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An improved purification method for the lysosomal storage disease protein β-glucuronidase produced in CHO cells

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ABSTRACT

Human β-glucuronidase (GUS; EC 3.2.1.31) is a lysosomal enzyme that catalyzes the hydrolysis of β-D-glucuronic acid residues from the non-reducing termini of glycosaminoglycans. Impairment in GUS function leads to the metabolic disorder mucopolysaccharidosis type VII, also known as Sly syndrome. We produced GUS from a CHO cell line grown in suspension in a 15 L perfused bioreactor and developed a three step purification procedure that yields ~99% pure enzyme with a recovery of more than 40%. The method can be completed in two days and has the potential to be integrated into a continuous manufacturing scheme.

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1. Introduction

Lysosomal storage diseases (LSDs) are a diverse group of more than 50 genetic diseases of metabolism [1] affecting approximately one in 7700 live births [2], characterized by lysosomal accumulation of macromolecules such as mucopolysaccharides, glycogen, and glycosphingolipids [3,4]. Several enzyme replacement therapies (ERTs), a class of therapeutic proteins used to treat patients in whom a particular enzyme is deficient, are currently FDA-approved for specific LSDs. A major hurdle in the manufacturing of ERTs like any other biologic drug is the cost of producing and purifying the therapeutic protein, which in turn leads to an exorbitant cost for patients [5]. Thus, there is a need for techniques to increase production and improve recovery throughout downstream processing.

Here we investigated human β-glucuronidase (GUS), a lysosomal enzyme that is essential for the clearance of glycosaminoglycans, and whose impairment leads to the LSD mucopolysaccharidosis type VII, also known as Sly syndrome [6]. The published method for purification of GUS produced in Chinese hamster ovary (CHO) cells involves four chromatography steps: blue sepharose, phenyl sepharose, DEAE anion exchange, and CM sepharose [7]. In our experience, each step resulted in loss of enzyme of approximately 30–70%, leading to an average total process recovery of 5–10%.

In these studies the objective is to improve the purification method for GUS and to investigate the effect on GUS quality. Since the internal pH of the lysosome is between 4.5 and 5.0, and GUS has an observed pH optimum of 4.0 [8,9], we hypothesized that reducing the pH below 5.0 may destabilize and precipitate contaminating proteins, while leaving GUS unaffected. By incorporating the pH precipitation we are able to decrease the number of steps and time in the purification scheme of GUS compared to the previous method and demonstrate that precipitation of contaminating proteins through manipulation of pH is a suitable starting point for purification of GUS.

2. Materials and methods

2.1. Cell culture conditions and production of GUS

GUS was produced over a 14 day run of a PBS15 Vertical-

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Wheel™ bioreactor (PBS Biotech, Inc., Camarillo, CA), in which GUS-secreting CHO cells were cultured in Ex-Cell® 325 PF CHO serum-free chemically defined medium (Sigma-Aldrich, St Louis, MO) with 6 mM glutamine, 1 mM pyruvate, 100 U/mL Penicillin and 100 μg/mL Streptomycin. The bioreactor was inoculated on Day zero at ~0.26 million cells/mL and operated in fed-batch mode for the first 6 days (37 °C, 50% dissolved oxygen and pH 7.0 maintained with a bicarbonate buffer). Samples were monitored daily for viable cell density, nutrient concentrations, total protein concentration and GUS activity. Glucose, glutamine, glutamate, lactate, ammonium, sodium, potassium, pH, PO_2, PCO_2, osmolality and viable cell density (VCD) were measured twice daily using a Bioprofile FLEX Analyzer (Nova Biomedical, Waltham, MA). Perfusion was started on day 6 using an XCell™ ATF 4 (Repligen, Waltham, MA) at a VCD of 2.5 million cells/mL. Bioreactor was harvested on day 14 that coincided with the highest concentration of measured GUS activity. Harvested medium was centrifuged (4000 × g, 15 min) to remove the cells and stored at −20 °C before use in the purifications and analyses.

2.2. Purification method A

This method is an adaptation of a previously published method [7] and serves as a comparison to the improved Method B. Purification Method A consists of consecutive chromatography on blue sepharose, phenyl sepharose and DEAE sepharose columns. One liter of culture medium containing GUS was concentrated to 60 mL using an Äkta Flux s (GE Healthcare Life Sciences, Marlborough, MA) using a hollow fiber ultrafiltration cartridge (Xampler Laboratory Membrane, 50,000 NMWC pore size, 1400 cm^2 membrane area, 0.5 mm fiber i.d., 66.7 cm cartridge length). It was buffer-exchanged with 2 L of 20 mM sodium phosphate buffer, 150 mM NaCl, pH 5.5. The sample was loaded onto a blue sepharose 6 Fast Flow column (18 mL bed volume; resin from GE Healthcare Life Sciences) for GUS affinity capture. This column chromatography was performed using a programmable peristaltic pump. The column was pre-equilibrated with exchange buffer and washed with the same buffer for 10 column volumes (CV). GUS was eluted using a pH 7.5 buffer (10 mM sodium phosphate, 800 mM NaCl) at a flow rate of 1 mL/min 10 mL fractions were collected. The fractions were analyzed for total protein content and GUS activity as described in methods below and those determined to contain GUS were pooled.

The pooled fractions were buffer-exchanged (5X; 10 mM sodium phosphate buffer, 1 M NaCl, pH 8.0) in Amicon® Ultra 15 mL centrifugal filters (NMWCO 100 kDa; Millipore, Billerica, MA). The sample was loaded (1 mL/min) onto a HiPrep Phenyl FF (High Sub) 16/10 (20 mL) hydrophobic interaction chromatography column (GE Healthcare Life Sciences) pre-equilibrated with exchanged buffer. The column was washed with the same buffer (2 CV) and GUS was eluted in three steps using an elution buffer (10 mM Tris-HCl, 1 mM β-glycerophosphate disodium salt, pH 8.0): (a) 0–60% gradient for 3 CV; (b) 60–100% gradient for 3 CV and (c) 100% elution buffer for 4 CV. 10 mL fractions were collected, and those containing GUS activity were pooled. This second and subsequent column chromatography steps were carried out with an Äkta avant 150 chromatography system (GE Healthcare Life Sciences).

The pooled GUS fractions were buffer-exchanged (3X, 10 mM Tris-HCl, 1 mM β-glycerophosphate, pH 8.0) as stated above. The GUS sample was then loaded (0.5 mL/min) onto a HiTrap DEAE Sepharose FF (16 × 25 mm) weak anion exchange chromatography column (GE Healthcare Life Sciences) pre-equilibrated with the exchange buffer and washed with the same buffer (2 CV). A gradient elution of 0–100% was performed (4 CV; 0.5 mL/min; 10 mM Tris-HCl, 400 mM NaCl, 1 mM β-glycerophosphate, pH 8.0), followed by a step elution of 100% eluent (6 CV; 5 mL fractions). Fractions containing GUS activity that appeared more than 98% pure by an SDS-PAGE were concentrated, pooled, buffer-exchanged (if necessary for analyses), aliquoted, and stored at −20 °C.

2.3. Assessment of protein precipitation by decreasing pH

Bioreactor samples containing secreted GUS were initially concentrated 10-fold and 200 μL of these samples were diluted 5-fold into either acetate or citrate buffers (final concentration 160 mM, pH range 4.2–5.0). Samples (n = 3) were incubated for either 1 h or 20 h at 4 °C. Precipitated proteins were separated by centrifugation (10,000 × g, 5 min). Enzyme activity and protein concentrations were determined for the supernatant as described under GUS activity assay and protein quantification, respectively. Recovery was calculated as percent of control (1X phosphate-buffered saline, pH 7.4) in which there was no measurable protein precipitation. Purification factor is defined as the ratio of specific activity of each sample to that of the control. In order to determine the optimal pH for precipitation of contaminating proteins from the GUS supernatant, another experiment was run similarly (n = 3, 1 h incubation at 4 °C) in which the pH range was extended down to pH 3.0 in citrate buffer.

2.4. Purification method B

One liter of the stored bioreactor harvest was pH adjusted from 7.0 to 4.2 using 200 mM citrate buffer, resulting in a final concentration of 50 mM citrate buffer in approximately 1.3 L. The medium was incubated for 1 h at 4 °C and centrifuged (3500 × g, 5 min). The supernatant was loaded (5 mL/min) onto a blue sepharose column (described in Purification Method A, run using a peristaltic pump) pre-equilibrated with 50 mM citrate buffer, pH 4.2. The column was washed with equilibration buffer (3 CV; 5 mL/min) and GUS was eluted using a pH 8.0 buffer (20 mM sodium phosphate buffer, 1 M NaCl, 10% glycerol;10 mL fractions). Fractions with GUS activity were pooled.

The pooled fractions were buffer-exchanged (5X; 20 mM sodium phosphate buffer, 1 M NaCl, 10% glycerol, pH 8.0) as described above and loaded onto a hydrophobic column (HiPrep Phenyl FF (High Sub) 16/10 (20 mL) GE Healthcare Life Sciences) pre-equilibrated (1 mL/min) with exchanged buffer. Chromatography was performed on an Äkta avant 150 chromatography system. The column was then washed with the equilibration buffer (2 CV; 1.5 mL/min) and GUS was eluted in three steps (10 mM Tris-HCl, 1 mM β-glycerophosphate disodium salt, 10% glycerol, pH 8.0): (a) 0–80% gradient for 1.5 CV; (b) 80–100% gradient for 2.5 CV and (c) 100% elution buffer for 4.5 CV. Fractions (10 mL) containing GUS activity that appeared > 98% pure by SDS-PAGE were concentrated, pooled, buffer-exchanged (if necessary), aliquoted, and stored at −20 °C.

2.5. GUS activity assay

GUS activity was measured using a high throughput assay at 25 °C with PNPG substrate as described before [10]. The formation of *para*-nitrophenol (PNP) was measured at 405 nm in a Synergy H1 Multi-Mode Reader (BioTek Instruments, Inc., Winooski, VT). The assay was performed by adding 20 μL of sample into the well and then 180 μL of 20 mM sodium phosphate buffer, pH 7.4, containing 1 mM PNPG and 10 mM β-mercaptoethanol. Samples containing high concentrations of low pH buffer were assayed in 500 mM phosphate buffer, to sufficiently buffer the assay mixture at pH 7.4. This was necessary since the GUS activity was calculated using the molar extinction coefficient of PNP, which is 9000 M^{-1} at pH 7.4 [11]. An increase in sodium phosphate up to 500 mM did not affect