Expression and Purification of Active Human Kinases Using Pichia pastoris as A General-Purpose Host

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Abstract

The heterologous expression of human kinases in good purity and monomer, soluble and active form can become a challenging feat. Most of the reported successful attempts are carried out in insect cells as a host. The use of E. coli for expression is limited to a few kinases and usually is facilitated by large solubility tags that can limit biophysical studies and affect protein–protein interactions. In this report, we evaluate the methylotrophic yeast Pichia pastoris as a general-purpose host for expression of human kinases. Methods: Six diverse kinases were chosen due to their therapeutic importance in human cancers. Tested proteins include serine/threonine kinases cyclin-dependent kinases 4 and 6 (CDK4 and 6) and aurora kinase A (AurKA), receptor tyrosine kinase erbB-2 (HER2), and dual specificity kinase mitogen-activated protein kinase kinase 3 (MKK3b). Two challenging targets, CDK6 and HER2, were fused to the highly basic, N-terminal domain of the secreted tyrosine-protein kinase VLK. Standard expression procedure in Pichia pastoris used. Purification was done using affinity chromatography, purity and identity of the proteins were confirmed and compared. Results: Some of the tested kinases expressed with good yield and purity and comparable activity to commercially available versins. Addition of the VLK domain improved expression and decreased aggregation of two of the challenging CDK6 and HER2. This can be attributed to a correlation we observed between the protein pI and its expression level in P. pastoris, where basic proteins are more likely to be expressed and purified. Conclusions: This study introduces P. pastoris as a promising host for expression of soluble and active human kinases.

Background

Protein kinases play a central role in cell regulation and signal transduction by selectively phosphorylating downstream effector proteins. Dysregulation of kinase activity as a result of mutations, altered expression levels or localization can lead to abnormal cellular behavior and cancer. Human kinases are important drug targets, comprising one of the largest groups of druggable targets in the human genome. *(1)* Structural studies on kinases have been essential for advancing drug design as well as elucidation of protein-protein interactions that affect catalytic activity of kinases and are often dysregulated in cancer. *(2)* *(3)* High purity preparations of enzymatically active, full-length kinases are essential for structural biology. Although the *E. coli* system has been the major workhorse for recombinant protein expression, it has only limited success so far in producing human protein kinases. *(4)* Thus, the baculovirus expression system (BES) has become the system of choice for many kinases because it can consistently produce soluble and active enzymes in insect cells. However, long turnaround time and high cost of insect cell expression systems are undesirable. Furthermore, due to its prohibitively high cost of isotope labeling, BES is not convenient for producing proteins for nuclear magnetic resonance (NMR). One notable example is the tyrosine kinase c-Abl, which saw a large increase in NMR studies after a bacterial expression method for its purification was developed. *(5)* Furthermore, the majority of kinases expressed with BES have fused bulky protein tags, most commonly maltose binding protein (MBP), thioredoxin (Trx), or glutathione-S-transferase (GST), which complicate the study of kinase protein–protein interactions and may impart non-native properties to the recombinant protein.

The methylotrophic yeast *Pichia pastoris* is a widely used host for recombinant protein expression, though it has not been routinely used for human kinases. *P. pastoris* is genetically easy to manipulate with a strong promoter to drive expression of introduced genes. The target proteins are expressed in high concentration in cultures that require simple conditions and affordable media for rapid growth. *(6)* As a eukaryote, *P. pastoris* is capable of correctly folding most eukaryotic proteins and producing them in a soluble and active form; yeast can incorporate post translational modifications, such as glycosylation, disulfide-bond formation and proteolytic processing. Relevant for production of kinases, this system has been successfully applied for the production of functional proteins that require phosphorylation for activity, which is quite relevant to kinases. *(7-9)* As a microorganism, *P. pastoris* is amenable to high-density fermentation and growth in a minimal medium, allowing easy scale up and isotope labeling. To date, the expression of kinases has been challenging in this system, perhaps because they phosphorylate intracellular proteins, leading to growth inhibition and toxicity to *P. pastoris*, among other hosts. *(10)* Active PINK1 has been expressed with a small purification tag *(11)*; however, other *P. pastoris*-expressed kinases utilized bulky tags *(12)* or failed to express in an active form. *(13)* To our knowledge, a general approach for expressing kinases in *P. pastoris* has not been reported.

Towards the goal of expressing minimally tagged, functional full-length kinases (or more specifically their full-length cytoplasmic domains) at a reasonable cost, we developed an expression system in *P. pastoris*. The system has proved its potential as a general-purpose, protein-production platform using a variety of kinases, including cyclin-dependent kinases 4 and 6 (CDK4 and 6),

aurora kinase A (AurKA), receptor tyrosine kinase erbB-2 (HER2), and dual specificity kinase mitogen-activated protein kinase kinase 3 (MKK3b). All six kinases were isolated in an active form, with varying levels of expression. Full-length AurKA and MKK3b expressed with the highest yield (1 mg/L culture and 0.5 mg/L culture, respectively), purity, and activity, while CDK4 and CDK6 were only isolable in the presence of CyclinD2. Interestingly, we noted that more basic protein AurKA expressed and purified more efficiently than the acidic CDKs. Fusing the basic N-terminal domain of the kinase VLK to CDK-6 improved expression; while the yield of CDK-6 was still low (0.05 mg/L culture), we have not previously been able to express and purify this protein in a soluble form. HER2 expression levels were comparable in BES and P. pastoris, but BES preferentially expressed shorter kinase-domain constructs, while P. pastoris expressed constructs containing domains N- and C-terminal to the kinase domain. Taken together, this study supports the use of *P. pastoris* for small-scale expression of kinases and paves the way for scale-up via fermentation.

Results

Strategy for kinase expression in *P. pastoris*

We applied the same workflow for expression and purification for all target kinases. Constructs were prepared using the Gibson assembly method and transformed into yeast for expression tests, as detailed in Materials and Methods. Each target kinase was tagged with the small cleavable purification tags Strep II (WSHPQFEK) or His10 (Figure 1, Table 1). Transformants in *P. pastoris* typically produced different amounts of recombinant protein. We therefore screened multiple (4-10) single clones of the transformants, and the clone exhibiting the highest expression level was selected for scale-up. (Supplementary Figure 1)

The expression conditions such as induction time, temperature and methanol concentration (for protein induction) were optimized for the highest protein production. For the first day, a temperature of 30 °C was chosen to optimize yeast growth and biomass accumulation in buffered glycerol-complex medium (BMGY). After 24 hours, protein production was induced by adding 0.5% methanol and growing in buffered methanol-complex medium (BMMY) for 48 hrs at 28 °C to maximize protein production. Cells were then harvested, stored at -40 °C or processed at the same day as detailed in the materials section. Following purification on three serially connected, 5 ml Strap-Trap columns, proteins were either subjected to a second purification step when needed or directly characterized by polyacrylamide gel electrophoresis (PAGE), western blotting, and kinase activity assay. Protein purity was estimated from SDS-PAGE and enzymatic activity was measured using the ADP-Glo Max *in vitro* kinase enzymatic assay kit (Promega). Activity was compared to commercially available kinases with reported activity measured using the same substrates.

Expression and purification of MKK3b

MKK3 (isoform b) is a cytosolic kinase widely involved in cellular signaling. This dual specificity kinase is part of the RAS/MAPK pathway, which plays a key role in regulating proliferation, differentiation and survival (14). The active enzyme was expressed with a small Strep II tag in *P. pastoris* with good purity as judged by SDS-PAGE (Figure 2). After elution from a StrepTrap column, higher molecular weight aggregates were detected by SDS-PAGE. To purify the monomeric form, the eluent was pooled, diluted in buffer (50 mM Tris HCl pH8.5, 150 mM NaCl, 5% glycerol and 1 mM TCEP) and injected onto a gel filtration column (HiLoad 16/60 Superdex 200). The fractions containing the monomeric soluble protein were collected and concentrated and the identity was confirmed by Western blotting with anti-Strep antibody (Figure 2F). The yield was 0.5 mg/L culture and the activity was comparable to the commercially available MKK3 using the same reported substrate, inactive p38 (SignalChem, Table 1). The enzyme was previously expressed with a bulky MBP tag and His tag in *E. coli*; the inactive protein required co-expression with its upstream activator MEKK-C to obtain 25% yield of active enzyme. (14,15) Two other reports expressed a constitutively active mutant in BES with no reported yield. (16,17)

Expression and purification of full-length AurKA

The mitotic kinase AurKA has recently gained interest as a target in several malignancies. (18) Typically, AurKA is recombinantly expressed as the catalytic domain, but we were interested in the potential functions of the disordered N-terminal domain. We therefore expressed and purified strep-tagged, full-length AurKA in *P. pastoris* using a single purification step (StrepTrap). Purified protein expressed as monomer, 95% pure on SDS-PAGE and active. The identity was confirmed by Western blotting with anti-AurKA antibody and the yield was 1 mg/L culture (Table 1, Figure 3). We did not try to activate AurKA by phosphorylation or by addition

of activating protein partner Tpx2. Nevertheless, activity was approximately 25-30% of commercially available kinase using the same substrate, MBP (18.7 nmol/mg/min vs 62-73 nmol/mg/min; SignalChem).

Expression and purification of CyclinD2 (CycD2), CDK 4 and 6

Cyclin-dependent kinases are involved in cell cycle regulation and are considered important targets in human cancers. The kinases are almost always co-expressed using BES with a cyclin to improve the kinase structural stability, folding and activity. (19) N-terminal Strep-tagged CDK4 and CDK6 were expressed in *P. pastoris* after co-transformation with N-terminal His6-CycD2 to obtain cyclin-CDK complexes. Complex formation was confirmed on small scale of 100 ml culture by dual-tag tandem affinity chromatography; the complex co-eluted from both StrepTrap and HisTrap columns as confirmed by SDS-PAGE and Western blotting with specific antibodies (Figure 4). Large scale, 1 L culture for protein expression was processed on a StrepTrap column with higher purity and yield for the CDK6-CycD2 complex than the CDK4-CycD2 complex (0.30 mg/L, 95% vs 0.14 mg/L, 50%, respectively; Figure 5, Table 1). Further purification by HisTrap for CDK4-CycD2 complex did not improve the purity. Both CDK4 and 6 showed slightly improved activity compared to the commercially available versions, which were co-expressed with cyclin D1 in SF9 insect cells using the same substrate, Rb protein (773 - 928). Activity was 14.4 nmol/mg/min vs 12 nmol/mg/min for CDK4/cyclin and 17.1 nmol/mg/min vs 8-9.3 nmol/mg/min for CDK6/cyclin (SignalChem).

Attempted expression of the single proteins CDK4, CDK6 and CycD2 in *P. pastoris* with several tags was not as successful. CDK6 did not express at all despite several rounds of transformation and expression tests. CycD2 expressed but was insoluble, while CDK4 expressed in a soluble form but with very low purity and yield (Supplementary Table 1). These results agree with most published reports describing the difficulties in expressing the individual proteins. ***(19)***

We noted that the poorly expressing CDK4/6 and CycD2 were acidic proteins, with pI values of 6.08, 5.74, and 5.25, respectively (Table 1), while MKK3 was closer to neutral (pI = 6.28), and full-length AurKA was basic (pI = 9.25). The N- terminal domain of AurKA contributed to the overall basicity of the protein, and we hypothesized that addition of a basic domain would improve expression levels. To test this idea, we added a highly basic 110 amino acid N-terminal domain from the secreted kinase VLK (Molecular weight = 11.4 KDa, pI = 11.7) to the kinase domains of CDK6 and HER2 (see below) and monitored the effect on expression. In contrast to CDK6-Strep, which did not express in *P. pastoris* in the absence of CycD2, His10-VLK-CDK6-Strep did express (Supplementary Figure 1). Though the fusion construct showed good expression, subsequent purification on a StrepTrap column produced a protein with very low purity. The eluent was pooled, diluted 10-fold in buffer (50 mM Tris HCl pH8, 300 mM NaCl, 10% glycerol and 1 mM TCEP) and injected to an equilibrated 5 ml HisTrap column. After washing three times with increasing imidazole concentration (20 - 80 mM), the protein was eluted using 250 mM imidazole with improved purity. The eluent was pooled and incubated overnight with TEV protease to cleave the His10-VLK portion, then passaged over a 1 ml His resin gravity column to elute CDK6-Strep in the flow through (Figure 6). The identity was confirmed using Western blotting with specific antibody and the obtained protein was soluble and reasonably pure, albeit with low yield (0.05 mg/L, 70% Table 1). Given the difficulty of expressing CDKs without their cyclin protein partner, this result gave an initial indication that adding VLK fusion protein to shift the kinase pI could improve expression.

Expression and purification of HER2 kinase domain

HER2 receptor tyrosine kinase is a well-known oncogene, whose cancer signaling is modulated by the catalytically inactive (pseudokinase) receptor HER3. ***(20,21)*** We designed several constructs encompassing the HER2 kinase domain with different sequence lengths and purification tags, with and without a point mutation V956R located in the its C-terminal lobe (Table 1, Supplementary Table 1). V956R blocks homodimerization of HER2, thus decreasing its activity and toxicity, consequently improving expression. ***(22)*** The kinase domain of HER2 (residues 705-1029) expressed in SF9 insect cells to the level of 0.15 mg/L culture, however, longer constructs including part of the juxtamembrane segment located N-terminally to the kinase domain (JMB) and the C-terminal tail (residues 691-1255) did not express in SF9. We therefore compared expression for short and long constructs in *P. pastoris* to determine whether yeast would allow expression of HER2 containing these protein-protein interaction domains.

Codon usage of the wild-type HER2 gene was optimized for *P. pastoris* without changing the amino acid composition (GenScript). HER2 kinase domain with the JMB and full-length C-terminal tail (residues 691-1255), with or without the V956R mutation and

VLK-N-terminal sequence, was then expressed in *P. pastoris*. With a one-step purification on Strep-Trap columns, wildtype HER2 expressed at a modest but reproducible level and purity (0.2 mg/L culture, 70%), with some aggregation (Figure 7). Adding the VLK fusion protein eliminated the aggregation but was expressed at lower yield and purity (0.05 mg/L culture, 40%); V956R mutant HER2 expressed at apparent yields comparable to wild type but with lower purity (40%) as manifested by the higher molecular weight aggregates that could not be eliminated in subsequent chromatography steps. By size exclusion chromatography the wild type construct separated in agreement with it being a monomer, and its identity was confirmed using SDS-PAGE and Western blotting with anti-HER2 antibody. Both the wild type and mutant HER2 had kinase activity comparable to their commercial counterparts (Table 1).

Expression of the shorter HER2 construct with the kinase domain lacking the C-terminal tail (residues 705-1029) proved more challenging. While SF9 insect cells had produced high purity, active, protein in a monomer form with acceptable yield, this construct expression in *P. pastoris* suffered from aggregation, low yield and purity (Supplementary Table 1 and Supplementary Figure 2). Hence, SF9 and *P. pastoris* gave opposite results, with the long construct that encompasses almost entire intracellular portion of the receptor behaving better in yeast cells and kinase-domain only constructs expressing better in insect cells.

Discussion

In the past two decades, several kinases have been targeted for anticancer therapy by inhibitors developed to bind their kinase active sites. More recently, drug discovery has also focused on inhibiting the protein-protein interactions that regulate homo/heterodimerization of kinases and kinase/substrate interactions. **(**23-25**)** These interactions can occur outside the kinase domain, increasing the need for studies of kinases in the presence of its regulatory domains. Recombinant expression and purification of such longer constructs, however, is typically challenging.

We evaluated the yeast *Pichia pastoris* as an expression system for the production of active human kinases with short affinity tags. Emphasis was placed on expression of kinases including regulatory domains and on kinases that have been difficult to express in *E. coli* or insect cells. *P. pastoris* combines the advantages of rapid growth and low cost with the ability to express eukaryotic proteins with proper protein processing and folding. Based on our reported activity of the expressed kinases, *P. pastoris* successfully phosphorylated the proteins resulting in full or partial activation without the addition of external mammalian lysates or specific kinase activators.

For fifteen variations of six proteins, we observed a range of protein yield and purity (Table 1, Supplementary Table 1). The expressed kinases were obtained in a monomeric, soluble and active form, usually after one-step affinity purification, which may be important for limiting aggregation. The yields obtained for even the most challenging targets were sufficient for enzymatic characterization. All targeted kinases had activity comparable to commercially available sources except for AurkA (Table 1); in this case the 2-3-fold lower activity level could be due to the absence of full phosphorylation-induced activation or due to the absence of activating proteins such as Tpx2.

We observed that kinases with basic or near-neutral pI (AurKA, MKK3) tended to have higher expression yields and suffered less aggregation than acidic proteins (HER2, CKD4). AurKA, in particular, represents a case study for the success of *P. pastoris* as an expression system. Previous AurKA reports expressed the kinase domain in *E. coli* and full-length AurKA in SF9, but the yields were not provided. **(***18, 23, 26,27***)** In our hands, full-length, His6-tagged AurKA did not express in a soluble form using Rosetta strain of *E. Coli* (data not shown). The same construct suffered from degradation after attempted expression in SF9 insect cells. Moving to the yeast *S. Cerevisiae* yielded soluble protein without degradation, but the yield was low (0.14 mg/L). Shifting to *P. pastoris* maintained solubility and purity, with yield improved by almost 7-fold (1 mg/L, Figure 8). Thus, while we cannot directly compare the yield of AurKA expressed in yeast and BES, the P. pastoris system was an effective host to express the full-length kinase.

Whether P. pastoris is the optimal expression host for MKK3 is unclear. While bacterial expression was faced with limited success, MKK3 has been expressed as a constitutively active mutant in insect cells at a yield of 8.6 mg/L culture **(***14-17***).** In our hands, expression yield of wild type MKK3 in P pastoris was lower (0.5 mg/L culture); however, we did not attempt to express the constitutively active mutant. Hence, when expressing a single kinase, it is worthwhile to test multiple expression systems, as some different mutants of the same kinase might be better expressed in E. coli or insect cells.

We succeeded with expressing kinases with low pI upon modifications of their expression constructs. For example, most of the intracellular domain of HER2 was obtained as an active and soluble monomer in moderate yields (0.2 mg/L, 70% purity) in *P. pastoris* but failed to express in SF9 insect cells. By contrast, the isolated kinase domain suffered from aggregation and low yields in *P. pastoris* but expressed and purified successfully in SF9 cells (0.15 mg/L culture). For the most challenging targets, such as CDK6, we hypothesized that expression would improve if the pI was shifted from acidic to basic by addition of a cleavable N-terminal domain of VLK. Indeed, VLK fusion did improve expression of CDK6; Although yield was low (purified yield 0.05mg/L culture, 70% purity), this approach offers the opportunity to obtain isolated CDK6 for protein–protein interaction studies.

Our expression system utilized batch fermentation of *P. pastoris* grown in shake flasks by methanol induced expression; however, *P. pastoris* can also be grown in continuous-feed fermentation bioreactors, which should provide 10-fold higher cell mass and commensurate increase in protein yield. ***(28)*** The small, cleavable purification tags and simple purification procedures used here provide an approach to express active kinases that include domains outside the kinase domain, facilitating biophysical studies of protein-protein interactions and crystallization. This is the first report to attempt using *P. pastoris* as a general-purpose host to compare expression of several types of active human kinases.

Methods

Chemicals and reagents

The vector pPICZ-A and Top10F' bacterial competent cells were from Invitrogen and specific antibodies were from Millipore and Cell Signaling. SF9 insect cells and ESF 921 media were from Expression systems. Strep-Trap, His- Trap and gel filtration columns (Superdex 16/600 200 pg) were from GE. All other chemicals unless otherwise stated were from Sigma Aldrich.

Design and construction of protein expression vectors

The genes of HER2 (691-1255) and hVLK were codon-optimized for protein production in *P. pastoris* and ordered from Genscript Inc. The genes encoding the full-length AurKA, CDK4, CDK6, MKK3 and G1/S-specific cyclin (CycD2) were described previously (29). For intracellular expression, the genes were PCR amplified and cloned downstream of the methanol- inducible alcohol oxidase promoter of the pPICZ-A vector backbone that carry a zeocin selection marker using the Gibson assembly method. Affinity tags were added to all protein genes to facilitate purification (Table 1, Figure 1). For CDK6 and HER2, expression trials included a fusion construct with the N terminal hVLK (Figure 1). The prepared recombinant reactions were used to transform Top10F' competent cells, and plasmid DNA was prepared using Zyppy™ Plasmid Midiprep Kit (Zymo Research) following manufacturer's protocol and sent for sequencing (Elim Biopharm) to confirm the constructs then used for *P. pastoris* transformation.

P. pastoris host strain for expression

The host for protein expression was derived from the protease-deficient strain SMD1163 (his4, pep4, prb1). The newly engineered strain, denoted SMD1163H (pep4, prb1; Mut+), was obtained by transforming the SMD1163 host with the pPIC3.5K plasmid, which was linearized with SacI overnight. This was done to improve growth rates and enable it to grow in absence of histidine in the growth media.

Transformation of *P. pastoris* expression hosts

A high-efficiency transformation protocol was followed to make *P. pastoris* electroporation competent. ***(30)*** The transformation of host cells was carried out using the pPICZ-A plasmids containing the kinase constructs linearized with PmeI to facilitate transformation. 20-30 µg of linearized plasmid was transferred to a pre-chilled 0.2 cm electroporation cuvette, mixed with 40 µL competent cells, and incubated on ice for 5 minutes. Electroporation was conducted on a Bio-Rad Gene Pulser II porator with pre-set parameters for *P. pastoris* (2000V, 200Ω, 25µF). After electroporation, cells were resuspended in a mixture of 1 ml 1M sorbitol and yeast extract peptone dextrose (YPD) and recovered at 30°C for 1.5 hours. 200 µL of the recover mixture was plated on a YPD-Sorbitol (YPDS) plate with 0.5 mg/ml Zeocin. The plates were incubated at 30°C without light for 3 days, until colonies appeared.

To select for multi-copy inserts, we employed the post-transformation vector amplification (PTVA) method. ***(31)*** Briefly, all colonies from YPDS plate with 0.5 mg/ml zeocin were scraped, pooled in 1 ml sterile water, and further diluted into 10 ml sterile water. 150

µl diluted suspension was plated on a fresh YPDS plate with higher zeocin concentrations (0.5 to 2 mg/ml). The plate was incubated without light for another 3 days until colonies appeared. If more than 100 colonies were obtained, the process was repeated with increasing zeocin concentrations until the number of colonies was between 10 and 20.

Expression of kinase targets in *P. pastoris*

Positive transformants were picked and colony PCR performed using standard AOX sequencing primers (Forward: GACTGGTTCCAATTGACAAGC, Reverse: GCAAATGGCATTCTGACATCC). After confirmation of gene integration into the yeast genome, single transformed colonies were screened for target protein expression by inoculation in 5 ml BMGY and grown overnight. Cells were pelleted, washed, and resuspended in 5 ml BMMY with 0.5% methanol and grown for 48 hours at 28°C for protein production, methanol was replenished after 24 hours. Samples were picked at 24 and 48 hrs to measure biomass and protein production levels to assess toxicity and optimum incubation time for protein production. Proteins were isolated by alkaline extraction, gel electrophoresis, and electro- transfer to PVDF membrane using iBlot™ and iBind™ systems (Invitrogen) per the manufacture instructions. Western blots were probed with anti-kinase antibodies and/or anti-tag antibodies and detected by chemiluminescence. The transformant with the highest expression level was chosen for large-scale 1 Liter culture protein production.

Protein production and purification

A single colony of the highest expressing transformant of each target kinase was used to inoculate 20 ml of BMGY medium with vigorous shaking at 30°C, for 16-18 hours. The cells were pelleted, washed, and resuspended in 500 ml BMMY medium and grown for 48 hours at 28°C for protein production. Cells were then harvested, resuspended in Buffer A (100 mM Tris HCl pH8, 150 mM NaCl, 10% glycerol, 1 mM TCEP, 0.01% Tween) freshly supplemented for lysis with 2 mM PMSF and protease inhibitor cocktail tablet and lysed in a Bead-beater (SPEC) following manufacturer's instructions. The cell lysate was clarified by centrifuging at 21000 rpm for 1 hour. The supernatant was filtered through a 2-µm prefilter and a 0.45-µm filter then loaded onto an ÄKTA FPLC system with 3x5 ml StrepTrap columns that had been equilibrated with Buffer A. After loading, the columns were further washed with 3 column volumes of Buffer A. The protein was eluted with 3 column volumes of Buffer B (Buffer A supplemented with 5 mM D-desthiobiotin).

The eluent was pooled, concentrated and buffer exchanged (Amicon Ultra Centrifugal Filters) into 20 mM Tris HCl pH 8, 75 mM NaCl, 2% glycerol and 1mM TCEP. The concentration, purity and identity of target proteins in the eluted fractions were estimated by measuring OD280, SDS-PAGE with Coomassie staining and Western blots. If further purification was needed as detailed in the results section, the fractions containing the target protein were pooled and diluted 10-fold with the appropriate buffer before loading to next column.

Protein activity assay

Kinase specific activity was measured using the ADP-Glo Max assay protocol. Briefly, 5 µl of the primary kinase reaction was prepared in a 384-well plate by adding 2 µl of a series of concentrations of the kinases (50 – 400 ng final protein) in 1X kinase buffer (Cell Signaling), 2 µl of 250 µM high purity ATP mixed with the appropriate substrate and 1 µl of 5X kinase buffer. The substrates were purchased from SignalChem and included: 0.5 µg/µl peptide substrate (AAEEIYAARRG) for HER2 constructs, 0.1 µg/µl Retinoblastoma protein (Rb) for CDK4-CycD2 and CDK6-CycD2 complexes, and 0.1 µg/µl Myelin Basic Protein (MBP) for AurKA. Activity of MKK3 was measured indirectly by using MKK3 to activate 0.2 µg/µl inactive p38γ (Thermo Scientific). Two µl of this reaction mixture was added to 1 µl of 5X kinase buffer and 2 µl of 250 µM high purity ATP, mixed with 0.1 µg/µl MBP. Reactions were allowed to proceed for 45 min at room temperature, then 5 µl ADP-Glo Reagent was added and incubated for 40 minutes to terminate the reaction and deplete the unconsumed ATP. Finally, 10 µl of ADP-Glo Max Detection Reagent was added and incubated for 60 min. The resulting luminescence was detected using a Tecan Infinite F200 PRO multimode plate reader. A calibration curve was constructed with high purity ATP/ADP mixture to quantify the ADP produced in the kinase reaction. The specific activity of each kinase was calculated by dividing the number of moles of ADP produced by the reaction time in minutes multiplied by the enzyme amount in mg. The resulting activity was compared when possible to the reported activity of commercially available kinases.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: All raw data generated during this study will be made available in supplementary information files.

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Authors' contributions: MA and YF designed the research, MHA and YF performed the yeast expression, LL performed insect cell expression, NJ, YF, and MA wrote the manuscript. MM and HF critically reviewed the work, suggested experiments, and reviewed the manuscript. All authors read and approved the final manuscript.

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Table

Table 1: Kinases Expressed in *P. pastoris*

Protein construct		Tags		Mwt	pI	*P. pastoris* expression Characterization data					
		N-term	C-term	(Kda)		Agg.	Yield	% Purity	Dispersity	Kinase specific activity (nmol/mg/min) Measured	Reported[∞]
HER2 (691-1255)	WT	His10	Strep	66.8	5.24	Low	0.20 mg/L	70%	monomer	15.713	10-
	WT with VLK	His10	Strep	77.4	5.88	N	0.05 mg/L	40%	monomer	ND[√]	
	V956R	His10	Strep	66.9	5.28	Low	0.23 mg/L	40%	monomer	11.4	
MKK3b		Strep	——	42.6	6.28	Low	0.50 mg/L	95%	monomer	32.642	24-
AurKA		Strep	——	49.1	9.25	Low	1.00 mg/L	95%	monomer	18.773	62-
CDK6 with VLK		His10	Strep	52.3	8.57	Low	0.05 mg/L	70%	monomer	ND[√]	
CDK4/CycD2		#	——	37.0/36.1	6.08/5.25 N		0.14 mg/L	50%	Co-eluted	14.4	12
CDK6/CycD2		#	——	40.2/36.1	5.74/5.25 N		0.30 mg/L	95%	Co-eluted	17.19.3	8-

∞ Commercially available sources (in text)

√ ND Not determined due to low yield and/or purity

N terminal His6 on CycD2 and N-terminal Strep on both CDKs

Figures

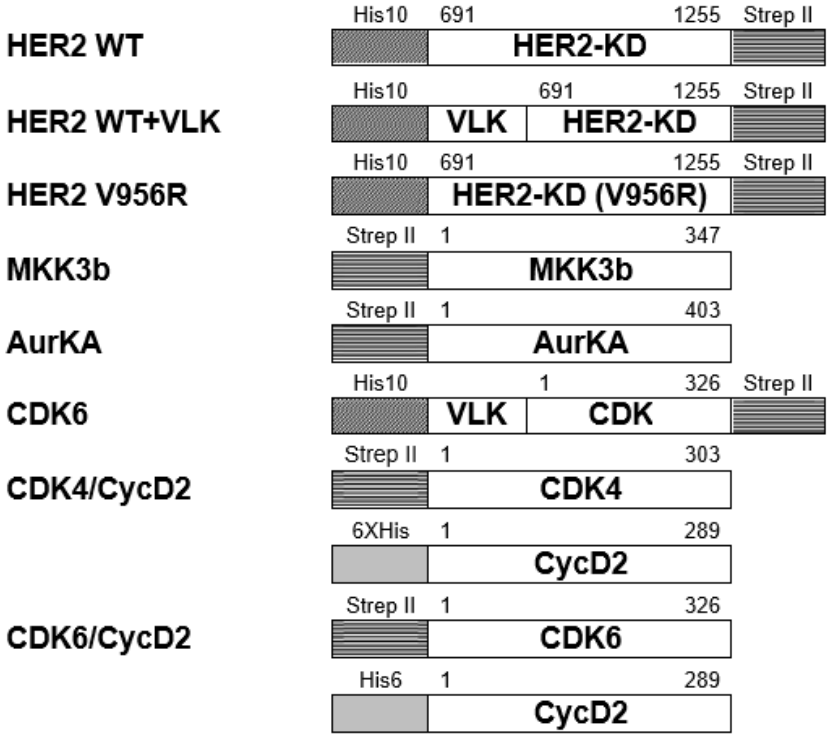

Figure 1

Constructs of kinases expressed in P. pastoris

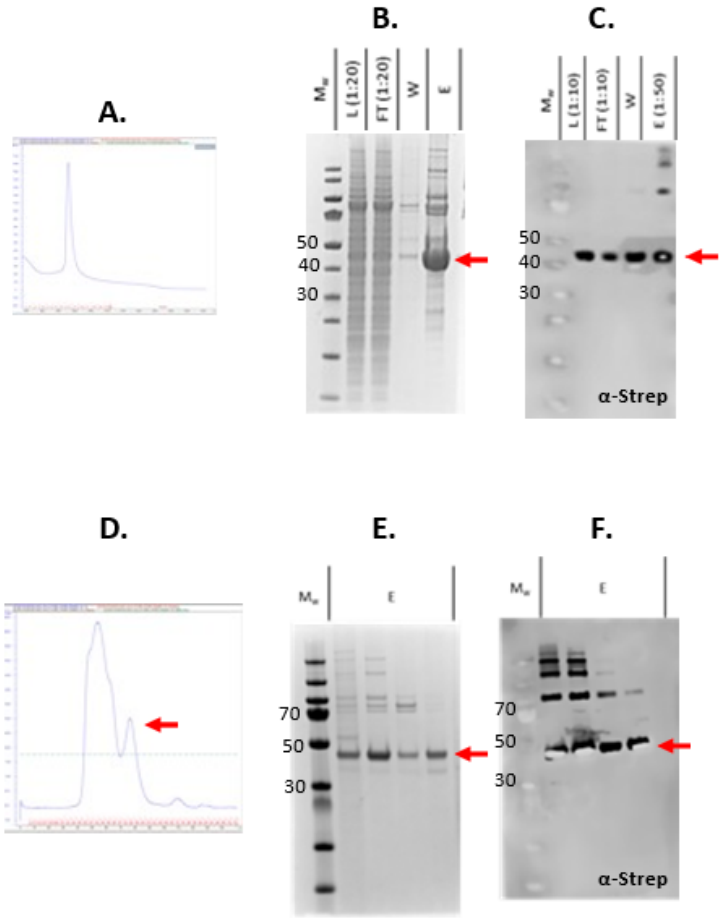

Figure 2

Purification of MKK3b: A) Chromatogram for Strep-Trap column (3x5 ml) elution, B) coomassie stain and C) Western blot for the purification. D) Chromatogram for second purification step through gel filtration column with arrow pointing to the last fraction containing the pure monomeric protein, E) coomassie stain and F) Western blot for gel filtration. Mw is the molecular weight marker, L is the clarified lysate, F is the flow through, W is the wash and E is the column eluent, dilutions indicated to avoid overloading the gel.

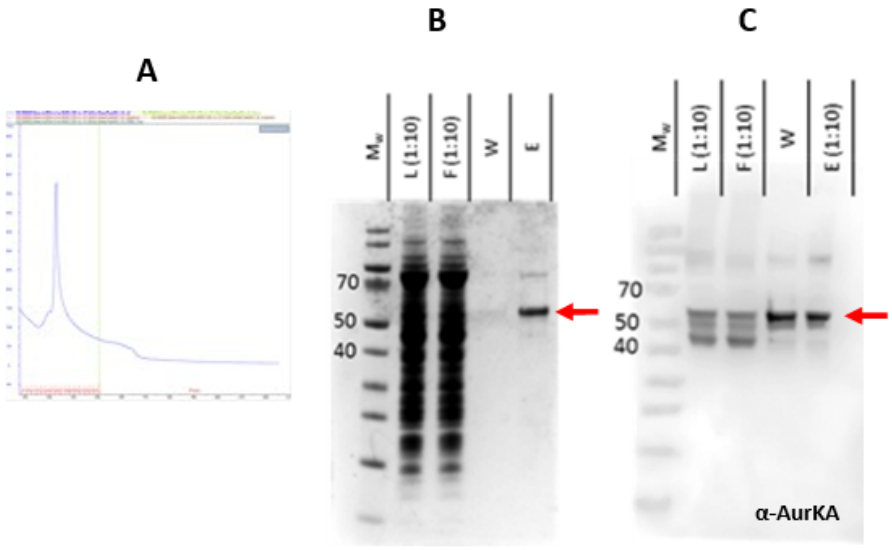

Figure 3

Purification of AurKA: A) Chromatogram of the purification through Strep-Trap column, B) coomassie stain and C) Western blot for the purification. Mw is the molecular weight marker, L is the clarified lysate, F is the flow through, W is the wash and E is the column eluent, dilutions indicated to avoid overloading the gel.

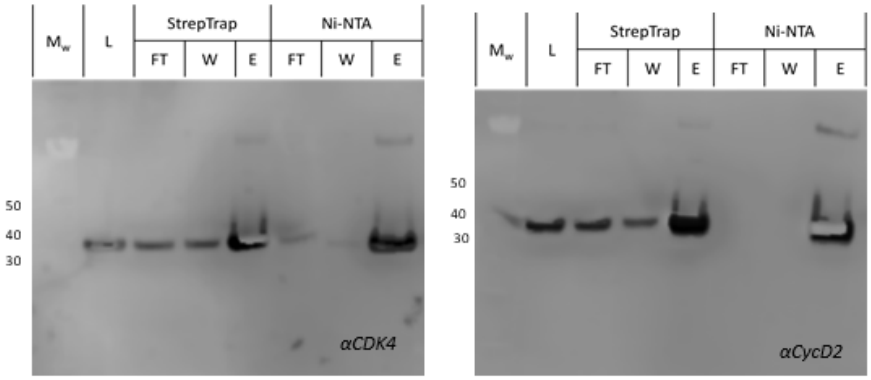

Figure 4

Western blots with specific antibodies for the small-scale purification of the CDK4/CycD2 complex showing co-elution of the two proteins from tandem purification steps on Strep-Trap and Ni-NTA columns. Mw is the molecular weight marker, L is the clarified lysate, FT is the flow through, W is the wash and E is the column eluent.

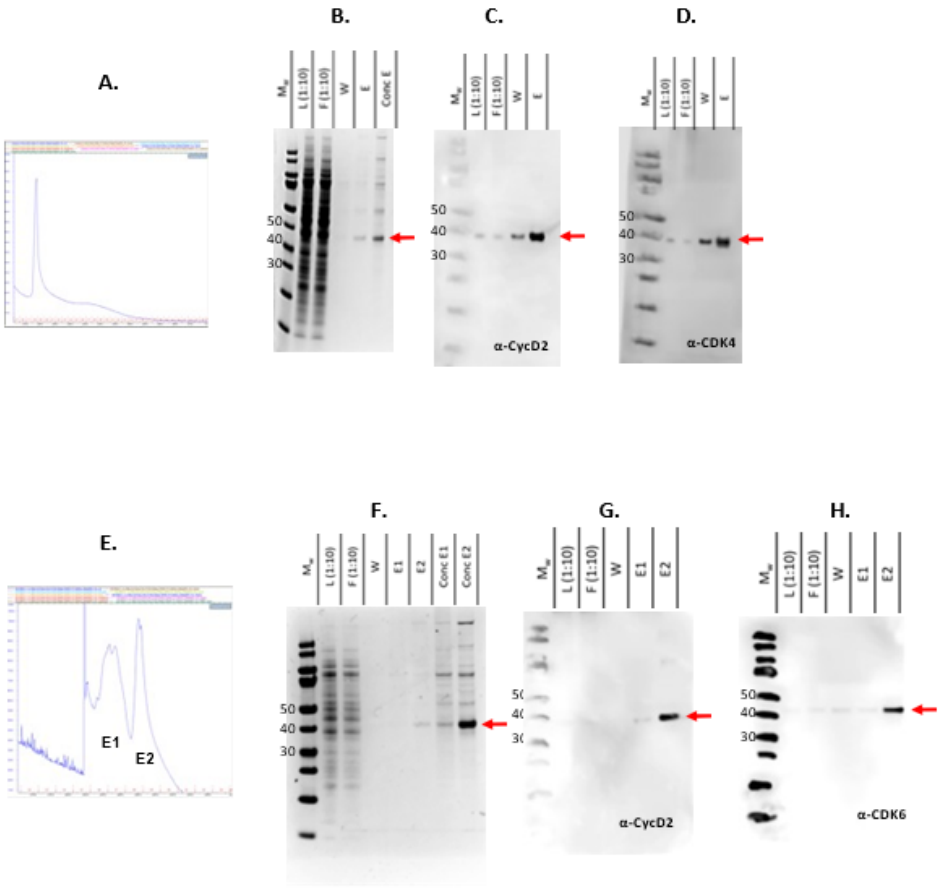

Figure 5

Purification of CDKs/CycD2 complexes: A, B, C, D) show CDK4/CycD2 and E, F, G, H) show CDK6/CycD2. A and E) Chromatograms of Strep-Trap column purifications, B and F) coomassie stains and C, D, G and H) are Western blots for the complexes' purifications using specific indicated antibodies. Mw is the molecular weight marker, L is the clarified lysate, F is the flow through, W is the wash and E is the column eluent, dilutions indicated to avoid overloading the gel.

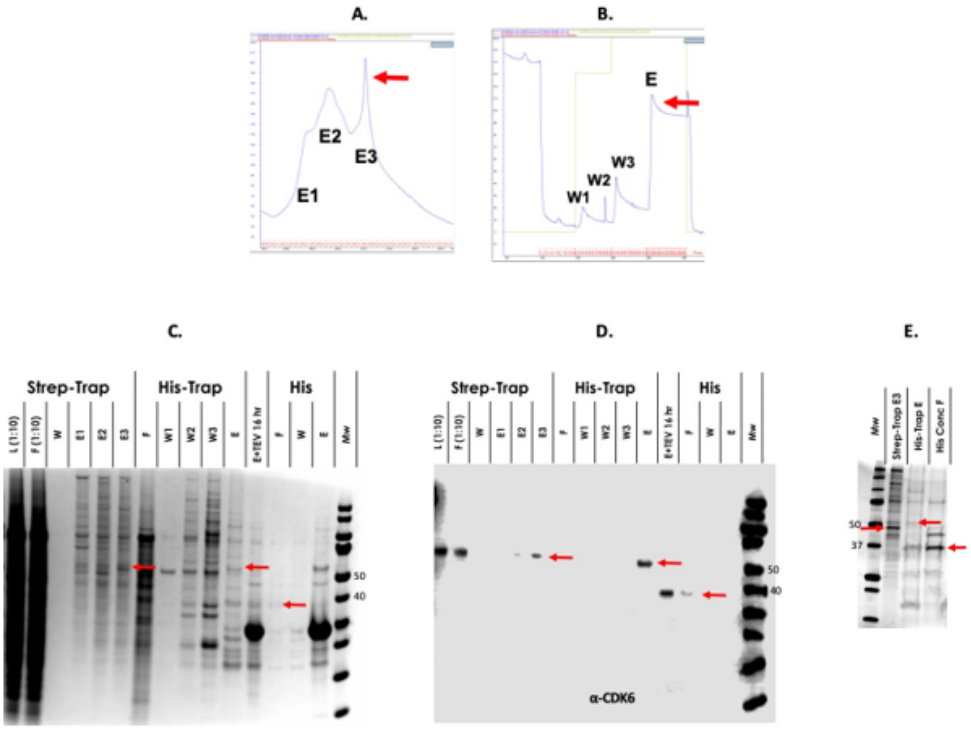

Figure 6

Purification of VLK-CDK6. A) Chromatogram for first step through Strep-Trap column, B) second step through His-Trap column, C) coomassie stain for the three steps including His resin D) Western blot for the same gel, E) concentrated elution from the three columns to show progression of purification. Mw is the molecular weight marker, L is the clarified lysate, F is the flow through, W1-3 are the progressive washs and E is the column eluent, dilutions indicated to avoid overloading the gel.

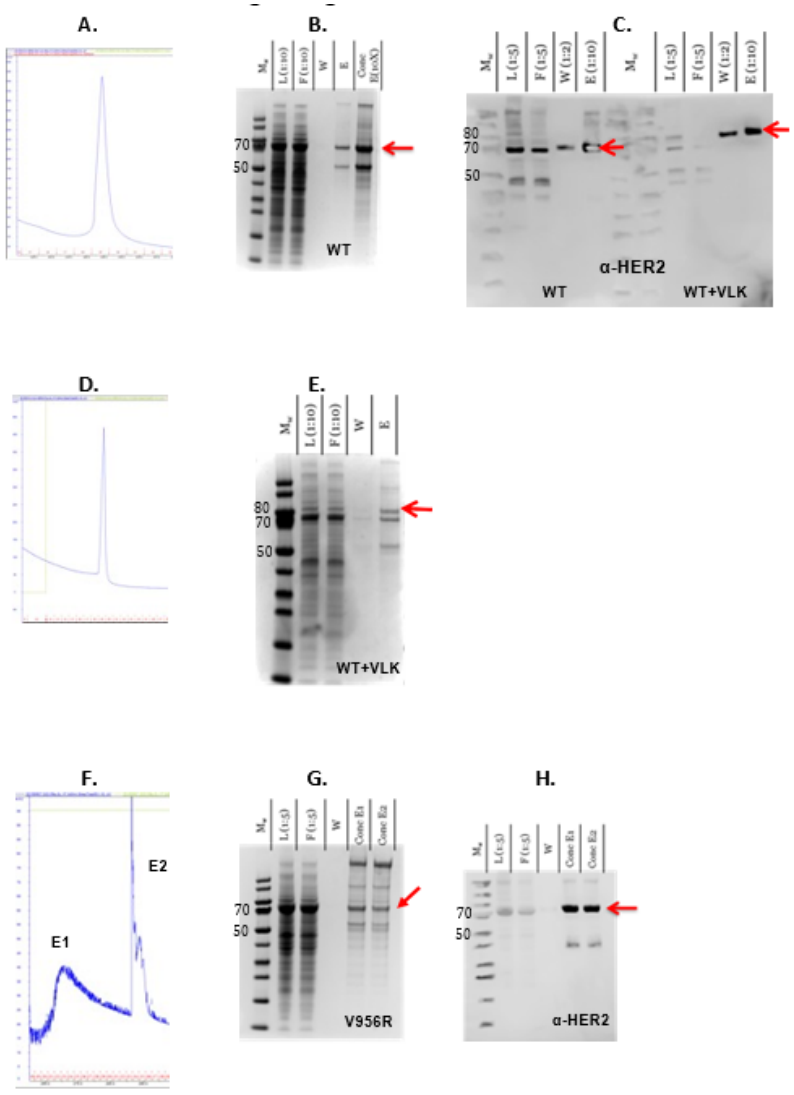

Figure 7

Purification of HER2 constructs: A, D and F) Chromatograms for Strep-Trap purification of WT, WT+VLK and V956R constructs, B, E and G) coomassie stains for indicated constructs, C) Western blot comparing the purification of the WT and WT+VLK, H) Western blot for the purification of V956R. Mw is the molecular weight marker, L is the clarified lysate, F is the flow through, W is the wash and E is the column eluent, dilutions indicated to avoid overloading the gel.

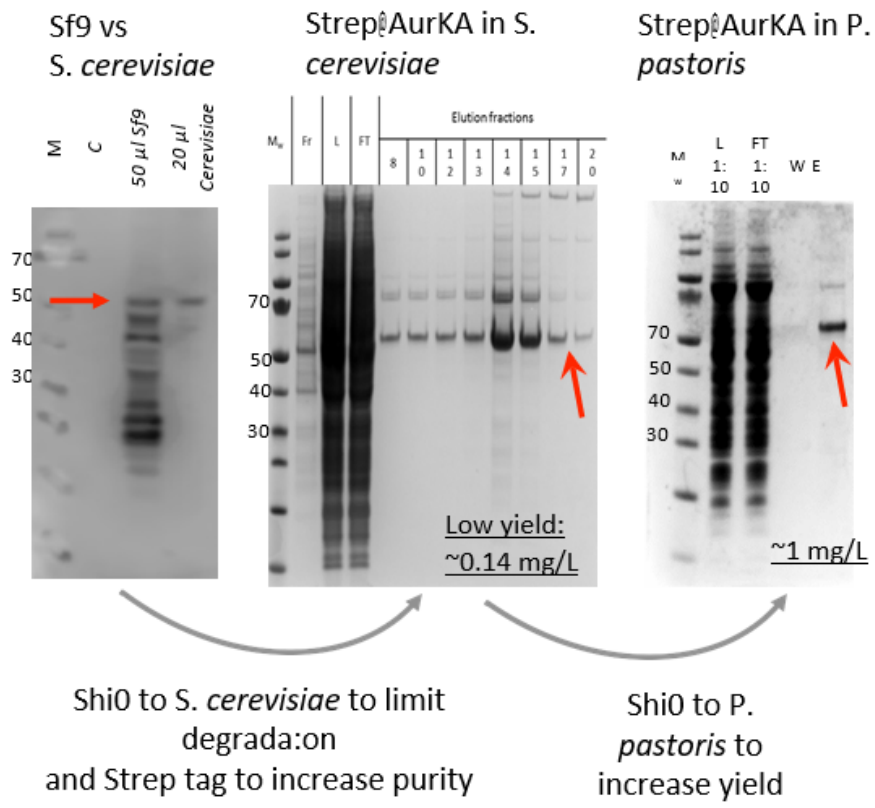

Figure 8

Comparison of AurKA expression in three different systems. A) Western blot for expression trial in SF9 insect cell versus S. cerevisiae, B and C) Coomassie stained gels comparing purification trials through Strep- Trap column under the same purification conditions for cell lysate from S. cerevisiae and P. pastoris, respectively. M is molecular weight marker, C is control, L is the clarified lysate loaded to the column, Fr is the filtrate from the concentrated lysate, FT is the flow through, W is the wash and E is the pooled column elution or its separate fractions.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTablesandFigures.pdf