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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.

Keywords

CHO; β-Glucuronidase; Enzymes; Purification; Precipitation; Bioreactor

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,

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Declaration

We declare that there are no actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations.

Contributions

EJFB performed the experiments and wrote the manuscript; SAK performed experiments, wrote manuscript; HP performed experiments; MA wrote the manuscript, CNM conceptualized the work, performed experiments and wrote the manuscript.

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-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 (5×; 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 (3×, 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 (1× 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 (5×; 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 the GUS activity or measured absorbance values (data not shown).

2.6. Protein quantification

Protein was quantified using Pierce™ Coomassie Plus (Bradford) Assay Kit (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. Assays were performed with bovine serum albumin (BSA) standards in a 96-well plate (clear bottom black plate; Corning) using a Synergy H1 Multi-Mode plate reader (BioTek Instruments, Inc.).

2.7. SDS-PAGE

The proteins were separated by SDS–PAGE on an 18 well 4–20% Criterion™ TGX™ Precast Midi Protein Gel (Bio-Rad, Hercules, CA) under reducing conditions and stained with Coomassie blue (PageBlue™ Protein Staining Solution, Thermo Scientific). Gels were imaged using an E-Gel® Imager system with an E-Gel® Imager Blue Light Base, an E-Gel®

Imager White-Light Conversion Screen, and an E-Gel® Imager Universal Filter (Invitrogen, Carlsbad, CA).

2.8. Capillary electrophoresis (CE)

CE was performed on a Beckman Coulter PA800 Plus pharmaceutical analysis system (Brea, CA) using manufacturer supplied assay kits, according to the protocols prescribed by the vendor. Purified GUS samples were analyzed by CE-SDS (capillary electrophoresis-sodium dodecyl sulfate) under both reducing and non-reducing conditions. In sample buffers, a 10 kDa internal standard was used. Under reducing conditions, BSA (0.14 μg/μL) was also used as an internal reference protein for quantification purposes. Capillary isoelectric focusing (cIEF) was performed with 3 M urea-cIEF gel sample buffer with internal pI (isoelectric point) standards of 5.5 and 9.5. This sample buffer was determined to be optimal to identify minor peaks. Five peptide standards of pI 4.1, 5.5, 7.0, 9.5 and 10 were also run using the same sample buffer to determine system suitability. GUS pI was calculated from a linear standard curve obtained from the migration times of pI standards from multiple runs that gave sufficient data points (n = 16).

2.9. Quantification of M6P level using Phos-Tag™ dye

Purified GUS samples from both methods that were quantified by capillary electrophoresis were used for estimating M6P levels by an SDS-PAGE (4–20% Criterion™ TGX™ Precast Midi Protein Gel, Bio-Rad, Hercules, CA) based phosphate staining method and a 96-well plate method (by immobilizing samples with 2% agarose) using sequential staining of Phos-Tag™Phosphoprotein Gel Stain and eLuminol™ total protein stain (GeneCopoeia™, Rockville, MD), as per supplier's protocol. In either the SDS-PAGE or 96-well plate method samples were stained with Phos-Tag™, imaged, destained and then stained with eLuminol™ and imaged again. Imaging of SDS-PAGE was performed with an E-Gel imager system (Phos-Tag™: UV light base; eLuminol™: blue light base) and quantified with GelQuant Express (Invitrogen). SDS-Polyacrylamide gel was subsequently stained with PageBlue™ and imaged with the E-Gel Imager system (blue light base with white light adaptor) to determine the size shift due to dephosphorylation and/or deglycosylation after treatment with Alkaline Phosphatase (AP; Sigma-Aldrich, St. Louis, MO) and/or PNGase-F (PNG; New England Biolabs, Ipswich, MA). These treatments (10 μg of sample protein) were also used to ascertain the specificity of the Phos-Tag™ stain and to ascertain whether the phosphate moiety was present on glycans or the sidechains of the amino acid residues. Measurements of the Phos-Tag™ and eLuminol™ signals in the 96-well plate were made in Synergy H1 Plate reader (Phos-Tag™ Ex/Em: 550/580 nm; eLuminol ™ Ex/Em: 460/600 nm) and these methods are described in more detail elsewhere (Ketcham et al., 2017 [12]). Ovalbumin is known to have two of its residues phosphorylated [13] and, therefore, determination of the phosphate content for both methods was performed by comparing the Phos-Tag™ and eLuminol ™ fluorescence intensities to the intensities of ovalbumin standards (1–15 μg), which allowed quantification of the amount of phosphoprotein and total protein. The amount of phosphoprotein was divided by the total protein to yield the average number of phosphosites per molecule, which is M6P in the case of GUS.

3. Results and discussion

3.1. Purification of GUS with method A results in low yield

GUS was purified from 1 L of culture medium harvested from the final day (day 14) of a 15 L bioreactor culture of GUS-secreting CHO cells. During the first step, the medium was concentrated and buffer-exchanged, which did not change the purity but resulted in a loss of over 30% of GUS (Table 1). The second step on the blue sepharose column also did not increase purity and again resulted in additional loss of GUS activity, despite no detectable loss in total protein. The subsequent step on phenyl sepharose (Fig. 1A) increased the purity by 2.5-fold of that of the initial culture medium but also resulted in a more than 50% loss in GUS activity. The final step on DEAE sepharose (Fig. 1B) resulted in GUS that was estimated to be 99% pure by an SDS-PAGE analysis (Fig. 2), but the final yield was only 6.7%. Hence, the yield was only 2 mg of GUS from 1 L of culture medium (Table 1), a very low yield for a therapeutic enzyme that needs to be manufactured in gram quantities to keep the cost low.

3.2. Precipitation of contaminants is optimal in pH 4.2 citrate buffer

We initially tested acetate and citrate buffers, pH values between 4.2 and 5.0, and incubation times of either 1 h or 20 h (Table 2). GUS precipitated in acetate buffer with decreasing pH, whereas GUS was recovered almost entirely in all citrate buffer samples, irrespective of pH. Additionally, there was no increase in purification factor when incubation time was increased from 1 h to 20 h for either buffer composition. These results led us to choose citrate buffer and 1 h incubations for further experiments at narrow pH intervals in a range of 3.0–5.0. GUS recovery in the supernatant was 100% for the pH range of 4.2–5.0, and gradually decreased from pH 4.0 to 3.0 (Fig. 3). The purification factor increased as pH was decreased from 5.0 to 4.2, and decreased as pH values were further reduced (Fig. 3). Thus, citrate buffer at pH 4.2 was selected as suitable for first step protein precipitation in Method B purification.

3.3. Purification of GUS with method B results in high purity and increased yield

GUS was purified using Method B from 14th day bioreactor harvest three separate times using 1 L medium each time and the results were averaged (Table 3). During the first step, contaminating proteins were precipitated by reducing the pH of the culture medium to 4.2 using 200 mM citrate buffer. This initial step resulted in a purification fold increase to 2.29 with only 1.7% loss on an average. The culture medium was then loaded onto a blue sepharose column which resulted in a 33% loss but a further purification fold increase to 3.74. In the final step, a pool of the GUS-containing fractions from the blue sepharose column was purified on a phenyl-sepharose column (Fig. 4) resulting in 99% pure GUS based on an SDS-PAGE analysis (Fig. 5). Overall an average GUS recovery of 41.8% obtained from Method B represents a significant improvement compared to 6.7% that was obtained using Method A. These results demonstrate that: (a) pH precipitation of contaminating proteins is an excellent method to enrich GUS in the culture medium without losing activity; (b) incorporating pH precipitation increases overall recovery and (c) in subsequent chromatography steps life of the chromatography resins may be extended due to decreased contaminating proteins.

Overall summary of the two methods of GUS purification is provided in Fig. 6. The flow diagram of the two purification schemes enlists the steps followed and the percentage of recovered GUS activity compared to the starting bioreactor harvest used in purification process. It is apparent that incorporating pH precipitation as a first step in Method B provided superior recovery at every key step that translated into better overall recovery and significantly reduced process time. In terms of GUS purity achieved, Method B was either comparable to Method A or better (Figs. 2 and 4), which was further investigated by capillary electrophoresis (CE) analyses as described below.

3.4. Characterization of purified GUS by CE analyses

Capillary electrophoresis is commonly employed for the analyses of therapeutic proteins as it provides more sensitive and quantitatively superior analysis of proteins compared to SDS-PAGE. Here we have performed CE analyses and compared the GUS samples purified by the two methods to investigate if inclusion of pH precipitation step in Method B has adversely affected the overall quality of the protein (Fig. 7). CE-SDS performed under reducing conditions showed that GUS migrates much later than BSA with an apparent size slightly more than 100 kDa, with qualitatively no significant difference between the GUS samples purified by the two methods (Fig. 7A). These data are in agreement with previous observations and corroborate that glycosylation of GUS renders it larger leading to longer migration time than non-glycosylated proteins of similar size [10]. Analysis of CE-SDS under non-reducing conditions showed that GUS samples purified by both methods have monomers and dimers, indicating the occurrence of inter-molecular disulfide bonds (Fig. 7B) which corroborates a previous report [9]. However, GUS purified by the improved Method B showed noticeably lower amounts of dimer (~30%) relative to the monomer. Arguably, including pH precipitation step not only decreased the overall purification time but also has the potential to improve the quality of the protein as seen with lower dimer content in the GUS sample purified by Method B.

Capillary isoelectric focusing (cIEF) was performed on the GUS samples purified by the two methods to investigate if the charge variants were significantly different in these samples. GUS migration pattern in cIEF demonstrated an apparent, relatively broad pI corresponding to 5.75–6.8 (Fig. 7C). Significant differences were not apparent between the GUS samples purified by the two methods based on their cIEF profiles.

3.5. Assessment of relative amounts of GUS M6P levels

Lysosomal enzymes are targeted via M6P-receptors [14] and, therefore, for the efficacy and potency of a lysosomal storage disease ERT product the level of M6P is a critical quality attribute (CQA). Typically, M6P levels can be assessed by either mass spectrometry [15] or by an anion exchange chromatography method that requires a pulsed amperometric detector [16]. Either of these approaches involves processing of the protein samples by multiples steps before quantification. Here we adopted Phos-Tag™, a phosphate specific binding dye [17] to assess the M6P content along with eLuminol™, a protein specific binding dye to assess the total protein content in an SDS-PAGE method that does not require pre-processing of the sample proteins. Deglycosylation with PNGasae-F or dephosphorylation with alkaline phosphatase of GUS samples eliminated Phos-Tag™ signal with or without a measurable

size shift, respectively (Fig. 8). Ovalbumin standard with its two phosphorylated amino acid residues [13] served to quantify the relative levels of M6P signal. As ovalbumin is a glycosylated protein without M6P, deglycosylation did not abolish the Phos-Tag™ signal but resulted in a measurable size shift. Dephosphorylation of Ovalbumin with alkaline phosphatase however abolished the Phos-Tag™signal without a noticeable shift in size. With this approach it was thus possible to identify if the phosphate moiety was located on the glycan or on the sidechains of amino acid residues. Thus, it was ascertained that the Phos-Tag™ signal indeed reported the M6P level of GUS and quantification was possible using Ovalbumin standards (Ketcham et al., 2017; 12). It was also possible to immobilize the purified GUS samples with 2% agarose in a 96-well plate, then sequentially stain and destain with Phos-Tag™ and eLuminol dyes and corresponding fluorescence signals measured in a plate reader. By these two methods we assessed the M6P levels to be comparable at around 1.7–1.9 moL/mol protein in the GUS samples purified by the two purification methods (Fig. 8) and found that the improved purification method B appears not to affect the CQA of the lysosomal enzyme GUS.

4. Conclusions

Here we describe an improved approach for the purification of GUS that decreases the time to less than half compared to the previously used protocol with vastly improved recovery and a comparable purification fold. This method begins with a pH adjustment of the cell-free culture medium containing GUS followed by 1 h incubation and a 5 min centrifugation to pellet down contaminating proteins. The clarified culture medium containing GUS at pH 4.2 is then purified on a blue sepharose column followed by a phenyl sepharose column to yield highly pure and functional GUS. Purity of the GUS samples from both methods is comparable by CE-SDS analysis under reducing conditions. However, CE-SDS analysis under non-reducing conditions showed that the relative amounts of dimers are less in GUS purified by the new improved method. This indicates that possibly pH treatment and thus decreasing process time reduce the unfavorable intermolecular disulfide linkages. Capillary IEF profiles of GUS samples showed comparable pI in the range of 5.75–6.8. The M6P levels are found to be comparable in the GUS samples purified by both methods to be around 1.7–1.9 moL/mol determined using a combination of phosphate specific and protein specific dyes. This purification method may be more broadly applicable to other LSD enzymes that share similar biochemical characteristics and critical quality attributes.

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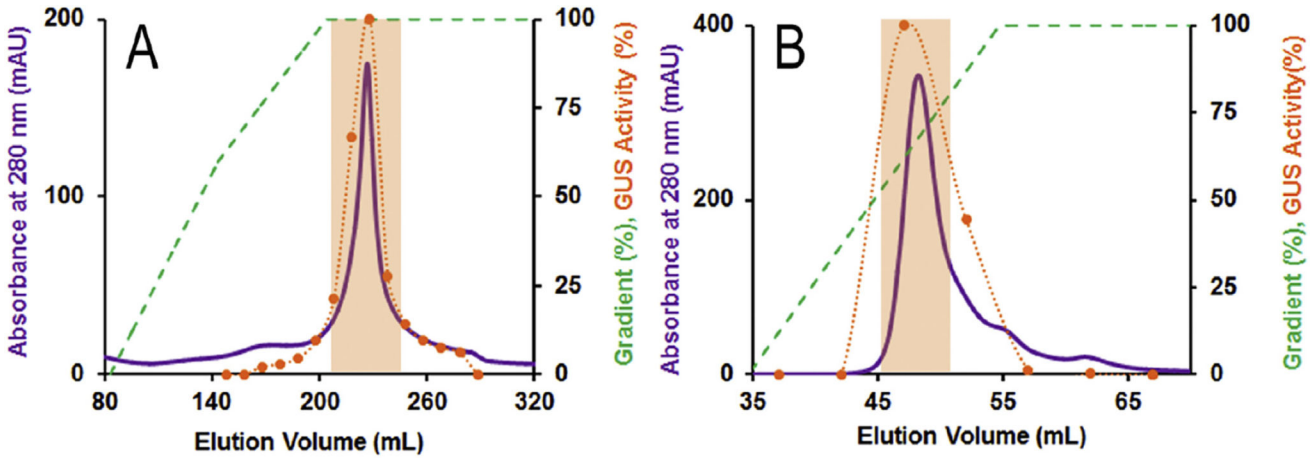

Fig. 1. Chromatographic elution profiles of phenyl sepharose and DEAE columns for Method A

A_{280} traces are shown in purple, GUS activity values for each fraction are shown as orange points as percentages of the fraction with the highest activity, and the percentage of gradient in green broken line. The portion of the A_{280} trace highlighted in the orange box represents the pooled fractions. (A) Phenyl sepharose column elution profile. (B) DEAE column elution profile. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

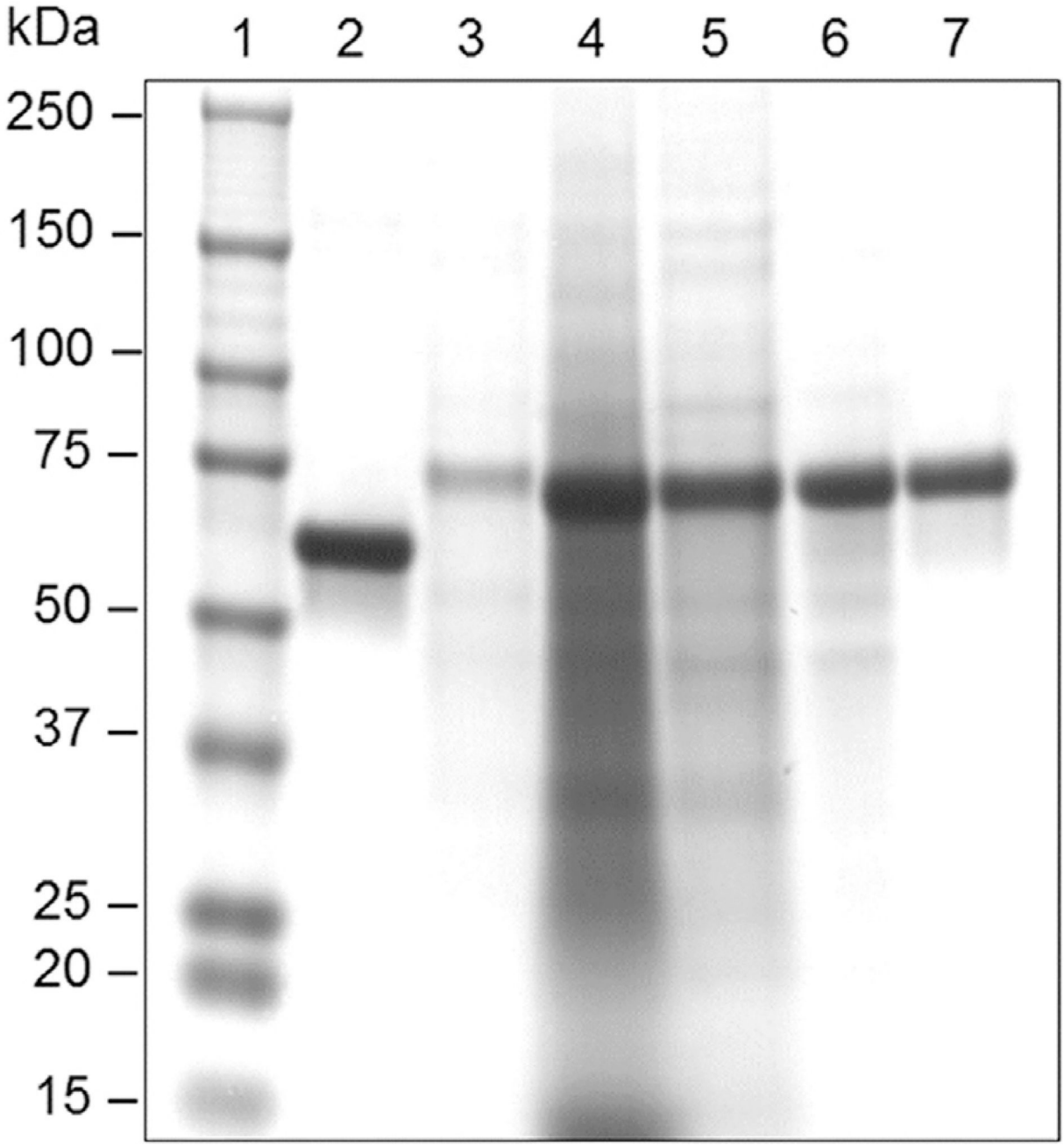

Fig. 2. SDS-PAGE analysis of Method A samples from each purification step

Lane 1, Precision Plus Protein™ Prestained Standards (Bio-Rad); Lane 2, BSA (4 μg); Lane 3, GUS in cell-free culture medium (2.8 μg); Lane 4, concentrated GUS loaded onto blue sepharose (13 μg); Lane 5, blue sepharose pool (10 μg); Lane 6, phenyl sepharose pool (4 μg); Lane 7, DEAE pool (2.3 μg).

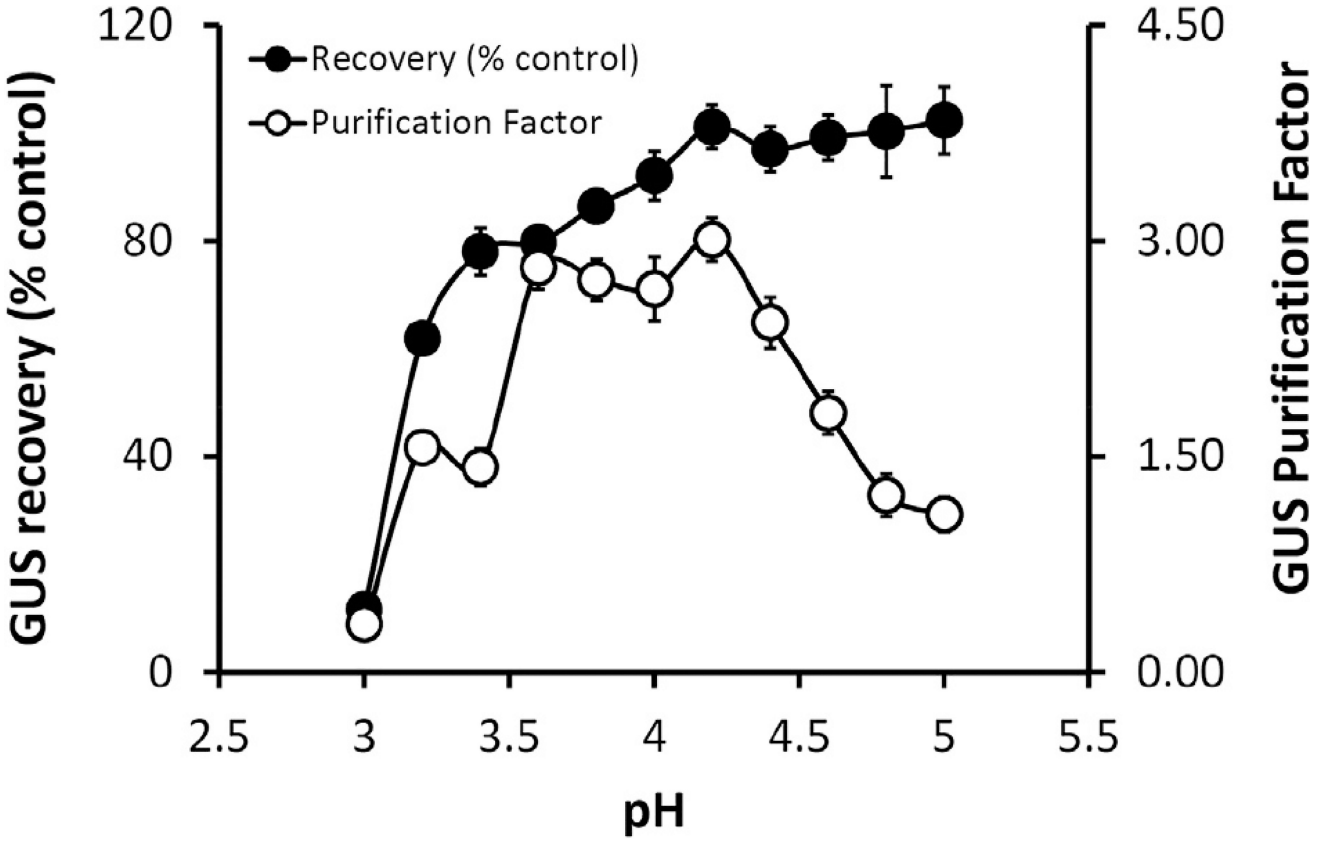

Fig. 3. GUS recovery and purification achieved with acidic citrate buffers

Recoveries of GUS activity were calculated as percent control and purification factors were calculated as ratios of specific activity of each sample to that of the control (pH 7.4 sample).

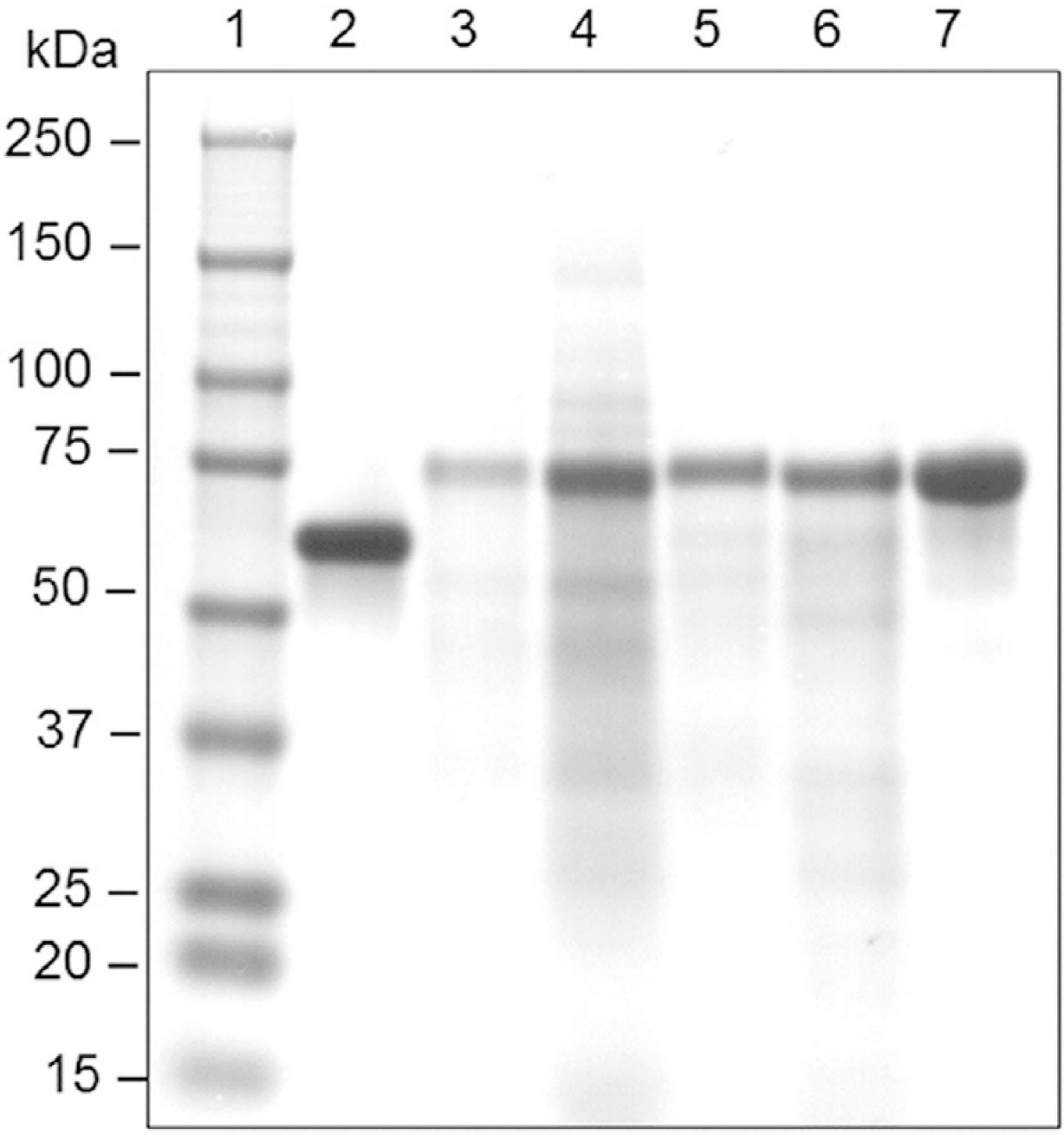

Fig. 4. Chromatography of GUS on phenyl sepharose column under Method B

(A) A_{280} trace is shown in blue, fraction numbers in orange, GUS activity as percent of the highest activity in orange data points and the gradient percentage in green broken line. The portion of the A_{280} trace highlighted in the orange box represents the pooled fractions. (B) SDS-PAGE analysis: impurities are seen in fractions 6–9 and fractions 10–17 display purified GUS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

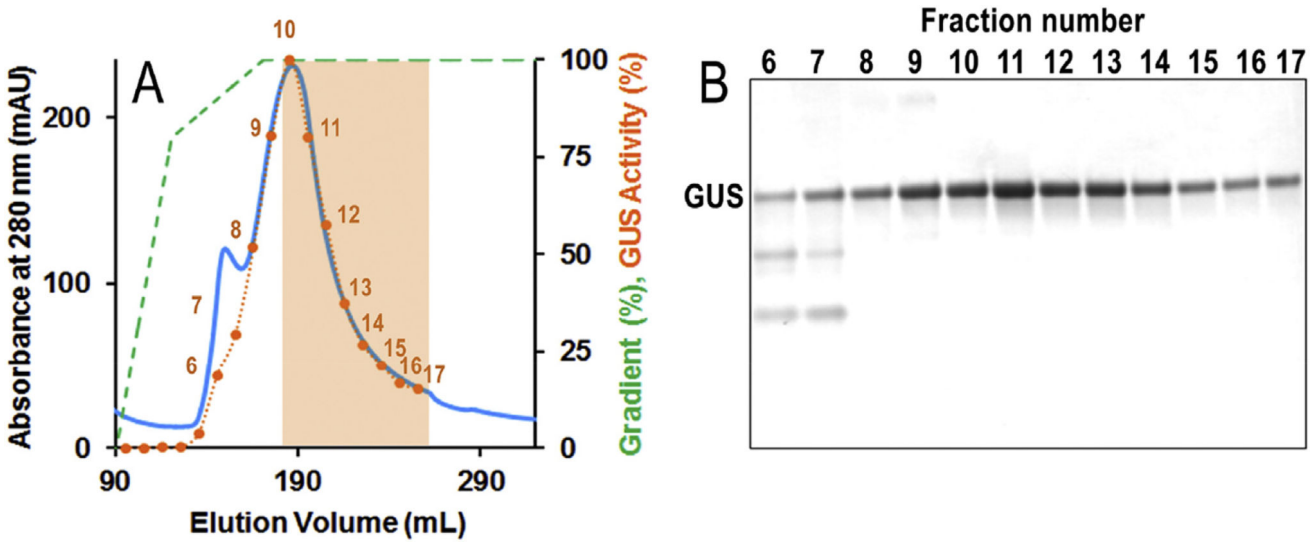

Fig. 5. SDS-PAGE analysis of Method B samples from each purification step

Lane 1, Precision Plus Protein™ Prestained Standards (Bio-Rad); Lane 2, BSA (4 μg); Lane 3, GUS in cell-free culture medium (2.4 μg); Lane 4, concentrated GUS in cell-free culture medium (10 μg); Lane 5, GUS in cell-free culture medium pH 4.2 (3.3 μg); Lane 6, blue sepharose pool (6 μg); Lane 7, phenyl sepharose pool (3 μg).

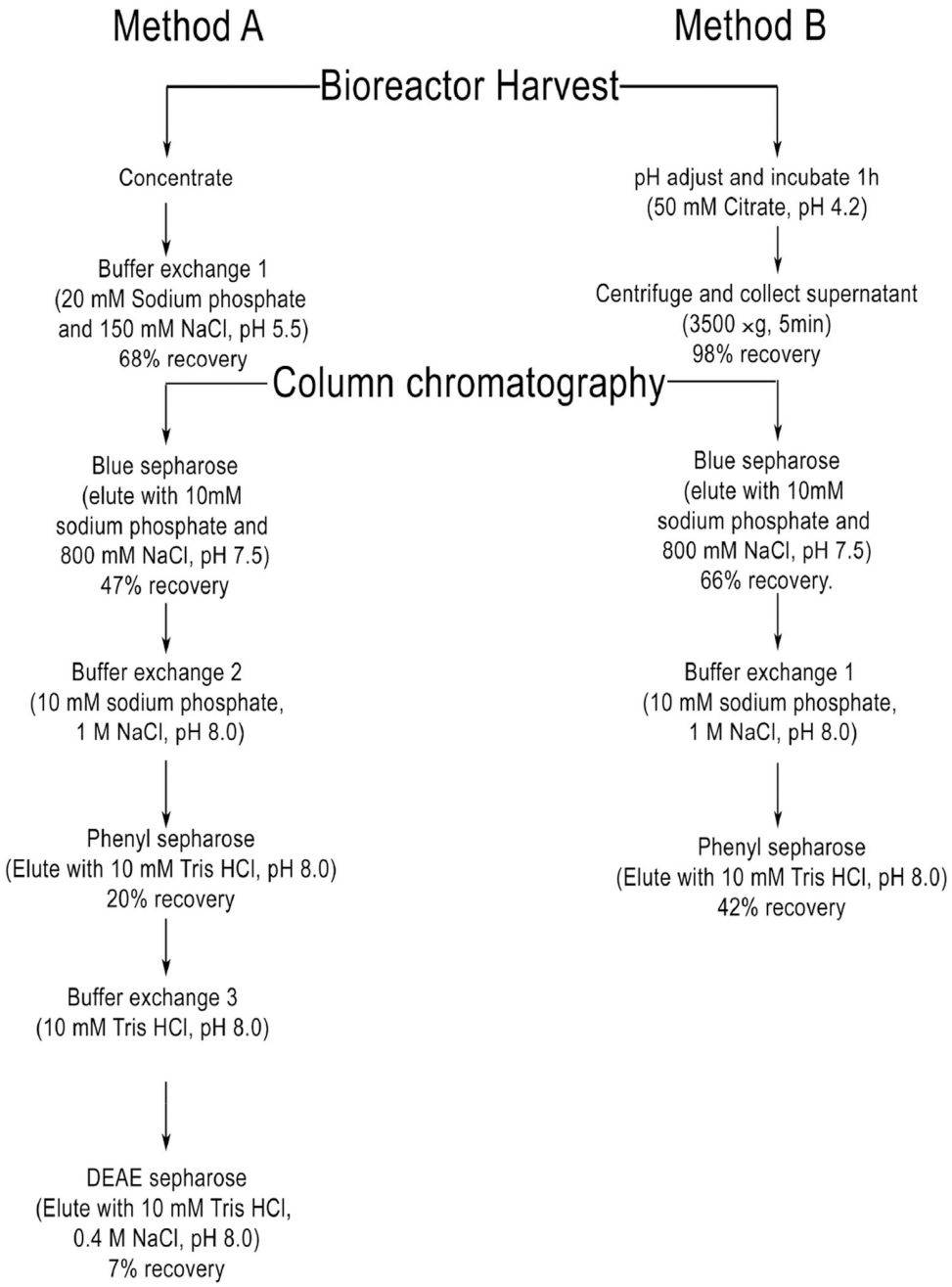

Fig. 6.

Summary of the two purification methods and recovery of GUS activity.

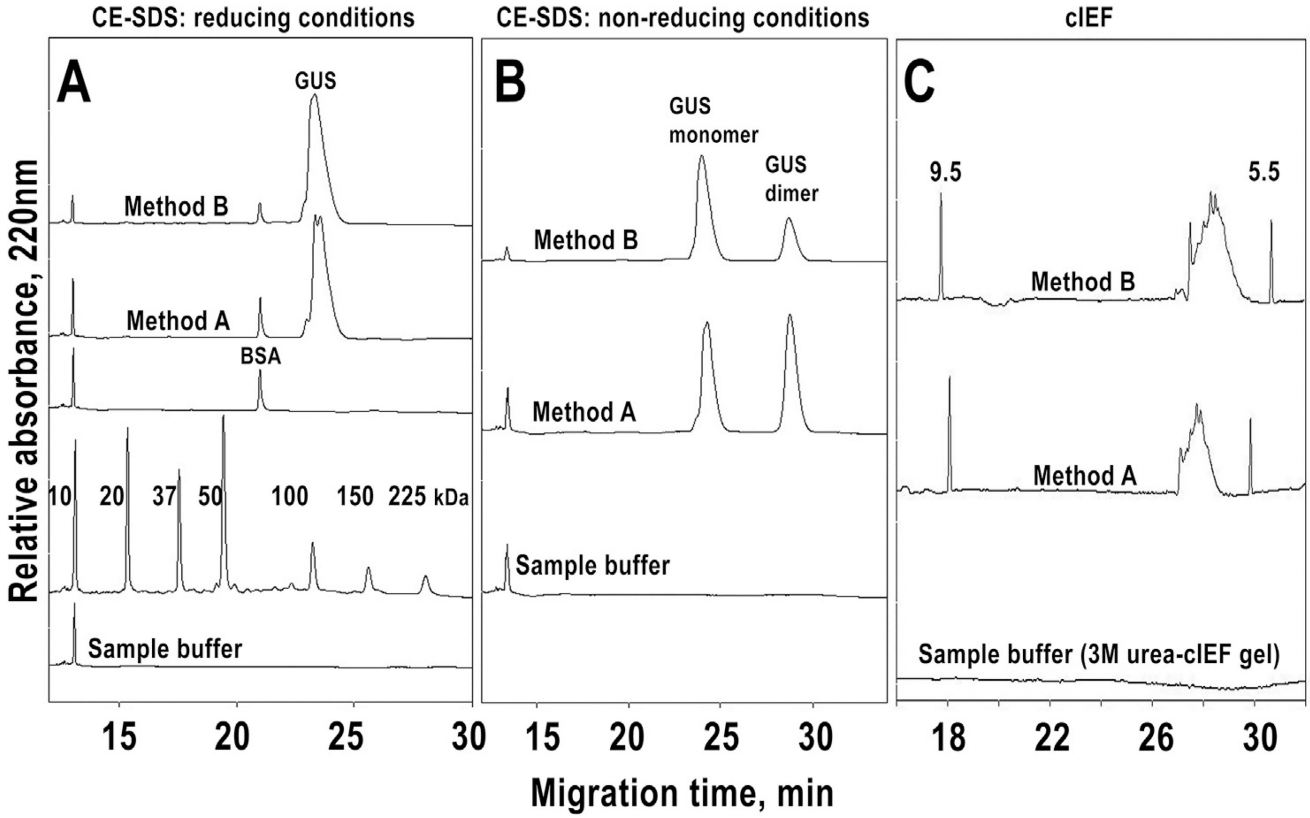

Fig. 7. Capillary electrophoresis characterization of purified GUS samples

All CE-SDS analyses were performed with a 10 kDa internal standard in the sample buffer; under reducing conditions BSA was also added (0.14 μg/μL) as a reference protein. (A) CE-SDS analysis under reducing conditions shows GUS migrates as a monomer after BSA with an apparent size of ~100 kDa. (B) CE-SDS analysis under non-reducing conditions shows presence of both monomers and dimers in purified GUS samples. (C) Capillary isoelectric focusing (cIEF) analysis shows GUS heterogeneity with a broad pI in the range of 5.75–6.8. Flanking sharp peaks are of internal pI standards, 5.5 and 9.5, respectively.

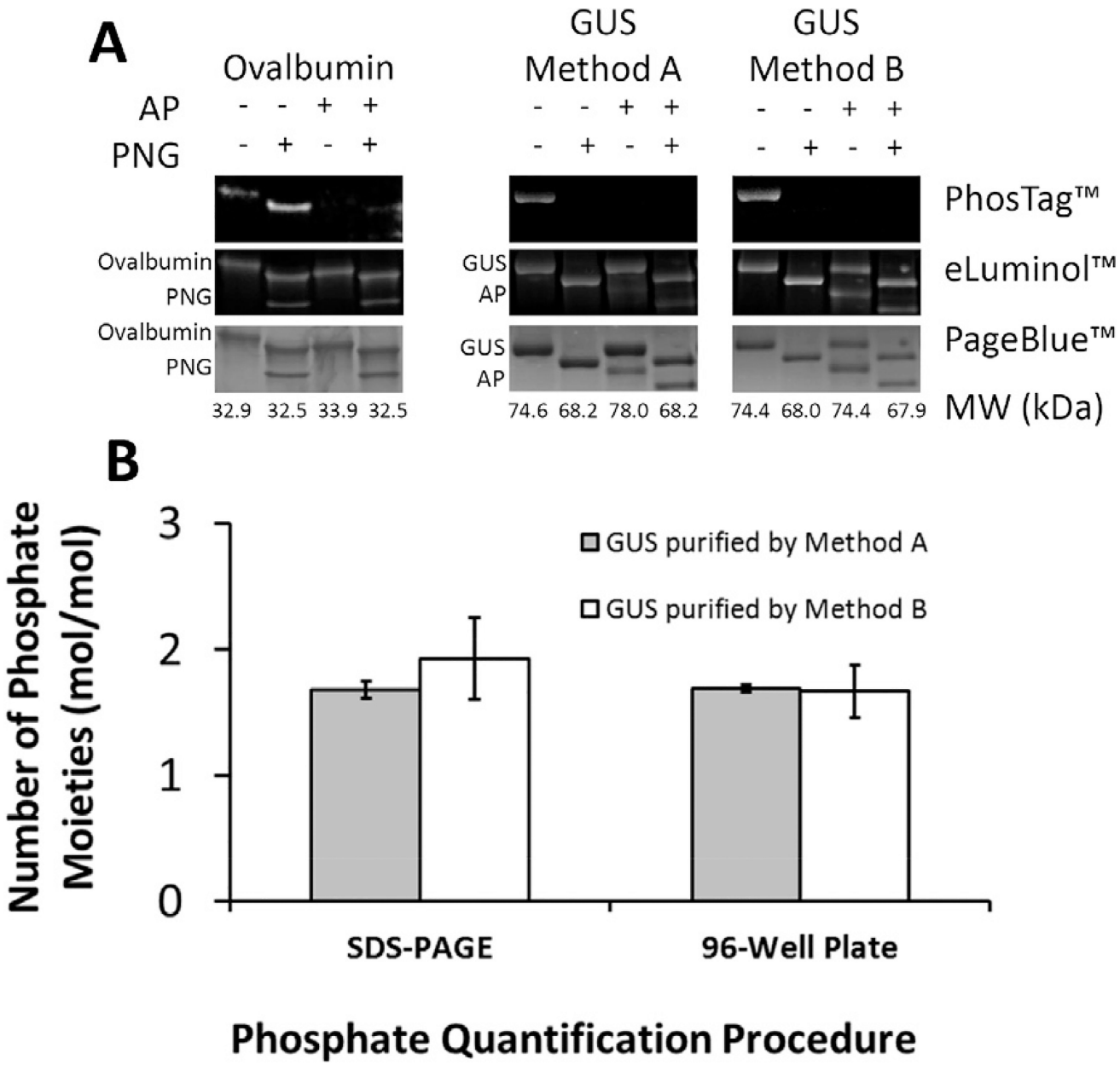

Fig. 8. Assessment of M6P level in GUS samples derived from purification methods A and B

Selective deglycosylation (by treating with PNGase-F; PNG) and dephosphorylation (by treating with alkaline phosphatase; AP) show that Phos-Tag™ signal came from phosphate moieties of the amino acid sidechains of Ovalbumin and M6P of GUS samples; PageBlue™ staining shows a size shift due to deglycosylation. PNG (slightly smaller than Ovalbumin) and AP (slightly smaller than GUS) are also indicated in the eLuminol™ and PageBlue™ stained gels (A). Image analysis and band intensity measurement due to Phos-Tag™ and eLuminol™ dyes in SDS-PAGE method yielded M6P levels comparable to the 96-well plate method (mean ± SEM; n ≥ 7) wherein fluorescence intensities were measured to determine

the phosphoprotein content (Phos-Tag™ signal) and total protein content (eLuminol™ signal) using Ovalbumin standards (B).

Table 1

Summary of purification of GUS by Method A.

Purification Step	Total Protein (mg)	Activity (unit[a])	Specific Activity (unit[a]/mg)	Yield (%)	Purification Fold
Culture Medium	133	4738	35.7	100.0	1.00
Concentration and Buffer-exchange	58	3235	33.3	68.3	0.93
Blue Sepharose	57	2255	31.9	47.6	0.89
Phenyl Sepharose	7	944	89.0	19.9	2.49
DEAE Sepharose	2	319	166.7	6.7	4.67

[a]One unit is defined as the amount of GUS activity required for 1 nmol of PNP formation from PNPG substrate per minute at 25 °C.

Table 2

Effect of pH and buffer composition on GUS recovery.[a]

Sample#	Buffer	Final pH	Timepoint (hours)	Recovery ± S.D. (%)	Purification Factor (Fold) ± S.D.
1	Citrate	4.2	1	101.8 ± 0.9	2.92 ± 0.14
2	Citrate	4.4	1	97.6 ± 1.8	2.46 ± 0.18
3	Citrate	4.6	1	99.8 ± 1.7	1.83 ± 0.12
4	Citrate	4.8	1	101.0 ± 7.5	1.25 ± 0.10
5	Citrate	5	1	103.0 ± 4.7	1.11 ± 0.06
6	Acetate	4.2	1	6.8 ± 0.5	0.45 ± 0.01
7	Acetate	4.4	1	18.2 ± 0.5	0.89 ± 0.15
8	Acetate	4.6	1	35.0 ± 1.0	1.15 ± 0.13
9	Acetate	4.8	1	53.2 ± 2.7	1.27 ± 0.07
10	Acetate	5	1	85.7 ± 5.4	1.53 ± 0.04
11	Citrate	4.2	20	95.7 ± 1.9	2.79 ± 0.07
12	Citrate	4.4	20	97.8 ± 4.8	2.32 ± 0.17
13	Citrate	4.6	20	96.2 ± 2.2	1.75 ± 0.07
14	Citrate	4.8	20	97.0 ± 2.1	1.30 ± 0.09
15	Citrate	5	20	100.2 ± 3.3	1.14 ± 0.02
16	Acetate	4.2	20	8.7 ± 0.7	0.50 ± 0.05
17	Acetate	4.4	20	21.9 ± 1.4	1.06 ± 0.24
18	Acetate	4.6	20	40.6 ± 1.3	1.40 ± 0.11
19	Acetate	4.8	20	58.2 ± 2.1	1.48 ± 0.10
20	Acetate	5	20	80.7 ± 1.2	1.37 ± 0.08

[a]GUS containing bioreactor samples (n = 3) were concentrated (~10 fold), pH adjusted and incubated at 4 °C. GUS activity was measured as described under Methods section and recovery is expressed as percent of control (pH 7.4).

Table 3

Summary of purification of GUS by Method B.

Purification step	Total Protein (mg)	Activity (unit[a])	Specific activity (unit[a]/mg)	Yield (%)	Purification fold
Culture Medium	163	5070	31.0	100.0	1.00
pH Precipitation	70	4985	70.9	98.3	2.29
Blue Sepharose	29	3350	116.2	66.1	3.74
Phenyl Sepharose	14	2121	157.1	41.8	5.06

[a]One unit is defined as the amount of GUS activity required for 1 nmol of PNP formation from PNPG substrate per minute at 25 °C.