



SUMO Gene Fusion Technology

ENHANCING FUNCTIONAL PROTEIN EXPRESION IN *P.PASTORIS*

Rationale for SUMO in Enhancement of Secretion in *P. pastoris*

SUMO (small ubiquitin related modifier), also known as Sentrin, are present throughout eukaryotes and highly conserved from yeast to humans; that encodes four SUMO genes (Hochstrasser et al. 2000) which has also been termed *SMT3* (Johnson et al. 1997). The yeast *Smt3* gene is essential for viability. Although overall sequence homology between ubiquitin and SUMO is only 18%, structure determination by nuclear magnetic resonance (NMR) reveals that they share a common three dimensional structure characterized by a tightly packed globular fold with β -sheets wrapped around a single α -helix (Bayer et al. 1998). SUMO molecules are encoded as C-terminus extension precursors and processed by endogenous SUMO proteases. Cells contain potent SUMO proteases that remove the C-terminal extensions. The C-terminus of SUMO is conjugated to ϵ amino groups of lysine residues of target proteins. Sumoylation of cellular proteins has been proposed to regulate compartmentalization of proteins, signal transduction, and cell cycle progression (Muller et al. 2001). Sumoylation and de-sumoylation is a very dynamic process regulated by SUMO ligases and SUMO proteases.

The first step in the secretion of proteins is efficient expression within the cell. Our approach of attaching a highly stable and compact SUMO structure to the N-termini of labile proteins is an important rationale for the development of secretory vaccines. We believe that the highly folded nature of SUMO will act as a chaperone to protect the fused protein as it is secreted into the media. It is also noteworthy that Blobel and colleagues discovered SUMO while pursuing the translocation of protein from nucleus through the nuclear pore membrane structure (Matunis et al. 1996). It is now recognized that sumoylation plays an important role in compartmentalization of proteins. Indeed, our most recent data on secretion of the difficult-to-secrete-protein, granzyme B, in *P. pastoris* suggests that SUMO is a potent signal for secretion of proteins (Figure 1).

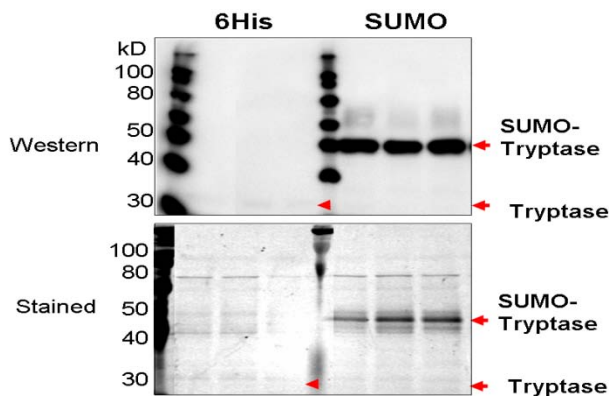


Fig.1. SUMO tag increases protein production in *P. pastoris*. Tryptase 2beta protease was fused to either 6His tag or to SUMO tag at its N-terminus and expressed as secreted protein in *P. pastoris*. Data from five different strains grown in 250 ml shake flasks was analyzed. After 48-h induction with methanol, raw media samples (10 ul) were analyzed by SDS-PAGE and Western blot. Protein estimation results from other experiments suggest that SUMO fusion facilitates at least a 50 fold increase in secretion of proteins (data not shown).

Coupled with our results that attachment of SUMO to the N-terminus of poorly expressed proteins dramatically enhances the level of expression in *E. coli*, yeast and insect cells suggest that the SUMO tag has universal properties in enhancing expression of recombinant proteins (Butt et al. 2005). The endoplasmic reticulum contains a quality control system that subjects misfolded or unassembled secretory proteins to rapid degradation via the cytosolic ubiquitin proteasome system. This requires retrograde protein transport from the endoplasmic reticulum back to the cytosol (Plemper et al. 1999). Attachment of SUMO to the N-terminus of secretory protein prevents miss-folding and chaperones the protein through ER. It is also likely that the

SUMO signal acts as an antagonist to ubiquitin and inhibits the retro-translocation of the protein back to cytosol. Thus a stage is set for one way flow of secretory proteins through ER.

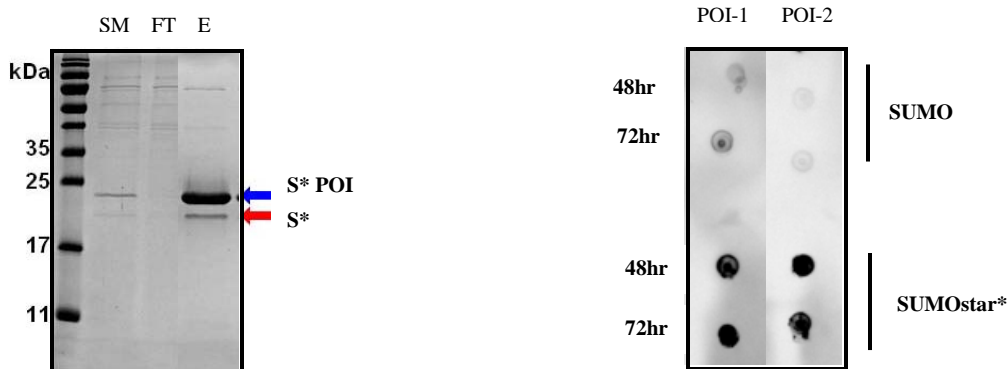
Role of *P. Pastoris* as a Factory for Antibody and Antibody Fusion Expression:

The ability to express biologically active proteins for research, biopharmaceutical or vaccine purposes has been hindered by the lack of a simple method that works for structurally diverse proteins. Secretion of protein is an inexpensive and simple way to overcome these challenges. *P. pastoris* is a methylotrophic yeast that was developed to convert methane to high protein animal feed, *P. Pastoris* is ideally suited to large scale fermentation. Continuous growth on methanol is carried out under very high cell densities sometimes exceeding 130 g L⁻¹ dry-cell-weight (Cereghino 2000). Recombinant protein production levels can reach grams per liter (Jahic et al. 2003; Brunel et al. 2004; Chen 2004).

The first stage in methanol metabolism is conversion to formaldehyde using oxygen and alcohol oxidase. The enzyme alcohol oxidase (two genes in *P. pastoris*) has poor affinity for O₂, so *Pichia* compensates by producing large quantities of the enzyme (30% soluble protein). Expression of exogenous proteins is therefore commonly placed under the control of the primary alcohol oxidase promoter (AOX1). Strains of *P. pastoris* have been developed with a disruption in the AOX1 gene as this has proven beneficial in the production of some heterologous proteins (Cregg 1987; Cregg 1988). These strains are referred to as Mut^S or methanol utilization slow, whereas wild type strains are Mut⁺.

Transformation of *P. pastoris* is based on homologous recombination. Popular vectors contain a section of the 5' AOX1 promoter and AOX1 transcription termination region. Once the vector has been created with the gene of interest, it is linearized and electroporated into *Pichia* cells. Through single crossover events the expression cassette is inserted upstream or downstream of the AOX1 gene. Integrations are detected by the incorporation of any number of selectable markers such as antibiotic resistance or metabolic rescue. Occasionally multiple copies of the gene are inserted in tandem, often leading to an increase in the production and/or activity of the protein of interest. In one study increasing the copy number of *LacZ* genes to 22 resulted in a 17 fold increase in β-galactosidase activity (Sunga and Cregg 2004). In another study four copies of *HbsAg* resulted in a fourfold increase in protein yield (Vassileva et al. 2001).

Expression of protein(s) of interest (POI) in *P.pastoris* using SUMOpro and SUMOstar™: representative examples



A. Purification of POI from *P.pastoris* condition medium

B. Dot blot analysis of condition medium samples. Immunodetection with anti-Flag-His6-POI

Alternative strategies are clearly required for the future in which mutational changes are readily adjusted for and new vaccine quickly produced in sufficient quantities. The cost for producing antibodies or fragments in mammalian cells is very high. In some cases the therapeutic dose of the protein is in excess of one gram per patient (Andersen et.al, 2004; Joosten 2003). Many promising recombinant antibody based projects are shelved due to a low protein secretion and the associated escalation of costs. It is clear that new technology is required for rapid and scalable production with low cost per gram protein. It appears that the SUMO system can enhance the level of production by up to 50 x fold in *P. pastoris*. If the cost of production can be decreased 10 fold while reducing strain optimization for new antibodies to three months, as compared to 18 months for CHO cells, this will lead to a breakthrough in antibody and vaccine production.

LifeSensors' SUMO mediated protein secretion system in *P. pastoris* is scalable to the production of hundreds of kilograms of protein that will meet the licensing requirements of FDA.

Purification of Proteins and Cleavage of SUMO to Generate Native Structures: *P. pastoris* secretes very few endogenous proteins so the primary protein component of the clarified media is a recombinant protein. For purification of SUMO tagged fusions, metal affinity chromatography on Ni Sepharose™ High Performance resin (Amersham) could be employed, (Malakhov, et al. 2004). Cleavage of SUMO from the fusion protein with a very robust SUMO protease allows generation of desired N-termini that is present in the native structure. a hallmark of the SUMO-fusion system (Butt et al. 2005). Additional steps might include ion exchange and size exclusion chromatography.

If the glycosylation analysis shows an addition of oligosaccharides by *P. pastoris* that result in modified structure and modified epitopes, the glycosylation problem could be solved by obtaining a modified strain of yeast in which the glycosylation system was humanized (Wildt and Gerngross 2005). Such strains have been produced, and shown to result in the addition of oligosaccharides commonly found in humans. Glycosylation in wild type *P. pastoris* is however very similar to humans and has been demonstrated to result in the production of therapeutic proteins which are safer for humans than those produced in other systems (Gerngross et al. 2004).

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