renin)	2.9	2.8	

C

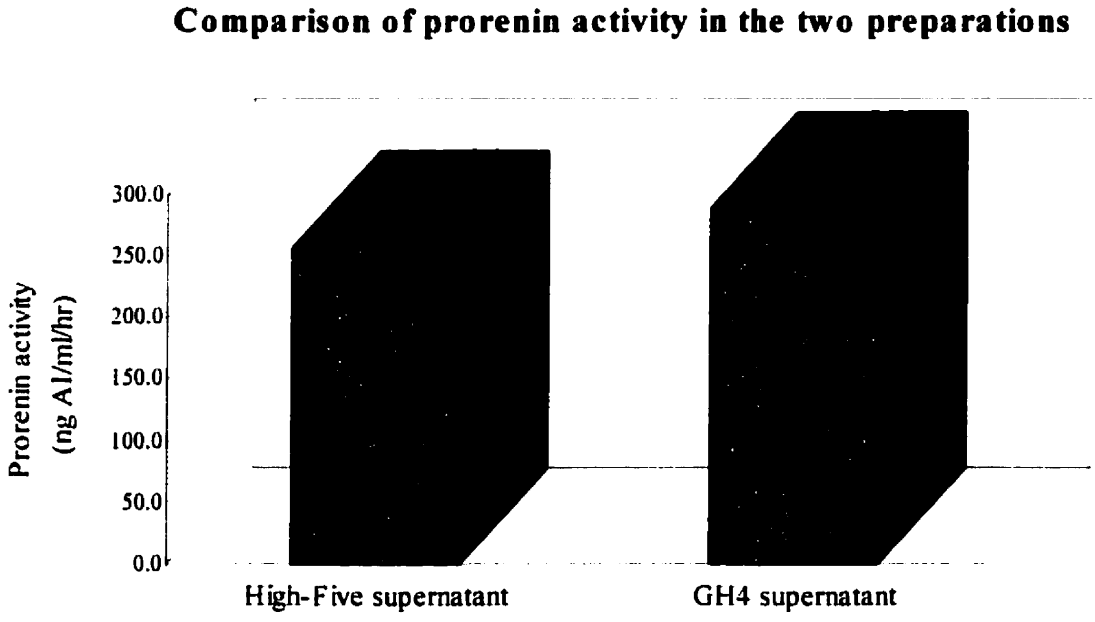


Figure 18: Comparison of the prorenin activity in two different preparations.

A: Western blot of the fractions recovered after ammonium sulfate precipitation and dialysis. A,D: Non-digested prorenin. B,C,E,F: Trypsin-activated prorenin. **B**: Prorenin activity measured in the fractions at the dilution 1/200 000 and amount of renin as determined by the Western blot. **C**: Graphic representation of the results. Black arrow: mammalian prorenin (~ 49 kDa). Open arrow: mammalian renin (~43 kDa). Black star: recombinant insect prorenin. Open star: recombinant insect renin.

had the same activity once converted to renin. Thus, although the prorenin produced in insect cells has a different molecular weight, chemical properties and charge, its enzymatic properties do not seem to be affected. Further, the fact that the recombinant renin from insect cells migrates at a lower position than the mammalian counterpart confirms that a different glycosylation pattern is probably responsible for the apparent lower molecular weight of prorenin in HF cells. If this would have been the result of the partial cleavage of the prosegment, both renins would have migrated at the same position. This observation has also been reported for other proteins expressed by the BEVS[55].

4. Discussion

4.1 A comparison of strategies used in recombinant protein expression

Production of numerous proteins has been tested in different systems before a suitable one was found and results have shown that the baculovirus system is one of the most efficient. In a very extensive study, Geisse *et al.* expressed the cytokine human Leukemia Inhibitory Factor (hu-LIF), a 43 kDa secreted glycoprotein, in five of the most commonly used eukaryotic systems: CHO, Sp2/0, Mel, COS and Sf9 insect cells[46]. Three different approaches have been developed to maximize production of the recombinant protein for these cell lines. In Chinese hamster ovary (CHO) and mouse myeloma (Sp2/0) cell lines, gene amplification is widely used[46]. A variety of treatments and agents can induce the amplification of genomic regions as circular extrachromosomal elements capable of autonomous replication that will eventually reintegrate into the genome as the selection process extends[46]. The developed approach involved the amplification of the dihydrofolate reductase gene (DHFR) that catalyses the conversion of folate to tetrahydrofolate, an important co-factor in the synthesis of glycine, thymidine phosphate and purine. Transfection of a plasmid containing the gene of interest in *cis* of the DHFR gene, which acts as selectable marker for cells grown in a medium deprived of nucleosides, and the use of the inhibitor methotrexate thus forces its amplification and results in the integration of many copies of the gene of interest. On the other hand, investigators have also taken advantage of natural enhancers of gene transcription or strong promoters[46]. In murine erythroleukemia cells (Mel), creation of an artificial dominant control region (DCR) from the globin gene locus where a foreign gene can be inserted has been shown to increase dramatically the expression of the foreign protein. In COS cells, expression of the large T-antigen from the insertion of an origin-defective SV40 genome, interacts with the SV40 origin of replication present in the transfected vector to increase its total copy number and the production of the protein[46]. Finally, insect cells are infected by a recombinant AcNPV where the gene of interest has replaced the highly expressed polyhedrin protein[55]. Results from this study have shown that the hu-LIF was expressed at high levels in all systems. Production of the protein in

amounts of 1 to 25 mg/L were measured but difficult to compare because different culture conditions were used[46]. Expression by the BEVS at 12 mg/L was the most advantageous in terms of protein amounts harvested versus the time required to set up the system[46]. In addition, hu-LIF derived from the CHO, Sp2/0 and Mel expression systems displayed on a SDS-PAGE a broad distinct band of 40 kDa or even a long smear for COS cells, reflecting probably heterologous glycosylation of the protein[46]. In contrast, multiple, albeit finer, bands were discernible for the BEVS and were associated with an incomplete glycosylation of the protein[46]. However, optimization of the culture conditions and testing of different cell lines was not carried out. Other investigators also compared the BEVS to yeast expression of their protein. Human interleukin-5 was produced in significantly higher amounts in Sf9 insect cells (2.7 mg/l) than in *Saccharomyces cerevisiae* JNY5 strain (12.5 μg/l)[66]. In addition, human C9 protein was expressed in Sf21 cells at approximately 7-fold the level found in COS cells and 70 times more than in *S. cerevisiae*[67]. However, much higher concentrations were found in the yeast intracellular fraction but the protein was completely inactive. In contrast, the C9 protein produced in COS or Sf21 cells showed indistinguishable hemolytic activities from that of the purified native protein. Interestingly, the insect recombinant C9 migrated at a lower MW on a SDS-PAGE[67].

4.2 Analysis of the factors that could explain the lower level of expression of prorenin when compared to polyhedrin and other proteins.

When compared to the level of expression of polyhedrin or other secreted proteins in insect cell cultures, the concentration of prorenin detected in the supernatants of High-Five™ cultures is low. Indeed, average expression of polyhedrin revolves around 1 mg/ml/1 x 10^6 cells infected[55] whereas prorenin concentration was estimated at 6-8 mg/L per 1.8 x 10^6 cells after 6 to 7 days of incubation (Figure 10, 11, 12 and 14). Also, the polyhedrin protein can be detected in the incubation medium by 12 hours post-infection and continues to accumulate for 4 to 5 days until cell lysis occurs[55]. In contrast, prorenin can only be detected in the supernatants by Western blot analysis 4 days after infection, although measurements of its concentration by radioimmunoassay - a more sensitive method - suggests its

presence as early as 24 hours PI (Figure 8). Thus, prorenin secretion appears to be delayed in comparison to polyhedrin and the level of expression that theoretically could have been achieved was in fact much lower. Numerous factors can explain such differences in the expression of the two proteins and they are analyzed one at a time below.

First, polyhedrin expression of wild-type AcNPV origin has been reported to be variable and differ with cells or tissues infected and with the quality of the cell media component[55]. Reports have shown that polyhedrin expression is not directly proportional to viral infection. The protein is highly expressed in *Spodoptera frugiperda* cell lines (Sf9, Sf21) but hardly produced at all in *Bombyx mori* infected cells[55]. However, the reason for this discrepancy is not understood for the moment. One possible explanation would be that cell-specific transcription factors or co-activators are required for the efficient transcription of the gene. Alternatively, the processing of polyhedrin could vary between the two cell lines and affect the degradation of the protein. Cell-specific factors are important in the transcription of genes and could explain in part why prorenin expression is higher in High-Five™ cells than in Sf9 cells. Some variation in the level of expression was also observed in this study between different preparations of media or depending on the condition of the insect cells. Older cultures tended to produce less protein.

Second, not all recombination events are equivalent. Early in the characterization of the BEVS, investigators noted that the level of expression of a foreign gene was variable depending on the viral clone that was selected in the purification process[55]. Since all viruses were derived from a single transfection, the only possible answer was that the homologous recombination events were not occurring at the same sites and that it was probably affecting the activity of the polyhedrin promoter[55]. Other investigators have also reported that false positive recombination events occurred in systems using the co-expression of ß-galactosidase, confirming the previous observations (P. Hugo, *personal communications*). In this study, this factor was probably not related to the lower level of expression of prorenin

because multiple clones were tested at each purification step and the most productive was retained. No false positive recombinant baculoviruses were found and the variability between the different strains were not important. Also, this factor did not contribute to the differences observed between the two cell lines since the same pPAPProR baculovirus preparation was used in these experiments.

Third, expression levels for multiple genes inserted in the same vector are often different and it has been suggested that the length and the nature of the leader sequence preceding the foreign gene could affect its transcription[55]. Most transfer vectors that have been constructed to date contain varying amounts of 5' and 3' viral DNA flanking the polyhedrin gene in addition to its promoter and the two homologous recombination sequences[55]. Multiple vector designs have been tested by Summers *et al.*[55] or Matsuura *et al.*[68] and some guidelines have been derived from these studies. First, the length of the leader sequence must be minimized. Insertion of foreign DNA sequences of more than 100 bp or a polylinker often resulted in a decrease in the transcription of the foreign gene[55]. Further, some elements around the transcription start site appear to play a regulatory role on the promoter[55,69]. The polyhedrin leader sequence is very AT-rich and it is possible that stronger interactions of multiple GC base pairs in the leader region of the inserted foreign gene could have an inhibitory effect on its transcription. Also, a significant increase in mRNA and protein expression has been reported when the gene of interest was fused in frame with portions of the amino-terminus of the polyhedrin gene[69], suggesting that elements 3' of the polyhedrin gene start site were important for maximal transcription and translation[69]. Protein levels were higher for derivatives in which the fusion was created more than 30 bases downstream of the polyhedrin ATG[69]. Hence, the sequence surrounding the first ATG of the gene of interest play a critical role in its transcription and translation as it has been reported in other expression systems. The position of the cloned gene in the transfer vector will affect productivity. This factor was not tested for each of the transfer vectors pBbac and pPbac but, although similar, the sequences surrounding the cloned gene are not identical. Also, replacement of the signal peptide has changed the sequence preceding that of prorenin and could also

contribute to the higher expression found in HF cells. In order to determine if this factor was related to the variation in prorenin secretion by each virus, quantification of mRNA expression by RNAse protection assays should have been carried out. Comparison with the level of expression of polyhedrin mRNA in a wild-type infection would determine if transcription of the foreign gene was a limiting factor.

Fourth, the nature of the promoter itself can play a critical role in the expression of a foreign gene in infected insect cells. Apart from its relative strength, the time of its activation and peak is important[55]. Some investigators have reported significant improvements in the expression and secretion of their gene by replacing the very late promoter of the polyhedrin gene with that of the basic protein (a weaker late promoter)[70]. They concluded that by advancing the time-frame of production of the human chorionic gonadotropin (hCG), more time was available before cell lysis for post-translational modifications to occur and for the processing of the multiple sugar moieties present on the molecule, resulting in higher secretion of a more biologically active protein[70]. However, the level of mRNA within cells between the two constructions was not compared and other factors mentioned previously could have contributed. Of interest, their recombinant hCG also exhibited a different electrophoretic mobility in SDS-PAGE as compared to native hCG like in this study. Their analysis attributed this difference to the level of glycosylation of the protein and they demonstrated that sialylation occurred in Sf9 cells in this case[70]. Thus, it appeared that Sf9 cells could not process large amounts of recombinant products very efficiently[70] which is in agreement with our own observations. It has been commonly observed that membrane-associated, secreted and highly glycosylated proteins are expressed in lower amounts in the BEVS in comparison with intracellular proteins[55].

Fifth, many investigators have reported that recombinant proteins expressed in insect cell lines infected with a baculovirus undergo the same post-translational modifications that are found in mammalian expression systems[55]. In all cases, their product was recognized by a specific antibody and was biologically functional[55]. Nevertheless, some differences have been noted and could explain the gap observed

between the expression of prorenin and that of polyhedrin. Glycosylation of foreign proteins in insect cells has been examined mostly by incorporation of radiolabelled sugars, by determining their sensitivity to endoglycosidases or by the addition of inhibitors of the glycosyl transferases such as tunicamycin[55]. Studies have shown that in the early phase of infection (12-36 hr.), there is a preponderance of high-mannose type oligosaccharides on the proteins whereas more complex forms can be detected later in the infection cycle (48-96 hr.)[73]. However, the trimming and the addition of terminal sugars appears to be limited[73,74]. The glycosylation of prorenin has been shown to be linked with the degree of secretion and clearance of the protein in mammalian cells[56] and could theoretically affect the secretion of prorenin in HF cells.

Sixth, the nature of the signal peptide plays a very important role in the correct insertion of proteins in the endoplasmic reticulum of insect cells and their secretion. The signal peptidase appears to be restrictive in the sequence surrounding the cleavage site as demonstrated by the higher levels of secretion of fusion products with the natural polyhedrin signal peptide[55]. Other investigators have also demonstrated that replacement of the native signal peptide of their protein was beneficial. Murphy *et al.* reported that using the baculovirus egt and p67 signal peptides instead of the native sequence of the HIV-1 gp120 improved both the expression and the secretion of their protein in Sf9 cells[59]. Up to 20 times more protein was produced in these cases and the ratio of intracellular versus supernatant protein content was dramatically improved[59]. Button *et al.* confirmed these results with a much better production of the truncated *Leishmania* surface metalloproteinase GP63 by using the p67 signal peptide[71]. Similar results were obtained by Tessier *et al.* when they replaced the natural signal peptide of the plant papain precursor with the one of honeybee mellitin[72]. However, other investigators were unsuccessful in similar attempts and it has been shown that improvement in the cleavage of the signal peptide of a foreign protein is not the only reason responsible for the higher level of expression[55]. Specific recognition sequences around the start codon could improve the binding of ribosomes and the translation of the protein. Alternatively, other factors like mRNA structure and stability, protein degradation in cases of misfolded products and the interaction

with intracellular chaperones have not been investigated in insect cells. These mechanisms have been shown to play an important role in bacteria and could also be implicated in insect cells. Further characterization of the BEVS will be required.

4.3 Prorenin glycosylation is not complex in Sf9 insect cells.

Aeed *et al.* analyzed the glycosylation of prorenin secreted by infected Sf9 cells cultured in TNM-FH[73]. As in this study, cells were infected at a MOI of 5 but supernatants were collected 48 hours post-infection after incorporation of tritiated mannose in the glycosyl moieties of prorenin. However, the yields obtained for the protein were not reported and can not be compared with our results. After pronase treatment to cleave the radiolabelled glycopeptides, they were fractionated on a Con-A Sepharose column and essentially all (97%) eluted as a single peak with the addition of α-mannoside, suggesting a very uniform pattern of glycosylation with trimannosyl structures[73]. Glycopeptides were then fractionated on PSA-agarose (a lectin binding fucosylated residues) where approximately two thirds were bound by the column and the rest was unaffected[73]. Finally, the glycopeptides recovered in the two fractions were digested by endoglycosidases and separated by HPLC. Almost exclusively trimannosyl structures were found in the small fraction whereas other structures of 6 to 8 mannosyl residues were detected in the other. Core fucosyl residues were only found in the first fraction[73]. Further, the elution profile of the residues suggested that no phosphorylation resulting in the generation of a lysosomal targeting sequence occurred in Sf9 cells[74]. In comparison with CHO and BW 5147 cells, Sf9 microsomial proteins did not associate with a mammalian ^{125}I-labelled mannose-6-phosphate receptor[73]. However, this result must be interpreted with caution because the species are evolutionarily distant. No activity of the UDP-GlcNAc lysosomal enzyme N-Acetyl glucosaminephosphotransferase was detected in these cells. Thus, the glycosylation of prorenin in Sf9 cells leads to the production of high-mannose complexes, some of which were linked by a fucosyl residue, that do not appear to have any charge and lysosomal targeting signal, in contrast to mammalian cells[73]. Our own observations suggest that a similar pattern is found in High-Five™ cells since prorenin was not retained on an anion exchange column at pH

7.4. The glycosylation of proteins observed in HF cells appears to be more complex than in Sf9 cells and could be contributing to a better secretion of prorenin[74]. Again, trimannosyl structures are predominant but in some cases galactose residues were added to create more complex structures. However, no sialic acids were present on the glycosyl moieties and these structures had no charge[74].

4.4 Renin expressed with the BEVS and in CHO mammalian cells have similar activities and structure.

In parallel with this study, Mathews *et al.* at Hoffmann-La Roche have also used the baculovirus expression vector system to produce a recombinant human renin without its propeptide in the Sf9 cell lineage[75]. In this construct, the natural signal peptide of prorenin was replaced by that of human gastrin-releasing peptide (hGRP), which has been shown to be effectively cleaved in insect cells. In a second case, renin was also produced directly in a CHO/dhfr- mammalian expression system by removing the targeting sequence and prosegment and by inserting instead the signal peptide of human interleukin-2. Further, the natural human preprorenin was also expressed in each systems. As one would expect, the level of production of renin devoid of its pro region in insect cells was extremely low (0.05 mg/L) and almost negligible in CHO cells (0.005 mg/L)[75], confirming the important role of the prosegment in the proper folding and secretion of the mature protein as discussed previously. In contrast, the concentration of prorenin in the supernatants of insect cell cultures and CHO cultures were much higher[75]. The level of prorenin produced by the baculovirus expression vector system was very low (0,5 mg/L) and did not surpass that found in the mammalian system (10 mg/L)[75]. These results confirm the observations reported when insect cells were infected with the pBPreProR baculovirus (Figure 7). However, the mRNA levels were not measured and the exact reason why the Sf9 cell line does not express at high levels the natural form of prorenin remains obscure.

Having access to large bioreactors, they were able to produce sufficient quantities of the protein in both systems to do a crystallographic analysis and a

biochemical comparison of the two prorenins. Recombinant mature renin was purified by affinity chromatography from 100 liters of the supernatant of infected Sf9 cells whereas prorenin was recovered from a CHO supernatant of a 23-liters airlift fermentor[75]. Purification of prorenin was accomplished in multiple steps and it was activated by the Arg-C protease[75]. Interestingly, the mature renin of infected Sf9 cells migrated at a lower molecular weight on a SDS-PAGE than the mammalian counterpart. Determination of the N-terminal amino-acid sequence of both proteins by Edman degradation confirmed the correct processing of the GPL signal peptide in insect cells[75]. However, the *in vitro* cleavage of the prosegment by the Arg-C protease occured between the Lys-Arg residues. These results suggested that glycosylation of the protein differed in these two systems[75]. To confirm this hypothesis, the authors compared the proteins radius using quasielastric light scattering and found that they were significantly different[75]. Crystallization of both mature renins demonstrated that the folding of renin in the BEVS was not affected and that irregularities in its three-dimensional structure were undetectable. Further, the catalytic activities of both products as measured by AI radioimmunoassay were almost identical but 2-fold lower than the activity of human renin purified from kidney extracts[75]. Also, inhibition curves with the renin inhibitor Ro 42-5892 and the immunoreactivity of all renin forms to two specific antibodies were indistinguishable[75]. All the results presented in this study are in agreement with the conclusions drawn from these recently published experiments. Prorenin produced in HF cells appears to be as functional as its mammalian counterpart and also has a lower molecular weight (Figure 18). Nevertheless, the level of accumulation of prorenin in our system is 15 times higher than the one reported in this case. Prorenin was harvested from HF monolayer cultures after 7 days whereas the authors of this study collected their supernatants after only 4 days[75]. Again sequences surrounding the start codon, the different processing of proteins observed between the two cell lines and the choice of a good signal peptide are primordial. This also emphasizes the importance of optimizing the system to obtain a good level of expression of the protein, specially for financial considerations.

4.5 Production of prorenin in serum-free media and TNM-FH.

Some investigators have also tested the possibility of replacing the TNM-FH medium with different serum-free media as it was done in this study. Ingley E *et al.* Observed a 10-fold decrease in the production of the human Il-5 when using a prepared serum-free medium[59]. Our own results suggest that production in the serum-free medium Baculogold™ would be suitable for a large scale production of prorenin with yields averaging 75% of the maximal amount of protein detected in TNM-FH (Figure 13). Considering the cost of this medium compared to prepared TNM-FH, the relative ease to separate prorenin from the major proteins found in the serum on a DEAE-Sephacel column (Figure 16) and the variability of the results depending on the adaptation of the cells to the new conditions, this approach will probably not be retained. However, our results are only preliminary and further characterization of the purification process is required. If too much prorenin is lost during the purification from the TNM-FH supernatants or if aggregation, denaturation or degradation problems arise, replacement by the Baculogold™ medium will become more advisable. Diminution of the number of purification steps by using the serum-free medium could reduce losses inherent to the purification process. Further, our results strongly suggest that the composition of the media is primordial to a good expression of the protein of interest. The yields obtained in the Baculogold™ medium were much higher than those of the SFM-II and HyQ media (Figure 13). Presumably, the long incubation period used in this study depletes the nutrients in these cases. Also, the cocktail of growth factors that are added stimulate the growth of HF cells in a different manner depending on the receptors present on the surface of these cells.

4.6 A strategic approach to prorenin purification

Design of a good strategy of purification is essential to maximize recovery and preserve the activity of the protein of interest. The approach chosen must take into account the biochemical properties of the recombinant protein and the scale of the process will depend on the amount of purified protein required and its concentration in the initial solution[76,77]. Different strategies have been used by investigators over the

years to purify renin but attempts that were made for prorenin proved to be much more difficult. In part, this was due to acid activation of the protein during the purification process or because of the presence of contaminating proteases[48]. In 1982, Inagami *et al.*[78] were only able to recover 6.3% or 30 µg of prorenin from 20 kg of hog kidney using 9 purifications steps of DEAE-sephacel, pepstatin-sepharose, octyl-sepharose, sephacel G-100, Affi-Gel blue and Con-A sepharose chromatography. The high number of steps involved in this process was probably responsible for losing a lot of the protein but also showed that the design of more selective methods would be advisable. Later, investigators tried to activate prorenin by acidification to benefit from affinity columns for renin using specific inhibitors[44] or pepstatin[48] but some of the material was not recovered and yields varied between preparations, suggesting that activation of prorenin in these cases was not completely reversible. On the other hand, some reports have shown that Octyl-sepharose[79], Cibacron-blue[79] and immunoaffinity chromatography[80] were valid steps for the purification of prorenin from human plasma but the concentration of the material in the original source was too low to attempt a large scale purification of the protein.

Our preliminary results suggest that prorenin could be separated from most of the other proteins present in the TNM-FH supernatant on a DEAE-Sephacel column (Figure 16). Indeed, recombinant BEVS prorenin was not retained by the column, presumably because of the absence of any charge on the glycosyl residues found on the surface of the protein. Further, no significant losses were found in this purification step (data not shown). Nevertheless, prorenin was often detected in two or three fractions and refinement of this technique is required to minimize the total recovery volume. Important losses were also associated with the ammonium sulfate precipitation steps. On average, no more than 70% of prorenin was recovered. Further, the dialysis fractions were often found to be turbid and it is possible that prorenin aggregated or precipitated during the progressive removal of the salt. Glycosylation of prorenin was found to be responsible for the multiple isoelectric forms found in the supernatant of GH_4 cells[56]. The possibility remains that the differential glycosylation of prorenin in HF cells produces a neutral charged protein

that could precipitate at the pH (7.4) of the dialysis buffer. More analysis will be required to confirm this hypothesis. Finally, the yields obtained from our preliminary purifications were comparable to that of GH_4 supernatants but refinement of the purification process should improve the yields obtained from the BEVS.

4.7 Future directions

Clearly, more investigation is required to find an answer to all of the questions that were raised in this study and to choose the best purification strategy possible. A lot of information about the biochemical properties of the recombinant prorenin produced in High-Five™ cells will be derived from these studies. Improvement of the recovery of prorenin from the ammonium sulfate precipitations will be important. Also, we plan to test other purification steps such as Cibacron-blue chromatography (a dye known to bind prorenin with good affinity), Octyl-sepharose chromatography (based on hydrophobic interactions), gel filtration-HPLC (molecular size) and chromatofocusing. Con-A affinity chromatography would also be an interesting choice since this lectin has been shown to bind with high affinity to high mannose oligosaccharides such as those present on recombinant proteins produced in insect cells[56]. Detail planning of the procedure will be important to insure a good continuity between each purification step[77].

Finally, optimization of scaled-up culture conditions will be critical for a good recovery of prorenin and to minimize cost. Rice *et al.* found that stirred vessels for suspension cultures were more advantageous than airlift fermentors because they can be used in small incubators and cost less. In both vessels, maximal cell density and the yields of protein obtained were comparable[81]. Another interesting perspective would be the use of small cell factories and large scale purification roller bottles since monolayers are formed in these vessels. Our experience suggest that aggregation of the insect cells in suspension cultures decreases their survivability and their infectivity.

5. Conclusion

This study reports the expression of human prorenin in insects cells infected with a recombinant baculovirus and suggests that this system could be used for a large scale production of the protein once a complete purification strategy will have been designed, tested and optimized. Twenty milligrams of pure prorenin or more would be required for the cystallization of the protein.

The first attempt at the expression of preprorenin under the control of the strong polyhedrin promoter was unsuccessful presumably because the native signal peptide was not processed correctly in Sf9 cells. Replacement of this sequence with the human Placental Alkaline Phosphatase (PAP) signal peptide improved the expression of our gene and the survivability of the insect cells. Different culture conditions as a function of the cell density, the period of incubation, the multiplicity of infection and the composition of the medium were tested to achieve maximal production of prorenin. The best results measured averaged **8 mg/L** when High-Five™ cells were plated at a density of 1.0 x 10^6 cells/ml and were infected by the recombinant pPAPProR baculovirus at a MOI of 6. Supernatants were harvested 6 to 7 days post-infection. Also, three serum-free media were tested but did not perform as well as TNM-FH. All of the parameters influenced the amount of prorenin that accumulated in the supernatant of HF monolayer cultures, albeit at a different extent, and thus emphasize the importance of the optimization of the system.

Comparison with prorenin produced in GH_4 cells demonstrates that both recombinant enzymes have similar catalytic activities and that they are recognized by the same antibody.

Preliminary experiences in prorenin purification have shown that ammonium sulfate precipitation of the recombinant protein was usefull for the concentration of the product but not in the selective removal of other proteins. On the other hand, the recombinant human prorenin produced in insect cells eluted at a low salt

concentration from an anion-exchange column whereas the mammalian prorenin was retained along with the other proteins. This could be explained by the absence of negatively charged sialic acids in the oligosaccharides of proteins secreted by insect cells and we intend to use this property of the insect recombinant prorenin to facilitate its purification.

6. Acknowledgments

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