Enzyme and Microbial Technology 89 (2016) 1–6

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Expression of human β-*N*-acetylhexosaminidase B in yeast eases the search for selective inhibitors

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ARTICLE INFO

Article history:
Received 9 November 2015
Received in revised form 29 February 2016
Accepted 7 March 2016
Available online 9 March 2016

Keywords:
β-*N*-acetylhexosaminidase
Hex B
Enzyme inhibition
O-GlcNAcase
Pichia pastoris

ABSTRACT

Human lysosomal β-*N*-acetylhexosaminidases from the family 20 of glycoside hydrolases are dimeric enzymes catalysing the cleavage of terminal β-*N*-acetylglucosamine and β-*N*-acetylgalactosamine residues from a broad spectrum of glycoconjugates. Here, we present a facile, robust, and cost-effective extracellular expression of human β-*N*-acetylhexosaminidase B in *Pichia pastoris* KM71H strain. The prepared Hex B was purified in a single step with 33% yield obtaining 10 mg of the pure enzyme per 1 L of the culture media. The enzyme was used in the inhibition assays with the known mechanism-based inhibitor NAG-thiazoline and a wide variety of its derivatives in the search for specific inhibitors of the human GH20 β-*N*-acetylhexosaminidases over the human GH84 β-*N*-acetylglucosaminidase, which was expressed, purified and used in the inhibition experiments as well. Moreover, enzyme-inhibitor complexes were analysed employing computational tools in order to reveal the structural basis of the results of the inhibition assays, showing the importance of water-mediated interactions between the enzyme and respective ligands. The presented method for the heterologous expression of human Hex B is robust, it significantly reduces the costs and equipment demands in comparison to the expression in mammalian cell lines. This will enhance accessibility of this human enzyme to the broad scientific community and may speed up the research of specific inhibitors of this physiologically important glycosidase family.

1. Introduction

Human β-*N*-acetylhexosaminidases (EC 3.2.1.52) are *exo*-glycosidases, which belong to the glycoside hydrolase family 20 (GH20) in the CAZy classification system (http://www.cazy.org). They are lysosomal dimeric enzymes composed of two subunits, α and β, which are encoded by the *HexA* and *HexB* genes, respectively. The subunits α and β are roughly 60% identical in their amino acid sequence [1,2]. These dimeric enzymes can be found as three different isoenzymes: Hex A (heterodimer of α and β), Hex B (homodimer of β) and Hex S (homodimer of α) [3,4]. Both subunits, α and β, catalyse the cleavage of terminal β-*N*-acetylglucosamine and β-*N*-acetylgalactosamine residues from a broad spectrum of glycoconjugates. The β subunit provides the hydrolysis of neutral substrates and is necessary for the binding of the G_{M2}-activator complex to Hex A. The α subunit is able to cleave even negatively charged substrates, such as 6-sulfated carbohydrates in keratan sulfate and in the most important natural substrate, the glycosphingolipid G_{M2} ganglioside [5,6]. Hex S is an unstable minor form and was found only in the patients with the Sandhoff's disease [2,7,8]. The GH20 β-*N*-acetylhexosaminidases act *via* a substrate-assisted catalytic mechanism, in which the oxazoline reaction intermediate instead of the covalent enzyme-substrate complex is formed [9].

Lysosomal β-*N*-acetylhexosaminidases play important roles in human physiology; their deficiencies cause severe neurodegenerative lipid storage disorders. Mutation in the gene *HexA* gives rise to Tay-Sachs' disease, which is best studied of this family of diseases, and mutation in the gene *HexB* leads to Sandhoff's disease, which is manifested as the failing of both Hex A and Hex B resulting in the accumulation of ganglioside G_{M2} in neuronal lysosomes. The prognosis in both diseases is very bad; they are usually fatal within 4 years of life [10,11].

At this time, there are some options for the treatment of lysosomal storage disorders, such as chemical chaperones, substrate deprivation, enzyme replacement therapy, gene therapy and bone marrow transplantation [8]. One possible route to enhance the

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http://dx.doi.org/10.1016/j.enzmictec.2016.03.003
0141-0229/

activity of β-*N*-acetylhexosaminidases bearing unfavourable mutations is the use of pharmacological chaperones, which are typically competitive inhibitors guiding the enzymes through the proper folding during their synthesis in the endoplasmic reticulum and consequently enabling the transport of the correctly folded protein into lysosomes. For this purpose, pyrimethamine has been identified as a suitable pharmacological chaperone of human Hex A in the screen of a library of FDA-approved compounds [7]. For the enzyme replacement therapy the active recombinant enzymes, whose activity is low or negligible in the patients, are used. Typically, these enzymes are produced in the mammalian cell lines, so that they naturally obtain the mannose-6-phosphate residues at the nonreducing end of the *N*-glycans, which are crucial for the contact with the receptors on the cell surface and for the incorporation into the cell [8,12]. Akeboshi et al. [8] expressed the recombinant Hex A in the methylotrophic yeast *Ogataea minuta*, which is the only microbial production of the human β-*N*-acetylhexosaminidase described in the literature so far; unfortunately, the yeast used is not commercially available. The *och1*-disrupted strain of *Ogataea minuta* (TK5-3) provided high yields of Hex A lacking the yeast-specific glycosylation, which makes this recombinant Hex A suitable for the enzyme replacement therapy. Alternatively, the glycosylation pattern of the yeast-produced enzymes for enzyme replacement therapy can be modified to generate mammalian-type *N*-glycans using a newly described glycosidase from *Cellulosimicrobium cellulans* [13].

Searching for highly selective inhibitors provides necessary tools for the study of the functions of the enzyme *in vivo*, as well as for the development of therapeutics with minimal side effects [14,15]. The general strategy is to design a compound mimicking the transition state with a special focus on the selective inhibition of just one of the two enzyme groups. In mammalian cells there is another GlcNAc-cleaving glycosidase, β-*N*-acetylglucosaminidase (*O*-GlcNAcase) from the family 84 of glycoside hydrolases, which is crucial for the regulation of a large number of physiological events. *O*-GlcNAcase displays strict specificity for a single *O*-GlcNAc unit bound to the protein [15–18]. Because of the variety of proteins modified, protein *O*-GlcNAc-ylation plays role in the etiology of metabolic diseases such as diabetes and neurodegenerative disorders like Alzheimer's disease (competition with phosphorylation in the process of tau-protein formation) [19,20]. Thus, the use of selective *O*-GlcNAcase inhibitor may offer a route to study these degenerative processes *in vivo* or may even slow down the progress of disease [14].

The oxazoline transition state analogue NAG-thiazoline was designed to prove the substrate-assisted catalytic mechanism of GH20 β-*N*-acetylhexosaminidases, showing a good inhibition potential with these enzymes [6,21]. Unfortunately, NAG-thiazoline was also found to inhibit *O*-GlcNAcase in a similar manner, which means that this inhibitor does not discriminate between the target enzyme groups [15]. In the past years, much effort was put into the design of selective inhibitors of the GlcNAc-processing enzymes, using NAG-thiazoline as the basic scaffold structure [15,22–25]. So far, the selective NAG-thiazoline-based inhibitor of *O*-GlcNAcase thiamet-G, which could be potentially used as therapeutics in the Alzheimer's disease, has been described and put into market [14].

Here, we present the facile, robust and low-cost production of recombinant human GH20 β-*N*-acetylhexosaminidase B in the commercial strain of *Pichia pastoris*. The produced enzyme was used for the inhibition assays with a series of potential inhibitors, *e.g.* derivatives of NAG-thiazoline. Moreover, the inhibition effects of the tested compounds were assayed also for the GH84 *O*-GlcNAcase, so that the selectivity of the inhibitors could be determined.

2. Materials and methods

2.1. Expression and purification of human β-N-acetylhexosaminidase B (GH20)

The sequence of the gene of human β-*N*-acetylhexosaminidase β subunit (GenBank: NM_000521; optimized sequence is presented in Fig. S1 in the Supporting information) was optimized for expression in *P. pastoris*, prepared synthetically (GENEray, China) and cloned into the yeast expression vector pPICZαA using the *Eco*RI and *Kpn*I restriction sites. The sequence of Hex β subunit was ligated to the vector without the predicted signal propeptide (aa 1–72). The expression construct pPICZαA-Hex B was electroporated to the yeast strain *P. pastoris* KM71H (Invitrogen, US) and the enzyme was extracellularly expressed following the procedure described in the manufacturer's instructions. First the expression vector was linearized by restriction endonuclease *Sac*I (New England Biolabs, US) and then the prepared competent *P. pastoris* cells were transformed with the linearized vector by electroporation according to the manufacturer's instructions (Electroporator, BioRAD, DE). The electroporated cells were grown in various concentrations under the selection pressure of zeocin (100 μg/mL) on YPD agar plates for 2–4 days at 28 °C.

For the production of the recombinant human Hex B the colonies were inoculated into 100 mL of BMGY or BMGH (EasySelect™ *Pichia* Expression Kit, Invitrogen, US) medium and incubated overnight with shaking at 28 °C. Then the cells were collected by centrifugation (3000*g*, 10 min, 20 °C) and the pellet was resuspended in 30 mL of BMMY or BMMH medium in a 300 mL baffled flask. The production of human Hex B was induced by methanol (0.5% v/v) addition every 24 h; the flasks were stirred on a rotary shaker at 28 °C and 220 rpm for three days; ca 160 colonies were screened this way prior to the stable Hex B-producing clone was found. For the large scale production of recombinant human Hex B we used 1 L of BMGH medium for overnight preculture. The cells were then collected and resuspended in 200 mL of BMMH medium and incubated at 28 °C on a rotary shaker with methanol induction (0.5% v/v) every 24 h for three days.

Recombinant human Hex B (dimer of β subunits) was purified from the culture medium of *P. pastoris* after three days of cultivation after methanol induction in a single step using cation exchange chromatography (Fractogel EMD SO_3^-, (Merck, DE) 10 mM sodium citrate–phosphate buffer pH 3.5). The proteins were eluted using a linear gradient 0–1 M NaCl (3 mL/min, 25 min), Hex B was eluted at the concentration of NaCl ranging from 0.35 to 0.45 M. The cells were separated by centrifugation (5000*g*, 10 min, 8 °C) and filtration, the obtained medium was diluted 3 times with water and the pH of the solution was adjusted to 3.5. Äkta Purifier protein chromatography system (Amersham Biosciences, US) was used for the purification.

Protein concentrations were measured using the Bradford assay (Bio-Rad, US) with bovine serum albumin as the standard. The final purity of the Hex B was checked by 10% SDS-PAGE.

For the deglycosylation of Hex B the *endo*-glycosidase Endo H (New England Biolabs, US) was used according to the manufacturer's instructions.

2.2. Expression and purification of human β-N-acetylglucosaminidase (GH84)

The gene of human *O*-GlcNAcase (GenBank: BC039583.2) was obtained from Prof. D. Vocadlo (SFU, Burnaby, BC, Canada). The gene was expressed in *E. coli* BL21(DE3) pLys, under the induction by IPTG (0.5 mM). After the cultivation at 16 °C, the medium was centrifuged (4000*g*, 15 min) and the cells were resuspended