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EVALUATION OF BUTYRATE INDUCED PRODUCTION OF A MANNOSE-6-PHOSPHORYLATED THERAPEUTIC ENZYME USING PARALLEL BIOREACTORS

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Abstract

Bioreactor process changes can have a profound effect on the yield and quality of biotechnology products. Mannose-6-phosphate (M6P) glycan content and the enzymatic catalytic kinetic parameters are critical quality attributes (CQAs) of many therapeutic enzymes used to treat lysosomal storage diseases. Here, we have evaluated the effect of adding butyrate to bioreactor production cultures of human recombinant β-glucuronidase produced from CHO-K1 cells, with an emphasis on CQAs. The β-glucuronidase produced in parallel bioreactors was quantified by capillary electrophoresis, the catalytic kinetic parameters were measured using steady-state analysis, and mannose-6-phosphorylation status was assessed using an M6P-specific single chain antibody fragment. Using this approach we found that butyrate treatment increased β-glucuronidase production up to ~3-fold without significantly affecting the catalytic properties of the enzyme. However, M6P content in β-glucuronidase was inversely correlated with the increased enzyme production induced by butyrate treatment. This assessment demonstrated that although butyrate dramatically increased β-glucuronidase production in bioreactors, it adversely impacted the mannose-6-phosphorylation of this lysosomal storage disease therapeutic enzyme. This strategy may have utility in evaluating manufacturing process changes to improve therapeutic enzyme yields and CQAs.

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Keywords

lysosomal storage diseases; enzyme replacement therapies; mannose-6-phosphate; enzymes; bioreactors; capillary electrophoresis

Introduction

Lysosomal storage disease (LSD) enzyme replacement therapies (ERTs) are a growing class of therapeutic proteins [1],[2]. More than fifty LSDs have been identified and they afflict ~1 in 5,000 people worldwide [3]. The genetic diseases result from impairments in lysosomal acid hydrolase production, stability or function, resulting in the cellular accumulation of substrates such as glycosphingolipids (Gaucher and Fabry's diseases), glycogen (Pompe disease) and mucopolysaccharides (Hurler and Hunter Syndromes) in patient tissues. These substrate accumulations result in pathogenic cascades [3]. Over the past two decades ERT products have become available for disease management and have extended patient's life expectancy. There are currently nine Food and Drug Administration (FDA) approved and twenty new LSD ERT products in clinical development. There is, however, a need to improve LSD manufacturing processes since patients often require routine administration of milligram quantities of protein for the duration of their life.

From a physicochemical perspective these enzyme products are complex and have multiple critical quality attributes (CQAs) that directly impact the biodistribution and potency of the product *in vivo*. In addition to the catalytic properties of the enzymes deficient in LSDs, mannose-6-phosphate (M6P)-containing oligosaccharides represents an important CQA since they are required for the uptake and targeting of the protein to lysosomes through the ubiquitous cation independent-mannose-6-phosphate receptor (CI-MPR) localized at the cell surface [4]. For our studies, β-glucuronidase was selected since it has all of the attributes of current FDA approved LSD therapies. It catalyzes the hydrolysis of β-D-glucuronic acid residues from glycosaminoglycans in lysosomes and functions in the catabolism of heparan, dermatan and chondroitin sulfates [5],[6]. It is an ~300 kDa lysosomal acid hydrolase which exists as a homotetramer composed of subunits that each contain four glycosylation sites located on asparagine 173, 272, 420 and 631. All four sites are linked to high mannose oligosaccharide structures and the glycans at asparagines 272, 420 and 631 are mannose-6-phosphorylated [7]. The absence or impaired function of β-glucuronidase leads to mucopolysaccharidosis type VII (MPS VII) in patients [8],[9].

Many methods have been employed to increase protein production in mammalian cells including bioreactor scale [10] and temperature changes [11–13], optimization of culture medium using small molecules [14], inhibition of histone deacetylases with agents such as butyrate [15–17], and genetic engineering of cell substrates [18–20]. Here, we have used bench scale parallel bioreactors and a protein production process that models bioreactor conditions to those used for commercial production of M6P dependent ERTs for LSDs. Capillary electrophoresis (CE), steady-state enzyme kinetic analysis and a newly developed M6P-specific single chain antibody fragment [21] were used to evaluate β-glucuronidase CQAs when culture media was supplemented to increase protein production. The strategy

can be executed in weeks, is quantitative, requires minimal material and scale, and utilizes analytics that can be performed on bioreactor harvest protein. Our results indicate that addition of butyrate to recombinant Chinese hamster ovary (CHO-K1) cells grown in a bioreactor resulted in a striking increase in β-glucuronidase production, but decreased enzyme M6P content. Thus, this approach has utility in evaluating manufacturing process changes for this important class of enzyme therapies.

Materials and Methods

Reagents and Cell Lines-

Ex Cell 325 PF CHO medium and sodium butyrate were purchased from Sigma (St Louis, MO). Trypan blue was from Fisher Scientific (Pittsburgh, PA). Minimum essential medium–Earles (MEM-Earles), G418 and 100X HT supplement were from Life technologies (Grand Island, NY). Para-nitrophenyl-β-D-glucuronide (PNPG) was from Gold Biotechnology (St. Louis, MO). GelCode Blue Safe protein stain and Pierce 660 nm Protein assay reagent were from Thermo Scientific (Rockford, IL). Chinese hamster ovary cell line producing recombinant human β-glucuronidase enzyme was obtained from William S. Sly, Saint Louis University School of Medicine [22]. The β-glucuronidase-CHO-K1 cells and parental CHO-K1 were adapted for growth in suspension in Ex Cell 325 PF CHO chemically defined medium using a method previously described [23].

Bioreactor Operations-

A frozen vial of 4 x 10^6 viable cells was expanded in a T_{25} flask, transferred to a T_{75} flask the following day and cultured for two days. Cells were then expanded in a 250 ml spinner flask for 3 days and then transferred to a 1 L spinner flask for expansion. 1.2 L parallel bioreactors (DASGIP BioTools, Shrewsbury, MA) were then inoculated (0.75 L medium at 2.5×10^5 cells mL^{-1}) to a final volume of 0.8 L. Bioreactors were operated in a fed batch mode for 8 days with parameters set to 37 °C, 50% dissolved oxygen (of air saturation), pitched blade impeller speed 125 rpm and pH 7.0 maintained with CO_2-bicarbonate buffer. Samples were taken daily for nutrient analysis, determination of viable cell density (VCD, million mL^{-1}) and enzyme activity measurements. Glucose and glutamine were fed to the bioreactors continually to maintain levels of 1.0 ± 0.5 g L^{-1} and 1.0 ± 0.5 mM, respectively. Butyrate was added to the bioreactors at a VCD of ~2.0 × 10^6 cells mL^{-1}. Glutamine, glutamate, glucose, lactic acid, ammonium, sodium and potassium ions were measured with a Nova Bioprofile 100 plus analyzer (Nova Biomedical, Waltham, MA). Glucose and glutamine were added to maintain at least 1.0 ± 0.5 g L^{-1} and 1.0 ± 0.5 mM, respectively. Viable cell density (VCD, million mL^{-1}) and enzyme activity were measured daily. Specific productivity (q_p) was calculated by dividing measured β-glucuronidase activity by viable cell-days (integral viable cell concentration, IVC) as described before [24].

Preparation of β-Glucuronidase Reference Standard-

The suspension adapted β-glucuronidase-CHO-K1 cells were grown in a 5 L bioreactor (Celligen 310, New Brunswick Scientific-Eppendorf, Enfield, CT) with operational parameters similar to those described for the 1.2 L bioreactors. After 9 days the harvest

was collected and β-glucuronidase reference standard was purified as described previously [22].

Processing of Bioreactor Harvests-

Bioreactor harvest supernatants were clarified (4000 ×g for 15 min), concentrated 100-fold into 10 mM Tris-HCl, 1 mM β-glycerophosphate, pH 8.0 using Amicon Ultra (EMD Millipore, Burlington, MA) concentrators (30 kD NMWCO) and stored at -80 °C. Total protein and β-glucuronidase protein concentrations were measured using Pierce 660 nm Protein assay reagent and capillary electrophoresis, respectively.

Enzyme Activity and Kinetic Analysis-

β-glucuronidase activity was measured at 25 °C using PNPG substrate [25]. Para-nitrophenol (PNP) product was measured at 405 nm [26] in a Synergy H4 plate reader (BioTek Instruments, Winooski, VT) with 1 unit of activity corresponding to production of 1 pmol PNP per minute. Activity in clarified conditioned medium was measured by diluting 10-fold into 20 mM sodium phosphate, 1 mM PNPG, 10 mM β-mercaptoethanol at pH 7.4. Kinetic parameters and enzyme specific activity were measured at pH 3.6 using 20 ng of β-glucuronidase in 200 μl of 115 mM acetate-formate, pH 3.6 and 0–10 mM PNPG. After 25 min, 120 μL of L-arginine and sodium bicarbonate (0.5 M each), pH 10 was added and absorbance at 405 nm was determined. Specific activity measurements were performed using 12.5 mM PNPG and calculated by dividing the units of activity by amount of β-glucuronidase protein. All measurements were performed in triplicate.

Capillary Electrophoresis-

Sodium dodecyl sulfate-capillary electrophoresis (SDS-CE) was performed on a Beckman Coulter PA800plus according to the manufacturer's instructions [27] with detection at 220 nm. β-glucuronidase was quantified in each analysis using 0.18 μg/μl BSA as an internal standard.

SDS-PAGE and Western Blotting-

Proteins were separated in 10% SDS-PAGE gels, and stained using GelCode Blue Safe protein stain. For Western blotting proteins were transferred to polyvinylidene difluoride membranes and probed with a single-chain antibody fragment against mannose-6-phosphate (M6P) [21]. Detection was performed using Vectastain ABC Elite kit (Vector Laboratories Inc, Burlingame, CA) and enhanced chemiluminescence.

PNGase-F Digestion-

Samples containing 12 μg of β-glucuronidase were treated overnight with or without PNGase-F (New England Biolabs, Ipswich, MA) in a volume of 50 μl according to manufacturer's instructions. Proteins were precipitated by addition of 150 μl of cold ethanol, centrifuged at 4°C (12,000 ×g for 10 min), and re-suspended in SDS-PAGE loading buffer. Samples were analyzed by SDS-PAGE and Western blotting with an anti-M6P antibody [21].

Statistical Analyses-

Non-linear regression analysis of Michaelis-Menten plots was performed to determine the steady state kinetic parameters and their limits at 99% confidence level. Post-hoc Dunnett's test was used to compare the mean values of samples of control and butyrate-treated bioreactors. Mannose-6-phosphorylation Western blot band densities were compared using a 't-test' for samples with unequal variances. Western blotting analyses of butyrate titrated bioreactor samples were quantified for M6P content and normalized to control (no butyrate) values. Linear regression fits with least squares analyses were performed on plots of butyrate concentration versus M6P content, and non-zero co-efficient of independent variables were ascertained by analysis of variance (ANOVA) and a t-test. JMP 9.0.0 software (SAS Institute Inc., Cary, NC) was used in all analyses.

Results and Discussion

Butyrate Increases β-Glucuronidase Produced from Recombinant Suspension CHO-K1 Cells in 1.2 Liter Parallel Bioreactors-

There is a need to maximize the production of therapeutic LSD enzymes in bioreactors while maintaining or improving the CQAs of the product, and strategies to address this issue in product development are required. Towards this end, an adherent CHO-K1-derived cell line that secretes recombinant human β-glucuronidase [22] was adapted for growth in serum-free chemically-defined medium [23] and a cell bank was established. Since butyrate has been previously shown to increase protein production in CHO cells in a number of bioproduction models [16],[28],[29] we evaluated its utility to increase production of human β-glucuronidase.

DASGIP 1.2 L bioreactors were selected for these studies since they can be simultaneously computer controlled with respect to pH, oxygen levels, temperature, impeller speed, and glutamine levels. In these experiments β-glucuronidase-CHO-K1 cells (2.0×10^8) were inoculated into the parallel bioreactors to establish a growing culture and two days later butyrate was added to the media. Samples were removed daily from the bioreactors and analyzed with respect to glutamine, glutamate, glucose, lactate, ammonium levels, osmolality (calculated value), cell density and viability, and enzyme activity (data not shown). As shown in Figure 1, 1.5 mM butyrate dramatically increased β-glucuronidase specific productivity (q_p) in parallel bioreactor runs. At day eight a 4-fold increase in specific productivity (q_p, μ units of enzyme activity/cell-day), compared to untreated control cells, was observed. At termination, the viable cell densities in the control and butyrate treated bioreactors were 15.3×10^6 and 3.9×10^6 mL^{-1}, respectively. Cell viabilities were similar between the butyrate treated and control bioreactors (>90%).

To demonstrate the validity of measurement of β-glucuronidase activity in the bioreactor conditioned medium, parental CHO-K1 cells were used as a negative control. Conditioned medium from the parental cells contained no detectable β-glucuronidase activity and spiking of purified reference standard β-glucuronidase into this conditioned medium confirmed (i) a linear activity response with respect to the amount of spiked enzyme and (ii) no effect of the conditioned medium on activity relative to assay reaction buffer alone (data not shown).

Thus, bioreactor harvests generated using the parental CHO-K1 cells did not interfere with measurement of β-glucuronidase activity in spiking experiments, and there was a linear relationship between added β-glucuronidase reference standard and catalysis of the PNPG substrate (data not shown).

Quantification of β-Glucuronidase Protein and Total Protein in Harvests from Butyrate Treated Parallel Bioreactors-

Next, we performed six bioreactor runs of β-glucuronidase-CHO-K1 cells in parallel in which three were treated with 1.5 mM butyrate on day 2, and three control bioreactors were not treated. Bioreactor operational parameters including temperature, impeller speed, dissolved oxygen and pH were tightly controlled throughout the runs (supplementary Fig. 1). Butyrate-treated cells exhibited less glucose and bicarbonate consumption, as well as reduced lactose production, compared to control cells, consistent with decreased cell number due to growth arrest (supplementary Fig. 2) [17]. On day 6, 7, and 8, 50 ml of bioreactor harvest was removed, buffer-exchanged and concentrated 100-fold. Cell viability was similar and ≥90% for all six parallel bioreactors at all time points.

To quantify β-glucuronidase protein in bioreactor harvests, a sodium dodecyl sulfate–capillary electrophoresis (SDS-CE) method was developed. In this method the concentrated harvests are diluted 10-fold into an SDS master mix that contained 0.18 μg/μl bovine serum albumin (BSA), boiled briefly, separated in the capillary based upon size, and elution is monitored at 220 nm. The BSA serves as an internal standard to quantify and normalize β-glucuronidase protein detected in each individual CE analysis.

As shown in Figure 2, when analyzed by SDS-CE β-glucuronidase is detected at the predicted eluting region of 24–26 min in bioreactor harvest from β-glucuronidase-CHO-K1 cells (top), but not in harvest from parental CHO-K1 cells. The highly purified β-glucuronidase reference standard was used to verify protein identity.

Figure 3 contains representative SDS-CE elution profiles for control and butyrate-treated bioreactor harvest samples. Summary data for total and β-glucuronidase protein levels in the three control (bioreactors 1, 2 and 3) and three butyrate-treated parallel bioreactors (4, 5 and 6) are shown in Table 1. The concentrations reported in Table 1 refer to bioreactor harvests prior to processing (i.e. 1X). The 1.5 mM butyrate treatment produced a statistically significant increase ($p<0.05$) in both total and β-glucuronidase protein content at day 6, 7 and 8. β-Glucuronidase levels were ~3.1-fold higher relative to untreated cells at termination of the bioreactor runs (day 8).

Butyrate Does Not Affect β-Glucuronidase Enzyme Kinetic Parameters-

Steady-state kinetic measurements using the Michaelis-Menten method describes the rate of enzymatic turn-over as a function of substrate concentration and provides measurements of Michaelis (K_m) and catalytic rate constants (k_{cat}) [30]. The specificity constant, k_{cat}/K_m, serves as the best parameter for comparisons of catalytic efficiencies between enzymes. The specificity constants using PNPG substrate for β-glucuronidase reference standard in control buffer or in processed bioreactor harvest from parental CHO cells were determined to be 24 ± 8 and 24 ± 7 sec^{-1} mM^{-1}, respectively (Table 2). The kinetic parameters K_m, and k_{cat},

and the specificity constant values obtained on harvest samples at day 8 from untreated (1, 2 and 3) and butyrate-treated bioreactors (4, 5 and 6) containing β-glucuronidase-CHO cells were compared and found not to be statistically different (Table 2). Thus, using butyrate to increase β-glucuronidase production in recombinant CHO-K1 cells does not affect catalytic properties of the enzyme.

Butyrate Decreases β-Glucuronidase M6P Content-

In CHO cells butyrate has been demonstrated to alter glycosylation of thrombopoietin [16], interferon-β [15], a recombinant fusion protein [31], but did not change glycans in tissue plasminogen activator [29]. The ability of many LSD therapeutic enzymes to target cellular lysosomes is dependent on levels of terminal M6P on N-linked oligosaccharides. Due to the importance of this product attribute, the effect of butyrate treatment on β-glucuronidase M6P content was evaluated using an M6P-specific single chain antibody fragment. Our Western blotting analysis using this Fab reagent produces a linear response from 0.5 to 5 μg β-glucuronidase reference standard per lane (data not shown). Analysis of constant amounts of β-glucuronidase protein in bioreactor harvests by Coomassie staining and M6P Western blotting (anti-M6P) demonstrated that butyrate-treated cells produced a β-glucuronidase with ~35% less M6P (bioreactors 4, 5 and 6) relative to control bioreactors (1, 2 and 3) (Fig. 4A, $p=0.035$). Digestion of the samples with PNGase-F to remove N-linked glycans resulted in loss of reactivity with the M6P-specific single chain antibody fragment and a reduction in size of ~7 kDa for all samples (Fig. 4B). Thus, butyrate had no obvious qualitative effect on β-glucuronidase polypeptide core or glycan structures, but specifically decreased mannose-6-phosphorylation of the N-linked oligosaccharides.

To better understand the relationship between butyrate concentration and β-glucuronidase production, enzyme specific activity and M6P content a titration of butyrate (0, 0.5, 0.75 and 1.5 mM) was performed in the parallel bioreactors. As shown in Figure 5, all tested concentrations of butyrate increased the specific productivity (q_p) of the cells and decreased the viable cell count compared to the control. To characterize the effect of butyrate concentration on β-glucuronidase production and CQAs we analyzed bioreactor harvests at day 6, 7 and 8 (Fig. 6). All of the concentrations of butyrate increased β-glucuronidase production by the cells, but no pronounced dose-dependent effect was observed. In contrast, butyrate treatment decreased β-glucuronidase M6P content in a dose-dependent manner, and a statistical analysis confirmed the negative trend. Thus, we found an inverse relationship regarding butyrate induced β-glucuronidase production and M6P content on the enzyme. Butyrate did not affect the specific activity of β-glucuronidase consistent with what we had observed for the kinetic parameters of the enzyme produced in 1.5 mM butyrate cultures.

The specific mechanism responsible for the butyrate effect on β-glucuronidase phosphorylation is not known. Two Golgi enzymes, an N-acetylglucosaminyl-1-phosphotransferase and an N-acetylglucosamine-1-phosphodiester-α-N-acetylglucosaminidase, are required for phosphorylation of lysosomal hydrolases mannose residues [32]. Therefore, it is plausible that overexpression of β-glucuronidase in the recombinant CHO-K1 cells saturates one or both of the two enzymes resulting in a decrease in the phosphorylation of each β-glucuronidase molecule. Consistent with our

findings with butyrate, the inducing agents propionate (3.4 mM) and DMSO (1.5% v/v) also increased β-glucuronidase production, 2.5- and 2-fold, respectively, and decreased β-glucuronidase M6P content (data not shown).

Conclusions

Due to the need to improve product yields in the manufacturing of LSD therapeutic enzymes, supplementation of the bioreactor media with butyrate to increase production of a M6P-containing lysosomal enzyme was evaluated with respect to its effect on the protein's CQAs. Butyrate significantly increased β-glucuronidase production from suspension CHO-K1 cells grown in serum-free medium. The effect of butyrate was further characterized using parallel bioreactor harvest samples, and measurements of enzyme quantity, kinetic parameters, and M6P content were assessed in rigorous head-to-head analyses. Butyrate had no impact on β-glucuronidase enzyme activity and kinetic parameters, whereas M6P content of the enzyme decreased, compared to controls. This study underscores the need for an early evaluation of product CQAs, and in particular, those related to glycosylation when selecting cell clones, optimizing bioreactor conditions, and considering changes in a manufacturing process. Further, we propose that this strategy can be used to provide a rapid assessment of the impact of bioreactor process changes on the quality of many biotechnology products and is applicable to the development of new innovative therapeutic protein products, and for biosimilars.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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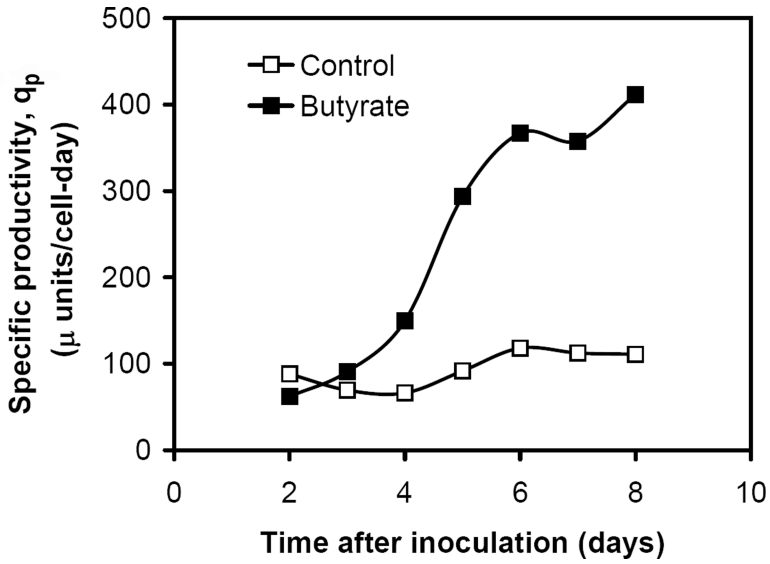

Figure 1.

Butyrate induced production of β-glucuronidase activity in 1.2 L parallel bioreactors. β-glucuronidase-CHO-K1 cells were cultured with medium alone (control) and butyrate (1.5 mM) was added at a viable cell density of ~2 million/mL on day 3. Enzyme activity was measured on samples daily and specific productivity (q_p) calculated as a ratio of activity to viable cell-days [24]. Data reflects mean ± standard error of mean of assays performed in triplicate. All standard errors were <6% of mean values and bars are not visible.

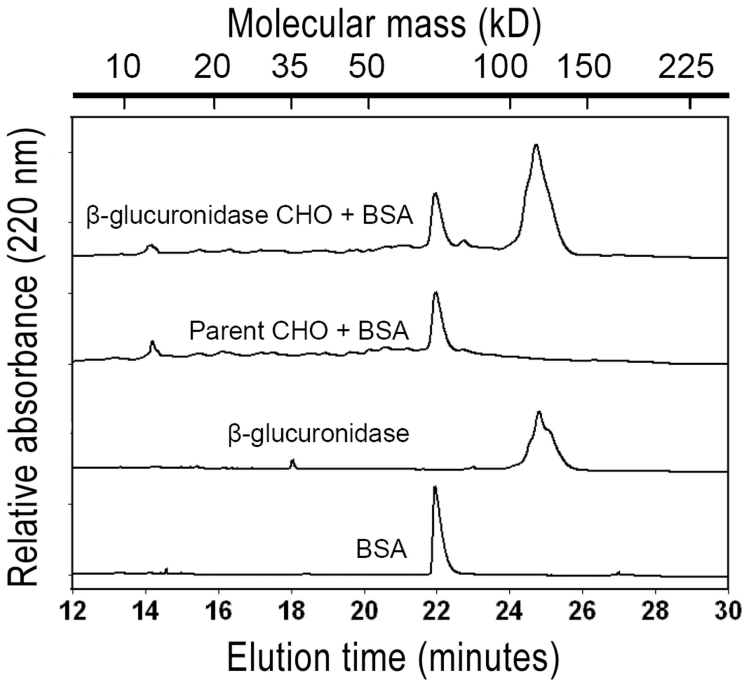

Figure 2.

Quantification of β-glucuronidase protein by sodium dodecyl sulfate-capillary electrophoresis (SDS-CE). Bioreactor harvests from β-glucuronidase-CHO-K1 and parental CHO cells were processed as described in Methods. Individual SDS-CE traces for these harvests diluted 10-fold into SDS master mix containing 0.18 μg/μl of BSA internal standard, BSA alone and purified β-glucuronidase reference standard (0.2 μg/μl) are presented..Elution times for molecular mass (kD) protein standards are indicated on top.

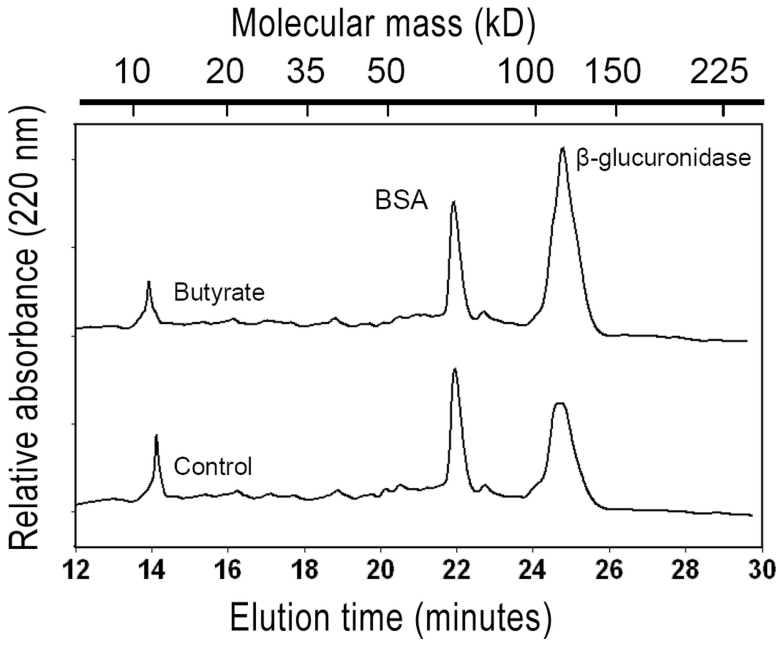

Figure 3.

Influence of butyrate on production of β-glucuronidase in bioreactors. Bioreactor harvest samples from β-glucuronidase-CHO-K1 cells treated without (control) or with 1.5 mM butyrate were analyzed by SDS-CE and representative traces are shown. After processing, bioreactor harvest samples were diluted 10-fold into an SDS master mix that contained 0.18 μg/μl BSA internal standard. Elution times for molecular mass (kD) protein standards are indicated on top.

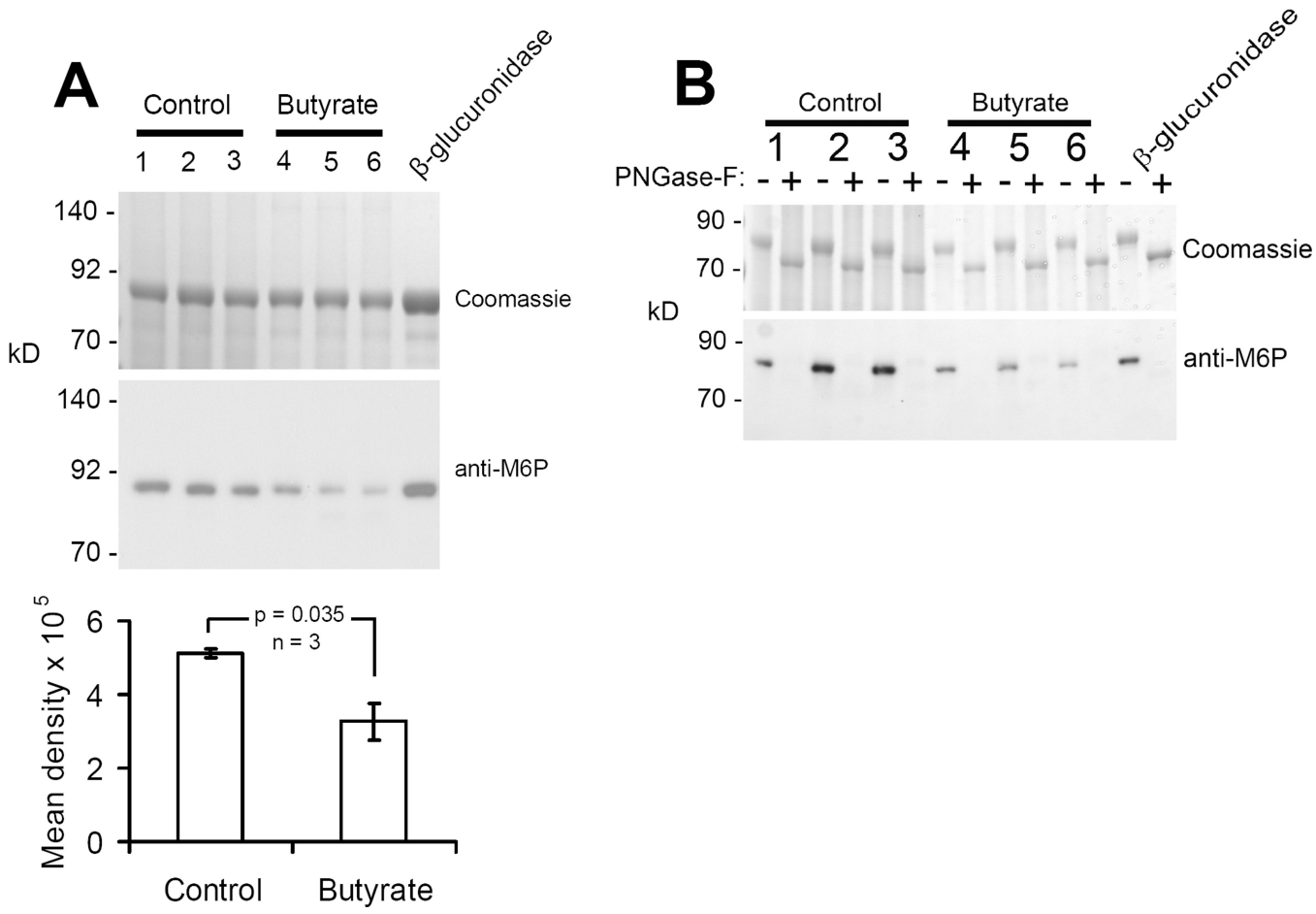

Figure 4.

Quantification of mannose-6-phosphate in β-glucuronidase by Western blotting. (A), Coomassie stained SDS-PAGE (10% Tris-glycine gel) analysis of 1 μg β-glucuronidase from control (bioreactors 1–3), butyrate-treated (4–6) day 8 bioreactor harvests and 2 μg of purified β-glucuronidase reference standard (far right) are presented. Middle, Western blotting for mannose-6-phosphate (M6P) from the identical samples (anti-M6P). Migration positions of protein standards (kD) are indicated on left. Bottom, comparison of the band densities in the middle panel by a t-test for samples with unequal variances. (B) Effect of PNGase-F treatment on β-glucuronidase. Day 8 bioreactor harvest samples containing 12 μg of β-glucuronidase from control (bioreactors 1–3) or butyrate-treated (4–6) β-glucuronidase-CHO cells were digested overnight with (+) or without (-) PNGase. Protein was precipitated with cold ethanol, resolvated in SDS sample buffer and one-fifth of the digest was analyzed by either Coomassie stained SDS-PAGE (top) or Western blotting for mannose-6-phosphorylation (bottom). Analysis of β-glucuronidase reference standard is provided in the far right lanes.

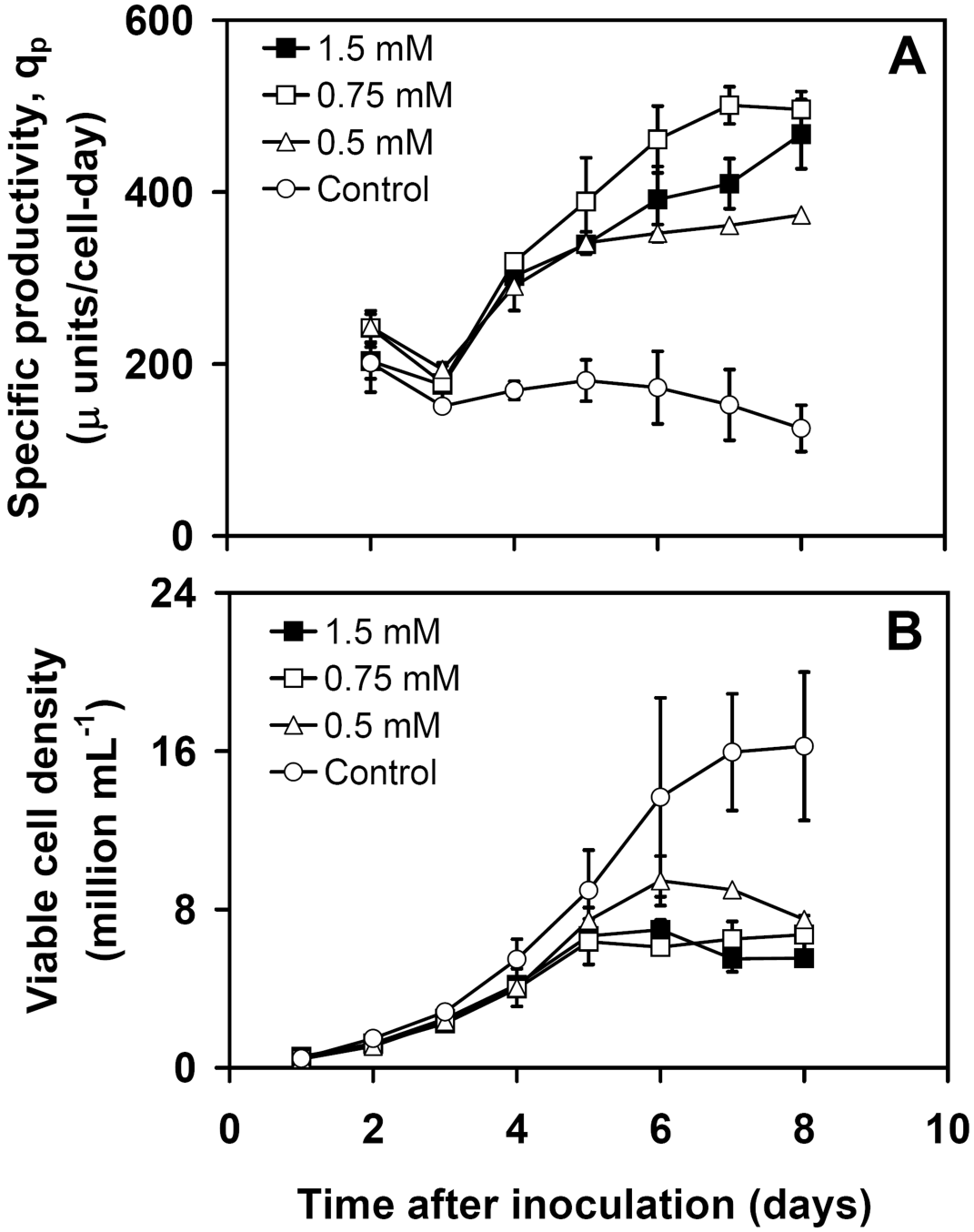

Figure 5.

Effect of butyrate concentration on specific productivity and viable cell density of β-Glucuronidase-CHO-K1 cells. Cells cultured in 1.2 L parallel bioreactors were untreated (control) or treated with 0.5, 0.75 or 1.5 mM butyrate at day 3. Specific productivity (q_p) at each day was determined as in Fig. 1 and are shown in A. Viable cell densities are presented in B. Data reflect mean ± half range error bars of two bioreactors for each concentration of butyrate.

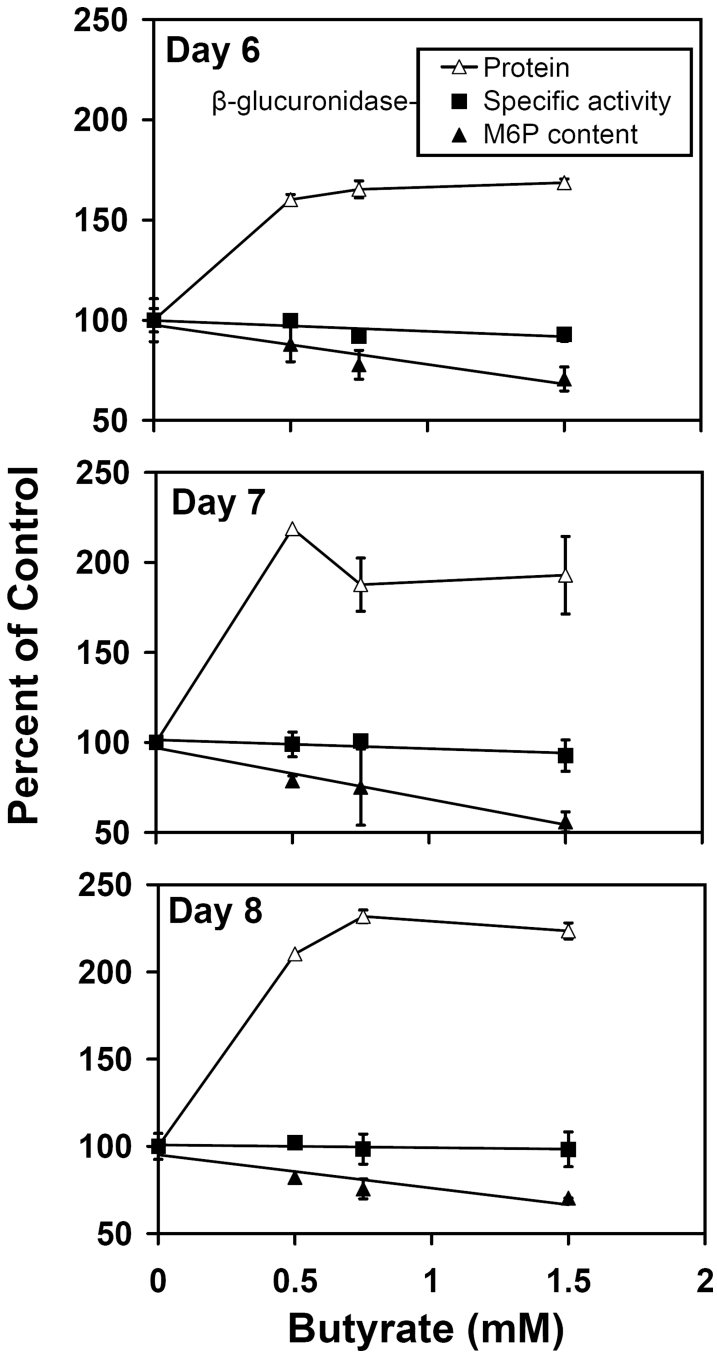

Figure 6.

Influence of butyrate concentration on β-glucuronidase production, specific activity and M6P content. Bioreactors conditions are as described in Fig. 5 and harvest samples were taken from bioreactors at day 6, 7, and 8. β-Glucuronidase concentration was determined by SDS-CE, relative M6P content by Western blotting and enzyme specific activity was measured using PNPG substrate. Levels are expressed as a percentage of untreated controls. Control samples contained 45, 55, and 63 mg β-glucuronidase/L at day 6,7, and 8, respectively. Averages of two bioreactors ± half range are presented. Negative trends for

M6P content with respect to butyrate concentration were indicated by regression ANOVA and a t-test for the slope ($p<0.03$).

Table 1.

Quantitation of β-glucuronidase and total protein in bioreactor harvests[a].

	Control			Butyrate		
		Protein (mg L^{-1})			Protein (mg L^{-1})	
Day	Bioreactor	β-glucuronidase	Total	Bioreactor	β-glucuronidase	Total
6	1	15.5	43.6	4	37.1	48.6
	2	17.4	43.5	5	41.2	65.8
	3	17.2	42.1	6	35.8	57.8
	Mean ± SEM	16.7 ± 1.0	43.1 ± 0.8		38.0 ± 2.8 (p = 0.001)	57.4 ± 8.6 (p = 0.045)
7	1	27.0	63.9	4	60.1	79.8
	2	27.7	61.0	5	47.3	67.2
	3	23.9	60.8	6	50.0	73.8
	Mean ± SEM	26.2 ± 2.0	61.9 ± 1.7		52.5 ± 6.7 (p=0.003)	73.6 ± 6.3 (p=0.036)
8	1	31.0	67.3	4	127.6	141.0
	2	30.2	71.7	5	73.9	106.8
	3	27.6	74.8	6	78.1	98.3
	Mean ± SEM	29.6 ± 1.8	71.3 ± 3.8		93.2 ± 29.9 (p=0.021)	115.4 ± 22.6 (p=0.029)

[a]β-glucuronidase concentrations were measured by capillary electrophoresis and total protein as described in Materials and Methods and reflect levels prior to harvest processing (1X); mean ± standard error of mean for each day are given. P-values derived from Dunnett's test comparing β-glucuronidase or total protein levels between control and butyrate-treated bioreactor samples for each day are provided in parentheses.

Table 2.

Summary of steady state enzyme kinetics parameters for β-glucuronidase in control and butyrate-treated bioreactor samples[a].

Sample	K_m (mM)	k_{cat} (sec^{-1})	k_{cat} / K_m (sec^{-1} mM^{-1})
1	0.8 ± 0.2	28 ± 2	34 ± 12
2	1.0 ± 0.3	30 ± 3	30 ± 13
3	0.7 ± 0.2	22 ± 2	31 ± 13
4	0.7 ± 0.1	20 ± 1	29 ± 6
5	0.8 ± 0.2	23 ± 2	29 ± 10
6	1.0 ± 0.2	23 ± 2	23 ± 7
RS in buffer	0.9 ± 0.2	22 ± 2	24 ± 8
RS in CHO-K1	1.0 ± 0.2	24 ± 2	24 ± 7

[a]Kinetic parameters (best fit ± half range of confidence intervals) were determined by non-linear regression of Michaelis-Menten plots at 99% confidence. Samples from control (1, 2 and 3) and butyrate-treated (4, 5 and 6) bioreactors at day 8 are compared with β-glucuronidase reference standard (RS) in buffer or in parent CHO-K1 harvest (bottom). All measurements were performed in triplicate using PNPG as substrate. A Dunnett's test indicated that no significant differences were observed between control and butyrate samples ($p>0.05$).