Biochemical Engineering Journal 112 (2016) 32–41

Contents lists available at ScienceDirect

Biochemical Engineering Journal

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

Regular article

Design and preparation of chimeric hyaluronidase as a chaperone for the subcutaneous administration of biopharmaceuticals

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ARTICLE INFO

Article history:
Received 13 December 2015
Received in revised form 6 March 2016
Accepted 28 March 2016
Available online 30 March 2016

Keywords:
Recombinant DNA
Fed-batch culture
Purification
Biomedical
Long-acting hyaluronidase
Subcutaneous administration

ABSTRACT

Subcutaneous (SC) delivery of biomacromolecular pharmaceuticals such as proteins often encounter barriers in the extracellular matrix, especially the hyaluronan (HA) network. In this study, chimeric hyaluronidases were designed, prepared and tested for assisting biopharmaceuticals in ID administration in mice as replacement of SC administration. The chimeras were hyaluronidase (rhPH20) conjugated with human serum albumin (rhPH20-HSA) and antibody Fc fragment (rhPH20-Fc). Expression of the new protein was undertaken in CHO cells cultured in a 5-L disposable bioreactor. Purification was carried out by a series of chromatographic methods to obtain high-purity products of 61 kDa (rhPH20), 79 kDa (rhPH20-HSA) and 190 kDa (rhPH20-Fc). The chimeric proteins rhPH20-HSA and rhPH20-Fc performed fairly well as spreading factors in short-term trypan blue intradermal (ID) infusion in comparison with recombinant hyaluronidase (rhPH20). They extended the channel opening from 24 h (rhPH20) to 85–120 h in vivo. The specific activity of rhPH20-Fc was 35,600 U/mg, higher than that of rhPH20-HSA (10,000 U/mg). Co-administration of rhPH20-Fc with two biomacromolecular pharmaceuticals, Stelara (150 KDa) and TNFRII-Fc-IL1ra (TFI, 250 kDa), through an ID route increased the bioavailability from 86% to 93% and from 64% and 97%, respectively, compared with rhPH20. The pharmacokinetic profile of ID administrated larger TFI was significantly improved through cooperation with the long-acting hyaluronidase.

1. Introduction

Subcutaneous (SC) administration for the delivery of biomacromolecular pharmaceuticals provides better safety and convenience than intravenous (IV) administration [1]. However, the bioavailabilities of SC-administered molecules would be lower than the IV route in general, especially for macromolecules [2], when in SC administration, the drugs have to pass through the complex three-dimensional extracellular matrix (ECM) of the dermis and then traverse capillaries or lymphatics in order to reach the general cardiovascular pool [3].

Because the hyaluronan network constitutes the main filler in ECM and serves as a barrier for drug delivery, its breakup could loosen SC space and therefore enhance molecular dispersion [4]. In the past, hyaluronidases extracted from animal tissues were used as chaperone factors [5]. However, undefined impurities and heterogeneous ingredients may cause inflammation. Thus, these hyaluronidases were gradually substituted by recombinant human hyaluronidase rhPH20, which was approved by the FDA in 2005 [6]. As reported, rhPH20 efficiently elevated absolute bioavailabilities of the small molecules ondansetron, morphine and ceftriaxone to 79% [7], 103% [8] and 107% [9], respectively. For biomacromolecular pharmaceuticals, however, the results were not as significant as for the small molecules. Absolute bioavailabilities ranging from 40% to 94% were reported with rhPH20, and required the incorporation of other substances, such as heparin [10–12].

It is possible that the effect of hyaluronidase on the subcutaneous delivery of biomacromolecules is not long enough. Frost [4] reported that the ECM changes induced by rhPH20 were almost reversible within 24 h. This period may be short for macromolecules, whose convection and diffusion are much slower than small molecules. Furthermore, biomacromolecules such as proteins

Abbreviations: SC, subcutaneous; IV, intravenous; ID, intradermal; HA, hyaluronan; ECM, extracellular matrix; BTH, bovine testis hyaluronidase; HSA, human serum albumin; rhPH20, recombinant human PH20; rhPH20-HSA, recombinant human PH20-HSA; rhPH20-Fc, recombinant human PH20-Fc; TFI, TNFRII-Fc-IL1 ra.

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http://dx.doi.org/10.1016/j.bej.2016.03.013
1369-703X/

are likely to be metabolized in the SC space. Retention in the SC space will lead to longer exposure to catabolic enzymes, while the promotion of intravascular uptake would reduce degradation. The extracellular matrix in the dermis has a complex composition and is made up of an intricate stereochemical structure, which the drugs have to pass through. Therefore, enough open time and space for the ECM "channel" may be helpful to minimize local entrapment and degradation, facilitating the macromolecule diffusion to the vascular compartment successfully. The question is why rhPH20 has so short an action time. Like other proteins, the hyaluronidase can be damaged in the subcutaneous environment through hydrolysis, enzymatic degradation, antigen-antibody reactions, or macrophage ingestion. Although the mechanism is not clear, it would be worthwhile to construct a new hyaluronidase with better durability than the current product against damaging factors, prolonging its role as a channel opener.

The strategy in this paper was to conjugate hyaluronidase with human serum albumin (HSA) or the IgG Fc portion. HSA is the most abundant protein in blood plasma and is widely used as a stabilizing agent for therapeutic proteins in drug formulations. It consists of three conserved homologous structural motifs, each carrying a binding site specific for affinity chromatography [13]. Fusion of heterologous peptides with the IgG Fc portion provides stability for natural proteins [14]. Both HSA and Fc fragments have been used for the conjugation of pharmaceutical drugs and are safe in vivo [15]. Bearing this in mind, we explored whether these reconstruction techniques could improve stability in the dermis and thus exert long-lasting effects. Two pharmaceutical proteins, Stelara (150 kDa) and TFI (250 kDa), were chosen as model molecules to co-administered for pharmacokinetics study. Stelara, also named as ustekinumab, is used to treat patients with relapsing-remitting multiple sclerosis [16]. TFI (TNFR2-Fc-IL-1ra) is a bifunctional ligand with enhanced anti-inflammatory effect [17].

2. Materials and methods

2.1. Materials

Escherichia coli strain Top10 and the cloning plasmid pMD18-T were purchased from Transgene Inc. (Beijing, China) and Takara Bio Inc., (Otsu, Shiga, Japan), respectively. The GC-rich vector pMH and CHO-S cells (routinely cultured in DMEM/F12 or B001 medium) were available in our laboratory. EcoRI and NotI restriction enzymes, T4 DNA ligase and the DNA gel extraction kit were from Takara Bio Inc., (Otsu, Shiga, Japan). All cell culture and transfection reagents were obtained from Invitrogen Corporation (Carlsbad, California, USA) unless specified. The His-tag affinity columns, SP Fast Flow, Q Fast Flow and MabSelect columns were purchased from GE Healthcare (Piscataway, New Jersey, USA). Goat anti-human IgG*HRP, mouse anti-His tag and rabbit anti-human PH20 antibody were obtained from Santa Cruz (Santa Cruz, CA, USA). Bovine testes hyaluronidase (BTH) powder was from Shanghai No. 1 Biochemical Pharmaceutical Co. (Shanghai, China). Nude Balb/c mice (6–8 weeks of age) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All animal experiments were in compliance with the Institutional Ethical Committee for animal care guidelines.

2.2. Gene construction and transformation

The plasmid templates for human hyaluronidase PH20, the Fc fragment of human immunoglobulin, human serum albumin, the murine Igκ chain leader sequence and the pMH3 plasmid as expression vector were obtained from AmProtein Inc. (San Gabriel, CA, USA). Primers, shown in Table 1, were designed using Vector NTI and synthesized by Sangon Biotech (Shanghai, China). The pMD18-T plasmid as cloning vector, EcoRI and NotI restriction enzymes, T4 DNA ligase and the DNA gel extraction kit were purchased from Takara Bio Inc. (Dalian, China). The *E. coli* strain Top10 was from Transgene Inc. (Beijing, China).

The scheme for gene construction is delineated in Fig. 1. The gene fragment of PH20 with the EcoR I site synonymously mutated was constructed by overlay PCR using the touch-down procedure with the F1, R1, F2 and R2 primers. Then, the rhPH20 sequence was added with the Igκ signal peptide for protein secretion by overlay PCR, with the F3, R3, F1 and R2 primers. Available rhPH20 with the Igκ signal peptide was then used as the template to overlay with HSA domain I and the Fc fragment of IgG, respectively, for constructing chimeric genes of rhPH20-HSA and rhPH20-Fc. The recombinant genes were all flanked on either side by EcoR I and Not I restriction sites through the PCR primer sequences. The lengths of the gene fragments were verified by DNA electrophoresis.

The chimeric genes were recycled and subcloned into pMD18-T through AT ligation. The mixture was transformed into Top 10 cells to select positive clones. The fidelity of the genes to their original design was verified by PCR, double enzymatic digestion of extracted plasmid with EcoR I and Not I, and DNA sequencing by Sangon (Shanghai, China). The recombinant cloning vectors were digested with EcoR I and Not I, and then the gene fragments were recycled and ligated to the pMH3 expression vector which was similarly digested and recycled. The resulting recombinant plasmids were transformed into CHO host cells along with salmon sperm DNA by electroporation twice at 160 V, 15 ms pulses using an electroporator (Bio-Rad, Richmond, CA, USA).

2.3. Clone screening and recombinant protein expression

For the screening of clones, the transfected cells were maintained in DMEM/F12 medium containing 10% fetal bovine serum (FBS: Invitrogen Life Technologies, Carlsbad, CA, USA) at 37 °C, 5% CO_2. Then, 50 mg/L G418 (Sigma-Aldrich, Shanghai, China) was added two days later. After one week of growth to form visible colonies, pipet tips were used to pick up the cell clumps and the contents were transferred into one well of a 96-well tissue culture plate (Corning, NY, USA). After that, single colonies were enriched through sub-culturing from 96-well plates into 24-well plates in D/F12 medium containing 100 mg/L G418 and subsequently into T25 flasks. The supernatants were collected from either the 96- or the 24-well plates or T25 flasks for the initial assessment of hyaluronidase activity by turbidimetry.

These adherent cultured clones were domesticated and propagated in serum-free medium B001 (AmProtein Inc., San Gabriel, CA, USA) in 40-mL flasks for 2 or 3 weeks. Batch cultures were carried to test the basic expression level for each clone. Clones with the highest expression level were expanded to 1 L spinner flasks, and then transferred to a 5 L bioreactor at an inoculation density of 2.0×10^6 viable cells per mL in B001 medium [18]. We chose single-use bioreactors to carry out cultivation process for their unique features, such as simple operation and low shear force. The parameters were 37 °C, pH 7.2, DO 10–30% and an air overlay of 0–100 cc/min. When the cell density reached $5–6 \times 10^6$, the temperature was reduced to 34 °C, and F001 (AmProtein Inc.) feed medium was added to maintain the residual glucose level at 2 g/L. After approximately 10 days of cultivation, the supernatants were harvested by centrifugation at 5000 rpm for 6 min for purification.

2.4. Purification of recombinant hyaluronidases

Chromatography columns (Q Sepharose FF, Phenyl Sepharose (low sub), SP FF, Blue Sepharose FF, Mabselect and Superdex-200) and the chromatography system (ÄKTA prime) were products of