Enhanced Expression and Secretion of SUMOstar Fusion Proteins in the *flash*BAC GOLD™ BEVS

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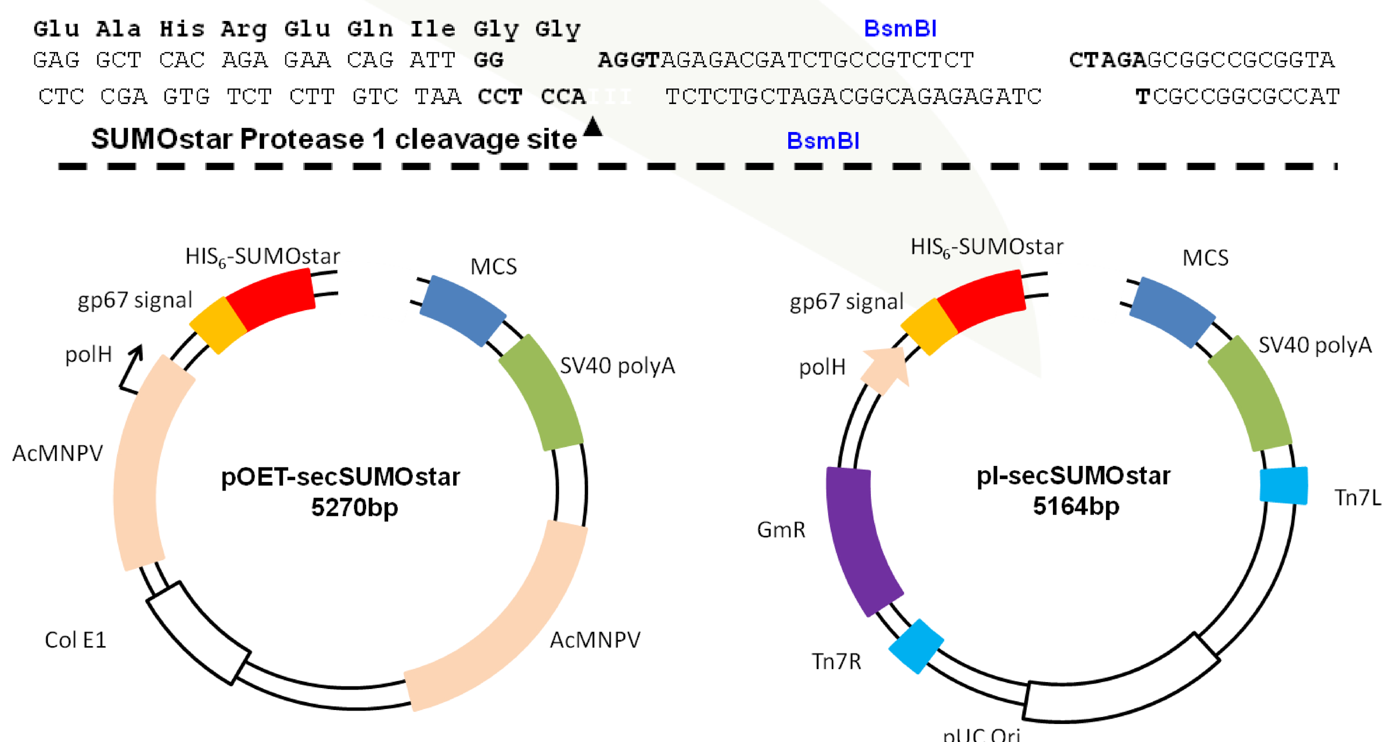
Abstract: The use of baculovirus expression vector systems (BEVS) have provided a cost-effective means for production of heterogonous proteins in a eukaryotic system for over 20 years. However, with some exceptions, the promise of BEVS having the production capabilities of *E. coli* combined with the post-translational abilities inherent to eukaryotes has yet to be fully realized. Considering the laborious and technical demands of traditional insect cell culture, mammalian systems (e.g. HEK293 or CHOK1) can often be a more attractive choice if overall protein yields are anticipated to be comparable. One "bottleneck" of particular concern in BEVS is that of efficient recombinant protein secretion. Endogenous secretion signal peptides often do not work well for the protein-of-interest (POI) and even the use of "universal" signal peptides derived from viral glycoproteins can yield less than satisfactory results. Recently, the use of a mutated *Autographa californica* multi-nucleopolyhedovirus (AcMNPV) genome for recombinant baculoviral construct generation, known commercially as *flash*BAC GOLD™, has been shown to enhance overall yields of secreted proteins in BEVS. The use of a deSUMOylase resistant form of the SUMO fusion tag, termed SUMOstar, has been demonstrated to convey the well established advantages of SUMO technology as seen in *E coli*, while having utility in eukaryotic systems. In order to investigate the potential of a BEVS combining both *flash*BAC GOLD™ and SUMOstar, we constructed expression cassettes allowing for the expression and secretion of heterologous proteins with either a HIS_6 tag alone, or as a HIS_6-SUMOstar fusion protein. These cassettes were cloned into expression vectors compatible with either *flash*BAC™ or Bac-to-Bac® (Invitrogen), and the systems were tested in parallel. Both interleukin-3 (IL-3) and interleukin-5 (IL-5) expression was greatly enhanced, over prolonged time, when expressed in the *flash*BAC GOLD™ system compared to Bac-to-Bac®. Expression of these interleukins as SUMOstar fusion proteins increased yields further, with a combined overall observed enhancement of 5 to 10-fold. The expression of KLK3/PSA also benefited dramatically from the combination of *flash*BAC and SUMOstar, with overall yields increased by as much as 20-fold, when compared to expression of HIS6-KLK3 in Bac-to-Bac. IMAC purification indicated levels of approximately 5 to 20mg per liter can be achieved without the need for bioreactor scale expression. In order to demonstrate the full utility of this combined BEVS, proteins were processed for tag removal, purified to homogeneity, and characterized. Precise mass determinations confirmed these proteins to have the desired N-terminus after tag removal. Functional characterization of KLK3/PSA revealed it to be an active protease. These candidate proteins demonstrate that the combination of *flash*BAC™ and SUMOstar have the potential to deliver high yields of native recombinant proteins in a cost-effective eukaryotic system.

Transfer Vector Construction



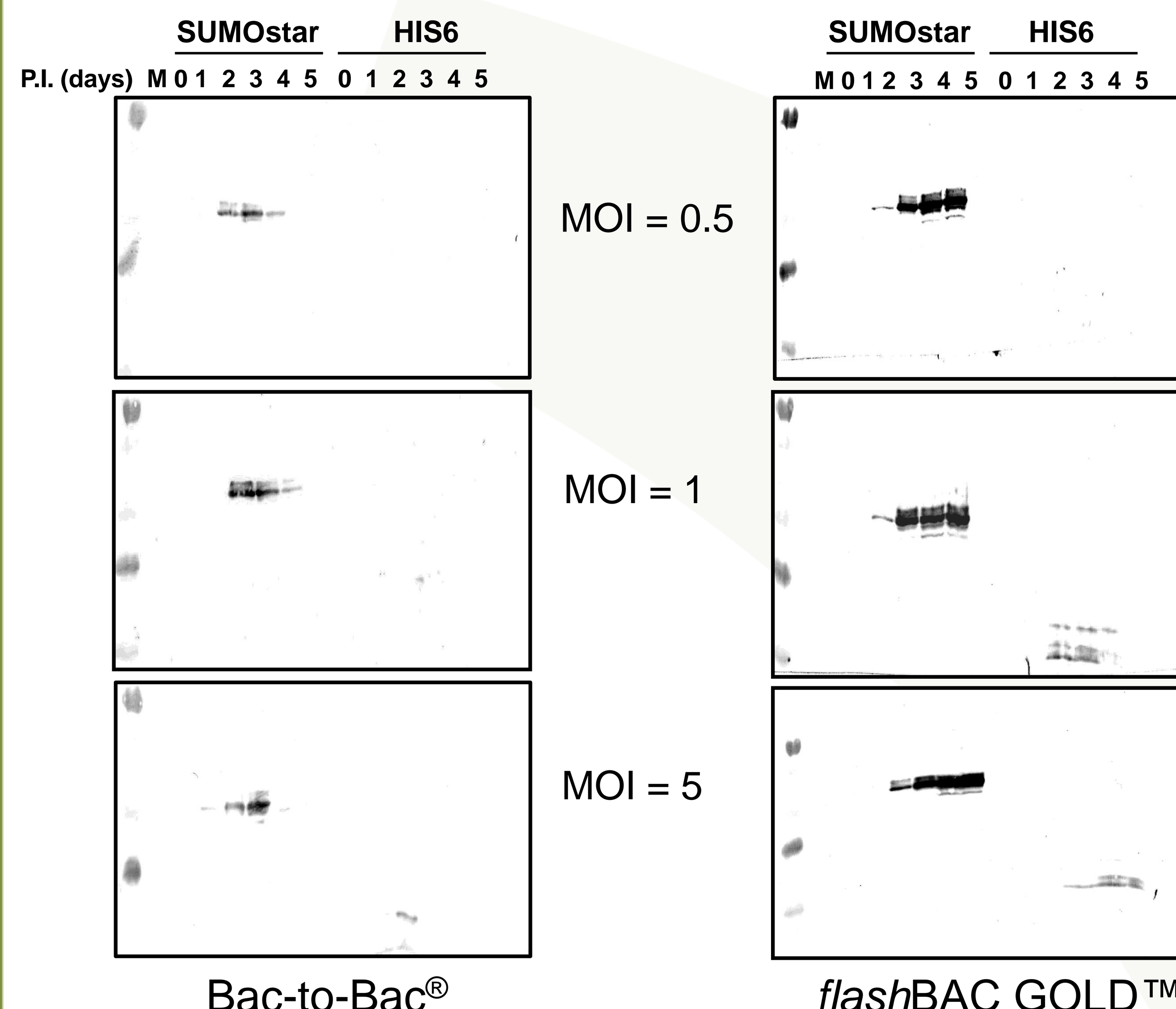
In-frame cassettes containing an initiation site optimized for BEVS, a gp67 secretion sequence, the HIS_6-SUMOstar tag, a MCS for directional cloning, and an SV40 polyadenylation signal were generated and cloned into a transfer vector compatible with either the Bac-to-Bac® (Invitrogen) or *flash*BAC™ expression systems. Transfer vectors containing a HIS6 tag alone were also constructed. Interleukin 3 (IL-3), interleukin 5 (IL-5), and kalikrien 3/prostrate specific antigen (KLK3/PSA) were cloned into all vectors.

Production and Propagation of Recombinant Virus

***flash*BAC™ GOLD BEVS generation.** Sequenced transfer plasmids (0.5µg) were combined with *flash*BAC™ GOLD viral genomic DNA and transfection reagent in Graces' unsupplemented insect media prior to application directly to adherent Sf9 cells. These cultures were allowed to incubate (27°C, protected from light) overnight, with transfections being quenched with the addition of fresh serum-free media containing antibiotics (SFM-AA). Viral stocks (**P1**) were harvested 4 days later. **P2** stocks were harvested 5 days post-infection (PI) of Sf9 cells grown in suspension (~1e^{6} cells ml^{-1}) with P1 stocks. P2 viral titers were typically >2e^{8} pfu ml^{-1}.

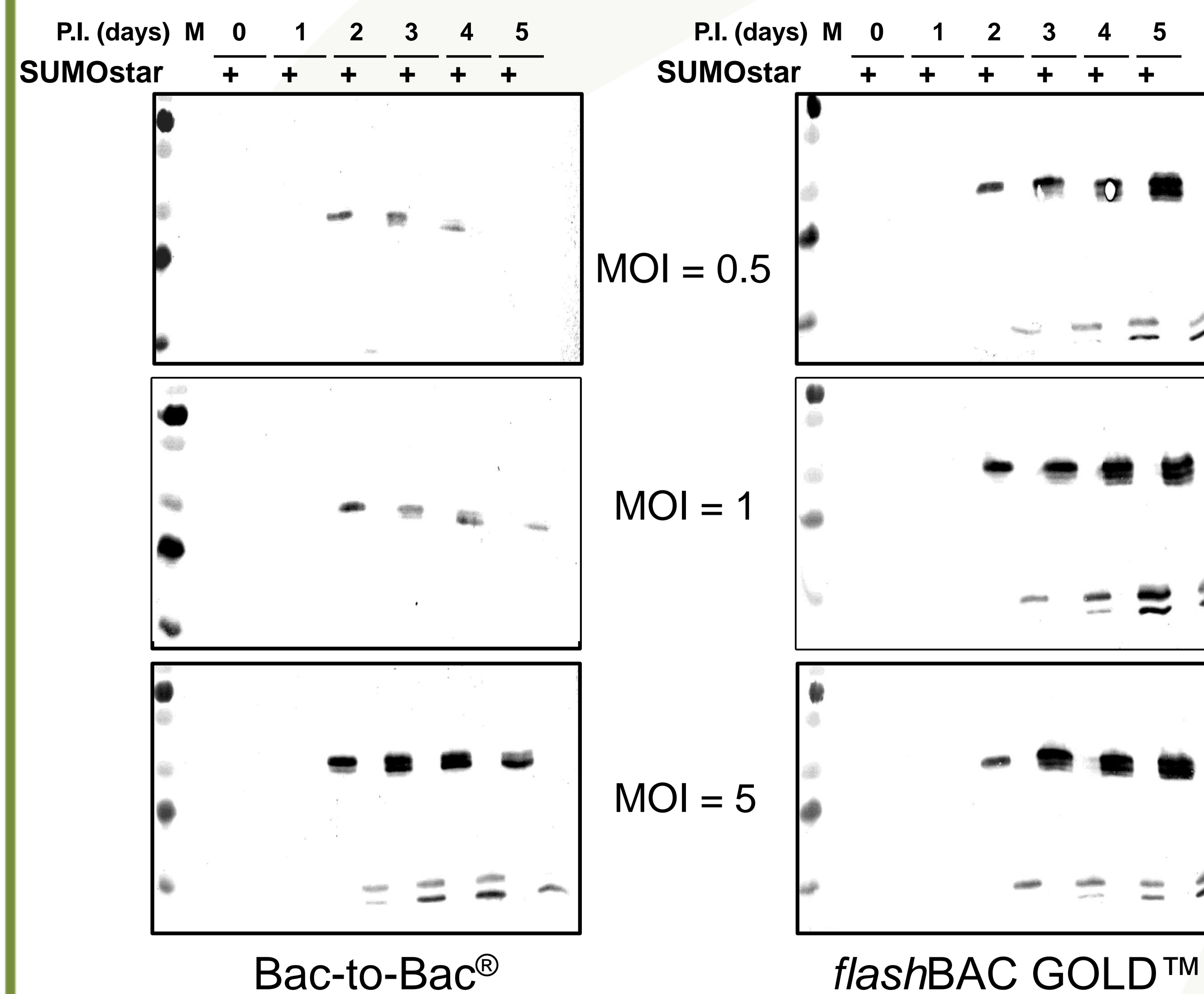
Bac-to-Bac® BEVS generation. Sequenced transfer plasmids were used to transform DH10BAC cells, for bacmid generation. Multiple bacmid colonies for each construct were chosen for purification and PCR screening. Positive bacmids were combined with transfection reagent in Graces' media and applied to adherent Sf9 cells. Generation and propagation of viral stocks (**P1 and P2**) were carried out essentially as described by Invitrogen. In some cases, **P3** viral stocks were generated to achieve sufficient titer (>2e^{7} pfu ml^{-1}).

Comparative Expression Studies with IL3



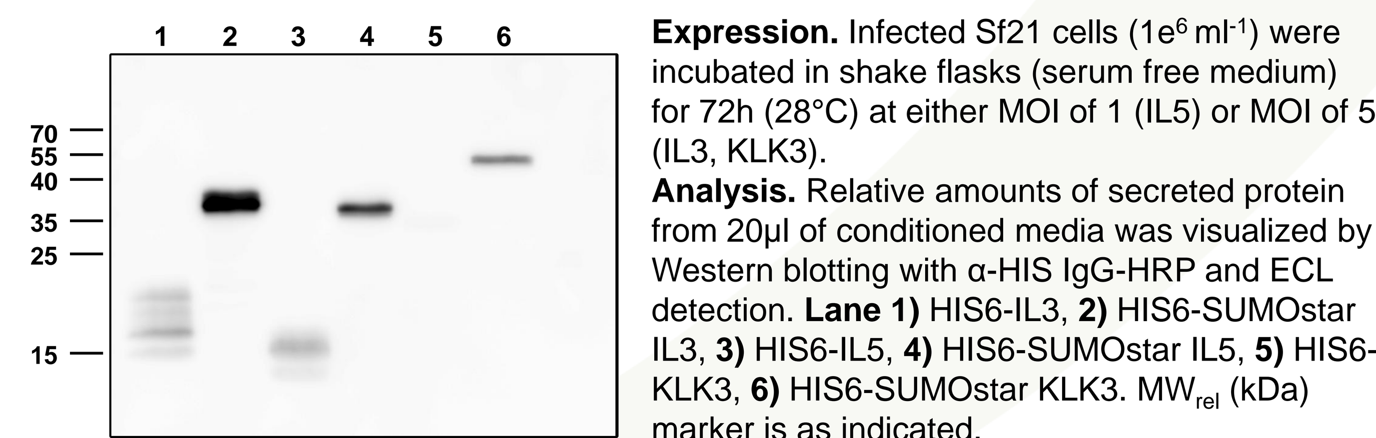
Expression. Sf21 cells (~1.4e^{6} cells ml^{-1}) were infected with *flash*BAC or Bac-to-Bac viruses (either HIS_6 or HIS_6-SUMOstar tag) at indicated MOIs and incubated (27C, 280 RPM) in 24-well deep well blocks. **Analysis.** Clarified conditioned media was concentrated, subjected to Western blotting, and detection with an anti-HIS IgG. **Results.** SUMOstar fusion resulted in enhanced secretion of IL-3 at all MOIs. The use of *flash*BAC resulted in enhanced secretion of all constructs over a prolonged time course.

Comparative Expression Studies with IL5



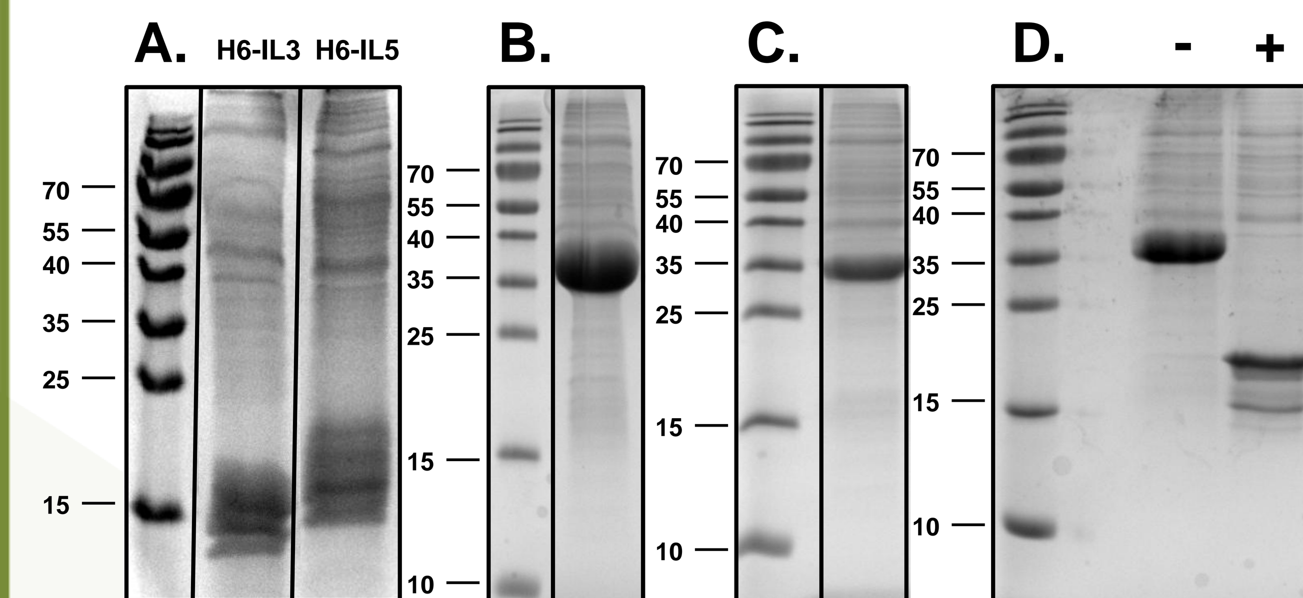
Expression and Analysis. Studies were carried out as outlined above for IL-3. **Results.** As with IL-3, IL-5 secretion was dramatically enhanced for SUMOstar fusions at all MOIs. The prolonged, sustained, secretion of these proteins as seen with the use of *flash*BAC likely results from the genomic deletions that reduce secretory traffic from AcMNPV proteins, improve cell viability, and reduce degradation of the protein of interest.

Scale up Expression of Recombinant Proteins



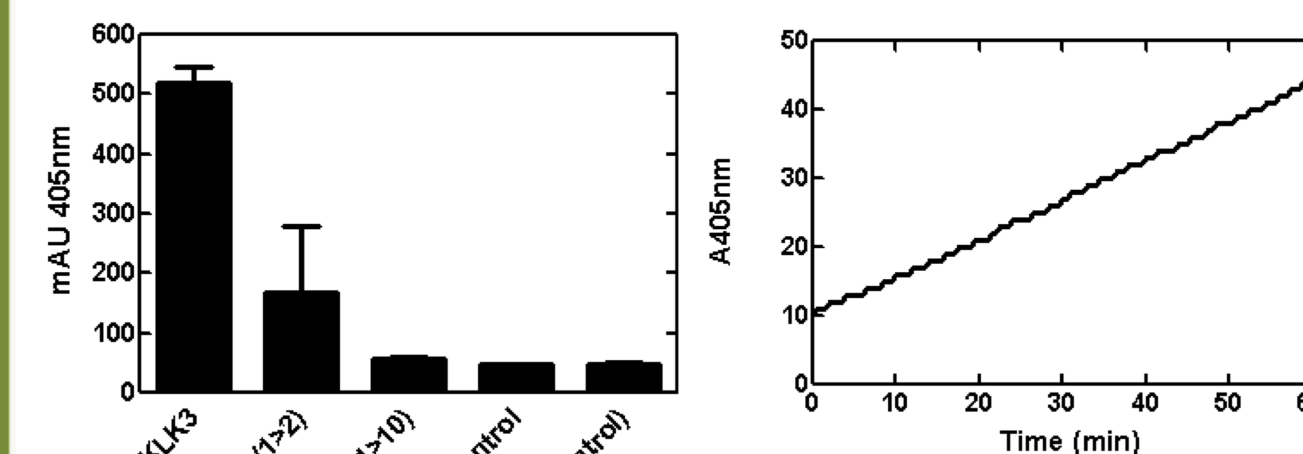
Expression. Infected Sf21 cells (1e^{6} ml^{-1}) were incubated in shake flasks (serum free medium) for 72h (28°C) at either MOI of 1 (IL5) or MOI of 5 (IL3, KLK3). **Analysis.** Relative amounts of secreted protein from 20µl of conditioned media was visualized by Western blotting with α-HIS IgG-HRP and ECL detection. **Lane 1)** HIS6-IL3, **2)** HIS6-SUMOstar IL3, **3)** HIS6-IL5, **4)** HIS6-SUMOstar IL5, **5)** HIS6-KLK3, **6)** HIS6-SUMOstar KLK3. MW_{rel} (kDa) marker is as indicated.

IMAC Purification of Recombinant Proteins



Methodology. 50ml of conditioned medium was dialyzed extensively versus 50mM HEPES, pH 7.5, 0.3M NaCl, (H/S) with1mM benzamidine. Medium was clarified by centrifugation and supplemented with leupeptin (5µg/ml) and E-64 (50µM). Protein was batch adsorbed (~16h) to IMAC resin (1ml). Resultant resin was collected in a gravity column, washed with 10ml of H/S, and washed with 10ml of H/S plus 10mM imidazole. Protein was eluted with H/S plus 300mM imidazole. Peak fractions (20µl) of **A.)** HIS6-IL3 and HIS6-IL5, **B.)** HIS6-SUMOstar IL3, and **C.)** HIS6-SUMOstar IL5 are shown analyzed by SDS-PAGE and coomassie blue staining. **D.)** HIS6-SUMOstar IL3 was dialyzed overnight versus H/S + 10mM BME in the presence of SUMOstar Protease 1. All steps were carried out at 4°C.

Activity of KLK3/PSA



Hydrolysis of Succinyl-Arg-Pro-Tyr-para-nitroanilide (Suc-RPY-pNA). Panel A. The activity of SUMOstar treated KLK3/PSA fusion protein was compared to control reactions containing SUMOstar treated IL5, or reaction buffer alone. Reactions were assumed to have run to maximum signal. **Panel B.** Monitored Suc-RPY-pNA hydrolysis by SUMOstar treated KLK3/PSA. All reactions were carried out in 20mM HEPES, pH 7.5, 300mM NaCl, 0.25mM Suc-RPY-pNA and monitored by absorbance at 405nm.

Summary of Purification and Characterization

Protein	Yield (mg)[a]	Yield (mg L^{-1})[b]	Comments
HIS6-IL3	0.22	4.4	Heterogeneous
SUMOstar IL3	1.39	28	>95% cleaved
HIS6-IL5	0.24	4.8	Heterogeneous
SUMOstar IL5	0.57	11	Homogenous
HIS6-KLK3	0.77	15	None
SUMOstar KLK3	0.37	7.4	Active

[a] Yields from 50ml of culture determined by either BCA or Bradford assay. [b] estimated yields based on 50ml culture

Conclusions: The secretion of candidate proteins in the flashBAC gold BEVS was greatly enhanced for 3 candidate proteins (IL-3, IL-5, and KLK3) relative to Bac-to-Bac. As this system obviates the need for bacmid preparation or plaque purification, the time to purified proteins is shortened by as much as two weeks, as well. Further enhancement was seen with SUMOstar fusion proteins compared to HIS-tagged proteins. As SUMOstar fusion provides an affinity tag that can be removed yielding a native N-terminus (except proline), its utility greatly aids in the speed and ease of purification from insect cell conditioned medium. Used in combination, these technologies enhanced the yields of all proteins tested 2 to 5-fold compared to conventional technologies, while providing a convenient method for the procurement of native protein.