Rapid Communication

A scalable insect cell-based production process of the human recombinant BMX for in-vitro covalent ligand high-throughput screening

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Abstract

Bone Marrow Tyrosine kinase in the chromosome X (BMX) is a TEC family kinase associated with numerous pathological pathways in cancer cells. Covalent inhibition of BMX activity holds promise as a therapeutic approach against cancer. To screen for potent and selective covalent BMX inhibitors, large quantities of highly pure BMX are normally required which is not a simple task with the currently available production and purification processes. Here, we developed a scalable production process for the human recombinant BMX (hrBMX) using the insect cells-baculovirus expression system. Comparable expression levels were obtained in small-scale shake flaks (13 mL) and in stirred-tank bioreactors (STB, 5 L). A 2-step chromatographic-based process was implemented, reducing purification times by 75 % when compared to traditional processes, while maintaining hrBMX stability. The final production yield was 24 mg of purified hrBMX per litter of cell culture, with a purity of >99%. Product quality was assessed and confirmed through a series of biochemical and biophysical assays, including circular dichroism and dynamic light scattering. Overall, the platform herein developed was capable of generating 100 mg purified hrBMX from 5 L STB in just 34 days, thus having potential to assist *in-vitro* covalent ligand high-throughput screening for BMX activity inhibition.

Key Words: hrBMX production, IC-BEVS, bioprocess development, hrBMX crystallization, cancer therapy

1. Introduction

Bone Marrow Tyrosine kinase in the chromosome X (BMX) is a member of the TEC family of non-receptor kinases [1] and plays an important role in a variety of critical physiological and pathological processes, including tumorigenicity, cells motility, adhesion, angiogenesis, proliferation, and differentiation [2–6]. Its involvement and integration in multiple and diverse cellular signalling pathways, has listed BMX as a potential therapeutic target for anti-cancer therapies, particularly for prostate cancer [3]. Compounds with selectivity against BMX are desirable, as these would offer many pharmacological advantages including selectivity for cancer cells and thus fewer side effects. [7,8]

The insect cell-baculovirus expression system (IC-BEVS) is a well-established platform for production of human recombinant proteins, including the TEC family kinases [9]. IC-BEVS has the advantage of being scalable, often resulting in short production times and high production yields [10]. Noteworthy, the ensuring proteins expressed in IC has many advantages as post-translational modification and protein stability, which cannot be achieved in other expression systems such as *E. coli*. [11]

IC-BEVS When undertaking protein expression in IC-BEVS, the best production condition is achieved by optimizing key parameters, such as multiplicity of infection (MOI), cell concentration at infection (CCI) and time of harvesting (TOH). [12] Purification of IC-derived proteins is challenging and should be carried in the minimum possible number of steps, increasing product recovery yields. Besides, recombinant protein expression can be engineered to contain affinity tags for chromatographic purification (e.g. N- or C-terminal HIS-tag) from their biological source; and for intracellularly expressed proteins, such as hrBMX [13], additional efficient cell-lysis and protein extraction methods should also be included. [14] To assist on process development and optimization, in particular, when the produced proteins are to be used in biophysical and structural studies (e.g., thermal shift assay, surface plasmon resonance, and X-

crystallography), it is essential to ensure protein sample quality and homogeneity. For this reason, it is crucial to perform a combination of complementary analytical methods, namely: SDS-PAGE, Western Blot (WB), Dynamic Light Scattering (DLS), and Circular Dichroism (CD) [15–17].

In this study, we describe (i) the optimal bioprocess conditions to produce hrBMX using IC-BEVS and stirred-tank bioreactors (STB), and (ii) the implementation of a 2-step chromatography-based strategy for the efficient purification of hrBMX. Homogeneity and chemical purity of purified hrBMX was confirmed by SDS-PAGE, WB, DLS and CD, meeting the standard for use in subsequent biophysical and structural studies.

2. Materials and Methods

2.1. Cell culture conditions

Spodoptera frugiperda derived Sf-9 cells (No. 89070101, ECCAC) were routinely cultivated in shake flasks every 3-4 days in SF900II SFM (Gibco) at 27°C and 100 rpm. Cell concentration, viability and size were estimated using Cedex HiRes Analyser (Roche Diagnostics).

2.2. Generation of recombinant baculovirus

The hrBMX gene was synthesized and assembled into pFastBac™ plasmid (Invitrogen) as described in Muckelbauer *et al.*, 2011. The correct transposition of the target BMX gene sequence was analysed by PCR, using primers specifically designed for the coding region of hrBMX catalytic domain 5′-GAGAACCTGTACTTCCAAGGC-3′ and 5′-TGTGGGCGGACAAAATAGTTG-3′. PCR product was evaluated by 1% agarose gel using 1 kb Plus DNA marker (ThermoFisher Scientific). Sf-9 cells were transfected with the recombinant bacmid using Cellfectin™ II reagent and Bac-to-Bac® Expression System (according to manufacturer instructions, Invitrogen) for generation of P0 recombinant baculovirus (rBAC). The ensuing rBac were sequentially amplified twice for generation of master virus stock P1 and P2 [18]. In both amplifications, rBac stocks were

harvested when cell viability was approximately 80% (corresponding to 96h post-infection, hpi), clarified by centrifugation (2 000 xg, 10 min) and stored at 4°C. Stocks were titrated by Cell Growth Cessation Assay and MTT techniques, as described in Roldão *el at.*, 2009.

2.3. Production of hrBMX in small-scale shake flasks

To determine the best conditions for hrBMX production, Sf-9 cells were infected at $1x10^6$ cells/mL in 13 mL shake flaks, using a MOI of 0.001, 0.01, 0.1 and 1 pfu/cell. Cultures were maintained until cell viability dropped below 50%. Samples were taken daily to assess cell growth and production kinetics. To assess intracellular hrBMX expression, cell pellets obtained after sample centrifugation (500 xg, 10 min) were loaded into a SDS-PAGE gel for analysis.

2.4. Production of hrBMX in stirred-tank bioreactors

The production of hrBMX was performed in a 5 L STB (Sartorius Stedim Biotech). The dissolved oxygen (DO) parameter was controlled at 30% automatically varying stirring rate (60-210 rpm) and temperature at 27ºC. Cells were seeded at a concentration of $0.6x10^6$ cells/mL, allowed to grow up to $1.0x10^6$ cell/mL and infected with MOI of 0.01 pfu/cell. Culture was harvested at 72 hpi, when cell viability reached 83%. Harvested bulk was centrifuged at 500 xg, for 15 min at 4ºC, and cell resuspended in five volumes of cold lysis buffer. Cells were disrupted by 3-consecutive cycles at 500 MPa using a high-pressure homogenizer (Avestin Inc.). The lysate was clarified by ultracentrifugation at 30 000 xg, for 30 min at 4ºC. The purification of the 6His-BMX protein (32.5 kDa) was carried out by loading the clarified lysate onto HisTrap™ FF column (GE Healthcare). Peak fractions were pooled and injected onto a HiLoad™ 10/300 Superdex™ 75 column (GE Healthcare). Collected fractions were concentrated to 10 mg/mL and stored at -80ºC. Buffer formulations were adapted from Muckelbauer *et al.,* 2011, with TRIS-base being used instead of HEPES. All purification steps were run at 4ºC.

2.5. Analytical methods

2.5.1. Conventional SDS-PAGE gel and Mn^{2+}-Phos-tag$_{TM}$ SDS PAGE

The electrophoresis was carried out according to Laemmli's method [19] and Kinoshita *et al.*, 2006 [20]. Gels consisted of 4.5% stacking and 12% separating gel. Capturing of hrBMX phosphorylation was conducted in normal SDS-PAGE gel by adding 50 μM of the Phos-tag$_{TM}$ ligand (FUJIFILM) and 1 mM of $MnCl_2$. Protein bands were visualized by Coomassie blue staining. Protein band intensity was determined using ImageJ (National Institutes of Health) and values were normalized with BSA band intensity (2 and 1 μg) to enable results analysis between gels.

2.5.2. Western Blotting (WB)

hrBMX samples were subjected to SDS-PAGE (4.5–12% gradient gels) and electroblotted to a PVDF membrane (Bio-Rad Laboratories), according to standard procedures [19,21]. After transferring, membranes were incubated with 6xHis-Tag mouse primary antibody at 1 μg/mL and with the goat anti-Mouse IgG (H+L) secondary antibody at 1:2000 (ThermoFisher Scientific).

2.5.3. Circular Dichroism

The CD spectra of the protein were acquired in a 195-300 nm wavelength range. Protein buffer exchange to PBS was carried out prior to CD. Samples were diluted to 0.25 mg/mL, experiments were made at 25ºC and the final spectrum was an average of three consecutive scans. A known denaturing concentration of TCEP (5mM) was also added to the sample to obtain the curve of denatured protein. Collected data was normalized by adjusting the values of ellipticity (θ) to molar concentrations.

2.5.4. Dynamic Light Scattering

Particle size characterization and detection of aggregates in purified samples was performed using samples diluted to a final concentration of 0.25 mg/mL. Measurements were run at 4ºC for 2 hours.

3. Results and Discussion

3.1. Optimization of infection conditions

A small-scale feasibility study was performed to determine the best MOI and TOH for hrBMX expression **(Fig. 1).** Our data shows that cell growth was inhibited after infection, more pronounced for the highest MOIs (0.1 and 1 pfu/cell) with peak concentration of 0.8-1.0x10^6 cell/mL at 24 hpi. Cells typically stop growing after infection [22] and this growth inhibition is related to the amount of virus infecting cell population, thus more pronounced for highest MOI. [12] A decrease in cell concentration and viability, together with an increase of 2-3 μm in cell size from 48 hpi on-wards, was observed for infected cultures when compared to non-infected control **(Fig.1, A-D)**. Interestingly, a peak in cell size of 19 μm was observed at 72 hpi, which corresponds to the maximum productivity of hrBMX. In fact, the correlation between cell size and protein expression has been described as a method to predict maximum productivity. [23] To quantify the amount of hBMX produced, MOI of 0.01 and 0.001 pfu/cell were analysed by WB (**Fig.1, E**). These conditions were selected based on (i) intracellular hrBMX expression (ii) cell viability higher than 80% at harvesting time (typical for intracellular recombinant protein expression). hrBMX expression was detected after 48 hpi, given that the expression of the foreign gene is driven by the *polh* promoter that is only transcribed during the late stages of infection (>18 hpi) [24]. The highest protein expression was observed at MOI of 0.01 pfu/cell at 72 hpi, which correlates with the literature [13].

3.2. Production and purification of hrBMX

The production of hrBMX was successfully scaled-up to STB, i.e. similar hrBMX production yields were obtained in shake-flask and STB **(Fig.1, F)**. IC-BEVS is being used in industrial settings for the production of several recombinant proteins [25], proving their easiness of used in small scale (optimization) and, later, scalability (production). Noteworthy, we reduced the overall number of steps and, consequently, total process time by 75% when compared to other purification processes [13] and

described for the first time a short purification process for hrBMX. Furthermore, based on buffer and additive screenings made prior to the starting of the experiments (data not shown), the buffering system was changed from HEPES to TRIS increasing hrBMX stability. Upon completion of the purification step, a yield 24.3 mg of purified protein per litre of culture was obtained.

3.3. Biochemical and biophysical characterization of hrBMX

Biochemical and biophysical characterization of protein samples prior to crystallization assays is essential to increase the likelihood of protein crystal formation [15]. Analysis of the native and Mn^{2+}-Phos-tag gels showed that hrBMX product obtained from IC is a mixture of non-phosphorylated and phosphorylated proteins **(Fig.2, E)**. Interestingly, we have reported the crystal formation of hrBMX, preferentially of the non-phosphorylated structure, which enabled to characterise at the atomic level the irreversible inhibition of the protein (Seixas and Sousa *et al*., submitted). DLS measurement showed a correlation of function with a typical curve for monodisperse samples, although some degree of aggregation was detected overtime **(Fig.2, F)**. This may account for the mutagenesis strategy we used, which consisted on the replacement of non-conserved residues by other residues that mimicked the crystal contacts of BTK, with the goal of creating packing opportunities for BMX crystal formation [13]. CD experiments **(Fig.2, G)** showed a pure protein in its native state, which was then ready for crystallization trials. The spectrum displays a curve typical of proteins that have a predominance of α-helices in their composition [17], which is in good agreement with the published structural information (PDB: 3SXR and 3SXS).

Altogether, our data demonstrated the successful development of a new bioprocess for hrBMX production in IC-BEVS **(Fig.3)**. Noteworthy, we were able to determine the first crystal structure of a covalently inhibited hrBMX. These new ligands are currently under investigation for BMX inhibition both in cell-based *in-vitro* and *in-vivo* experiments (Seixas and Sousa *et al*., submitted).

4. Conclusions

This report describes optimal conditions to produce hrBMX in STB using the IC-BEVS and proposes a rapid and efficient purification protocol to assure high product quality. It also provides substantial information regarding the biochemical and biophysical characterization of hrBMX that could be used as future guidelines for follow-up studies. In particular, we expect this new protocol to efficiently produce high purity hrBMX to accelerate the study of hrBMX inhibition. In turn, the new knowledge generated will guide the design of ligands with improved potency and selectivity.

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Figures captions

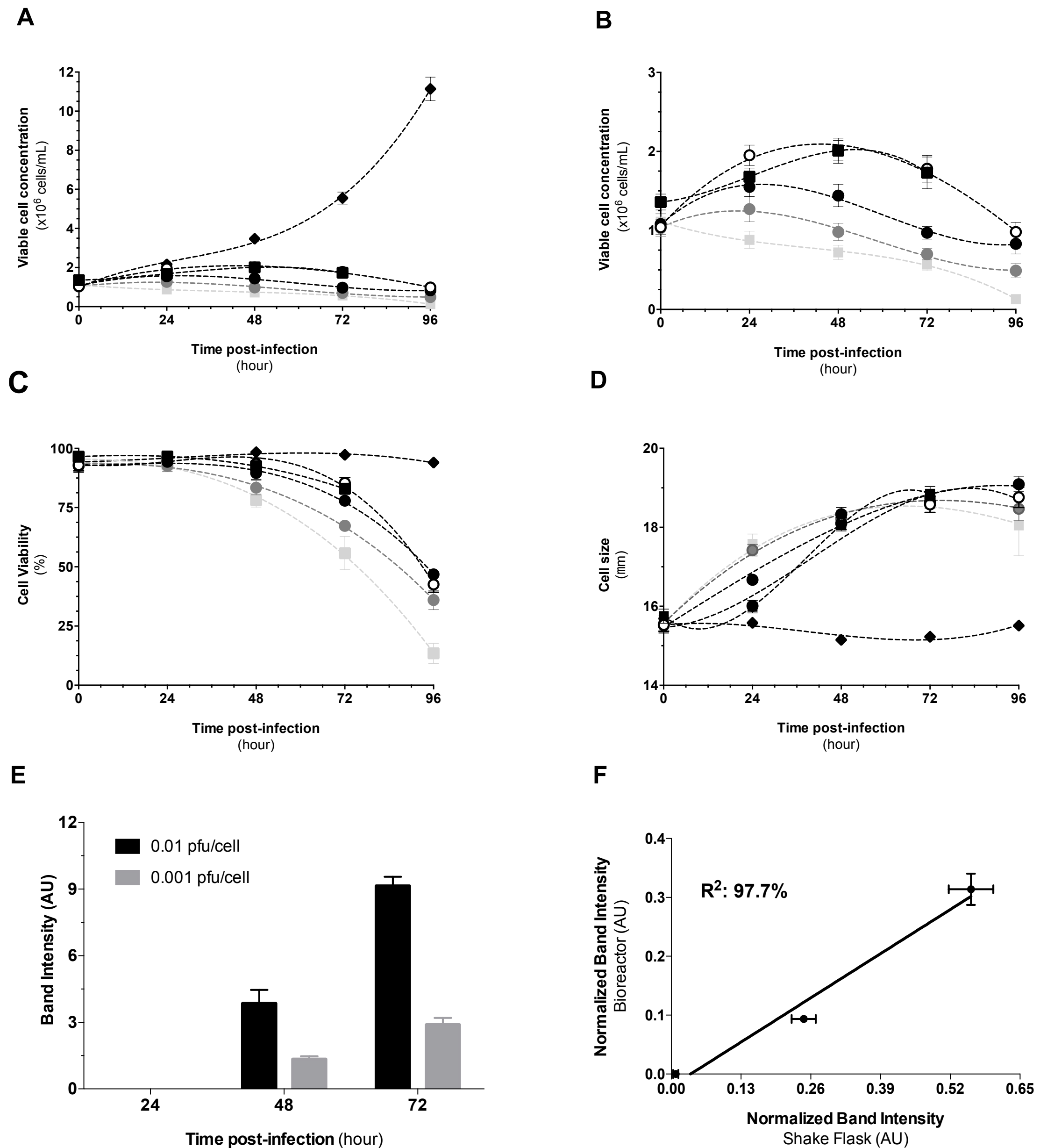

Fig.1 - Production and product quantification of hrBMX. Cell growth kinetics after infection: viable cell concentration **(A)**, insight of viable cell concentration graph **(B)**, percentage of cell viability **(C)**, cell size **(D)**. CTL – control culture (i.e. without infection) (black diamond). MOI of 0.001 pfu/cell (black square), MOI of 0.001 pfu/cell in shake flask (white circle), MOI of 0.01 pfu/cell in shake (black circle), MOI of 0.1 pfu/cell in shake (dark grey circle) and MOI of 1 pfu/cell in shake (light grey square). **(E)** Product quantification from WB band intensity. **(F)** Product quantification from SDS-PAGE band intensity, normalized with BSA band intensity values (1µg). Data are mean ± standard

deviation obtained from at least three independent measurements (n=3). Lines represent the tendency of the experimental data.

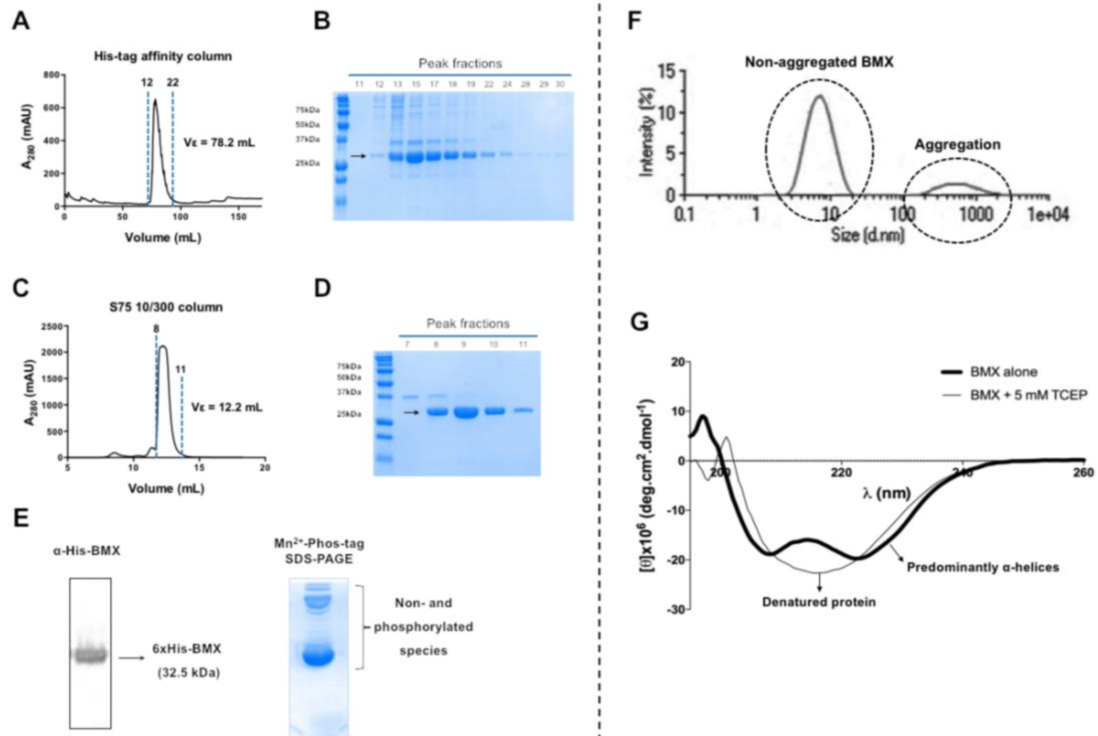

Fig.2 - Purification and characterization of the purified hrBMX. (A) His-tag chromatography. **(B)** SDS-PAGE gel analysis of the collected fractions from the nickel column. **(C)** Size-exclusion and **(D)** SDS-PAGE analysis of the collected fractions. **(E)** WB and Mn^{2+}-Phos-tag SDS gel of the final product. **(F)** DLS spectrum of hrBMX, where the relative intensity (%) is displayed as a function of the diameter of the protein particle. **(G)** CD spectrum of hrBMX alone (native state) and with 5 mM TCEP (denatured protein).

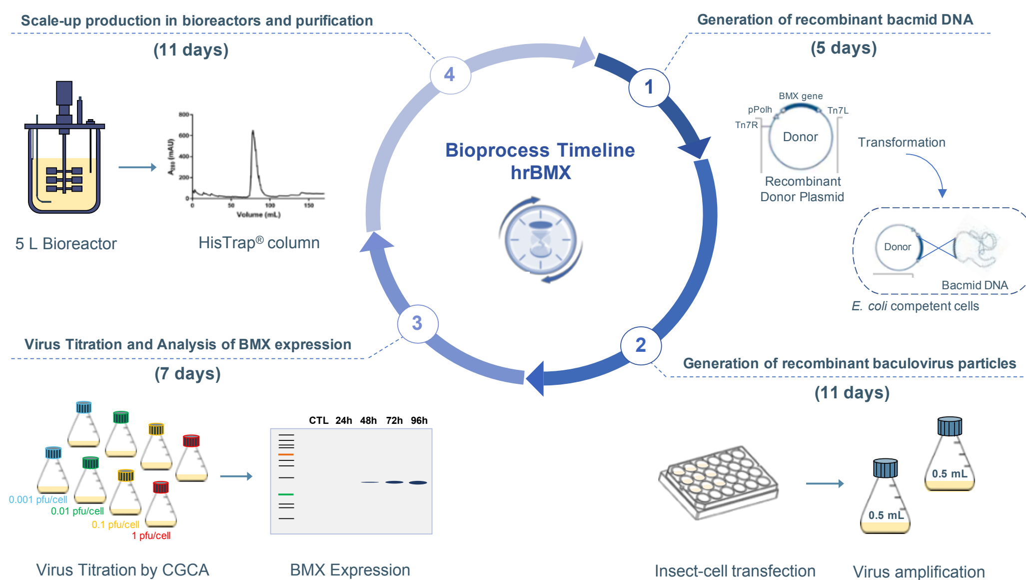

Fig.3 - Bioprocess workflow of the human recombinant BMX. The hrBMX gene was cloned into a baculovirus shuffle vector in competent *E. coli* cells and expression of the target protein was carried out by transfecting Sf-9 insect cells to produce stock recombinant baculovirus particles. Viral stock concentration was determined by the CGCA and MTT titration methods, and viruses were used to infect insect cells in stirred-tank bioreactors for large-scale BMX production.