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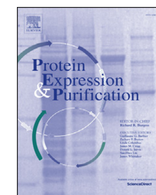

Heparin-binding peptide as a novel affinity tag for purification of recombinant proteins

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ABSTRACT

Purification of recombinant proteins constitutes a significant part of the downstream processing in biopharmaceutical industries. Major costs involved in the production of bio-therapeutics mainly depend on the number of purification steps used during the downstream process. Affinity chromatography is a widely used method for the purification of recombinant proteins expressed in different expression host platforms. Recombinant protein purification is achieved by fusing appropriate affinity tags to either N- or C- terminus of the target recombinant proteins. Currently available protein/peptide affinity tags have proved quite useful in the purification of recombinant proteins. However, these affinity tags suffer from specific limitations in their use under different conditions of purification. In this study, we have designed a novel 34-amino acid heparin-binding affinity tag (HB-tag) for the purification of recombinant proteins expressed in *Escherichia coli (E. coli)* cells. HB-tag fused recombinant proteins were overexpressed in *E. coli* in high yields. A one-step heparin-Sepharose-based affinity chromatography protocol was developed to purify HB-fused recombinant proteins to homogeneity using a simple sodium chloride step gradient elution. The HB-tag has also been shown to facilitate the purification of target recombinant proteins from their 8 M urea denatured state(s). The HB-tag has been demonstrated to be successfully released from the fusion protein by an appropriate protease treatment to obtain the recombinant target protein(s) in high yields. Results of the two-dimensional NMR spectroscopy experiments indicate that the purified recombinant target protein(s) exist in the native conformation. Polyclonal antibodies raised against the HB-peptide sequence, exhibited high binding specificity and sensitivity to the HB-fused recombinant proteins (~10 ng) in different crude cell extracts obtained from diverse expression hosts. In our opinion, the HB-tag provides a cost-effective, rapid, and reliable avenue for the purification of recombinant proteins in heterologous hosts.

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1. Introduction

Recombinant proteins have gained major interest in the world of pharmaceuticals and therapeutics. Commercial applications of recombinant proteins include therapeutics, diagnostic tools, drug design, and instruments in proteomics [1,2]. The advent of recombinant DNA technology has facilitated the overexpression of target proteins in heterologous host cells and has revolutionized the field of protein biotechnology. Tandem development of affinity chromatography contributed to the rapid and efficient purification of recombinant proteins expressed in different host cells with much larger yields *per se* [3–5]. Typically, purification by affinity chromatography requires an affinity tag fused to the recombinant protein of interest [6]. In general, affinity tags used for protein purification are expected to possess the following properties: (a) high binding specificity to a resin coupled to one of the metal/substrate/protein affinity partner(s) but not limited to; (b) versatility to be fused with a wide range of target recombinant proteins; (c) non-interference in the folding of the target recombinant protein to retain its biological active conformation; and (d) amenability

Abbreviations: HB, heparin-binding; GST, glutathione-S-transferase; GSH, reduced glutathione; IPTG, isopropyl thiogalactopyranoside; PMSF, phenyl methyl sulfonyl fluoride.

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for selective and easy release from the target recombinant protein either by chemical or enzymatic cleavage methods. The most commonly employed affinity tags include polyhistidine (His-tag), glutathione-S-transferase (GST), maltose-binding protein (MBP), thioredoxin, and several others [4,7]. The currently available affinity tags have specific limitations in their use due to: 1. Incompatibility with the conditions at which the recombinant target protein is stable and biologically active; 2. Degradation/aggregation of the affinity tag; 3. Instability under denaturing conditions; 4. Cost-effectiveness of the overall purification process involving the affinity tag. Because of the various shortcomings of the existing affinity tags, there is a strong need for the development of a novel affinity tag that avoids most of the drawbacks. Recently, peptides have shown promise as purification affinity tags in that they are not only more chemically stable than large protein tags but also exhibit lower toxicity to the host cells [8].

Heparin, the most negatively charged poly-sulfated polyanionic member of the glycosaminoglycans, is mainly found on the surface of the cells and in the extracellular matrix region. It plays an important role in different key cellular processes. Several proteins are known to have strong heparin-binding properties [9–14]. Critical analysis of heparin-binding pockets in these proteins revealed a specific distribution of positively charged amino acid residues, which are primarily involved in electrostatic interaction with the negatively charged heparin. Such electrostatic interactions have been exploited to purify several positively charged proteins using cation exchange chromatography [15,16]. Studies performed by Cardin and Weintraub have shown that heparin-binding proteins (HBPs) contain consensus sequences or strings known as heparin-binding motifs that are responsible for their interaction specifically with heparin [17]. Some of the common putative heparin-binding segments were XBBXBX, XBBBXXBX, and XBBXXBBBXXBBX, where B is one of the three basic amino acids (arginine, lysine or histidine) and X is any of the other 17 natural amino acids [18–21].

Heparin-binding proteins such as fibroblast growth factors [22], antithrombin [23], hepatic and lipoprotein lipase [24,25], fibronectin [26], chemokines [27,28], and others have specific intrinsic affinity to bind strongly to heparin. Owing to their inherent strong binding affinity to heparin, HBPs can be readily purified to homogeneity using a single step by heparin-Sepharose affinity chromatography without the requirement of additional affinity tags. Based on the available knowledge of the amino acid sequence and structural determinants of heparin-binding affinity, in the present study, we have designed an overexpression vector that encodes a novel 34-amino acid heparin-binding (HB) peptide affinity tag fused to the nucleotide sequence of a non-heparin-binding target protein(s). The usefulness of the HB-tag in the single-step purification of different non-heparin-binding recombinant target proteins has been successfully demonstrated. In addition, polyclonal antibodies raised against the HB-tag have been shown to provide an avenue for the sensitive and specific detection of HB-fused recombinant proteins. In our opinion, the HB-tag is an immensely valuable affinity tag that can facilitate the simple, efficient, and reliable purification of recombinant proteins expressed in heterologous expression hosts.

2. Materials and methods

Low-molecular-weight heparin was purchased from Sigma (St.Louis, MO). Heparin-Sepharose and GSH-Sepharose resins were purchased from GE Healthcare Bio-sciences (Pittsburgh, PA). All other general reagents were purchased from VWR Scientific (Radnor, PA). All antibodies were purchased from Genescript (Piscataway, NJ) using our specifications.

2.1. Solid-state synthesis of HB-peptide

Using FastMoc® chemistry, HB peptide was synthesized on a rink amide resin (RAM™) mainly used for synthesis of peptide carboxamides with a modified protecting linker 4-((2,4-dimethoxyphenyl) (Fmoc-amino)methyl) phenoxyacetic acid attached to the bead. The protecting group, 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (PBF) present on the peptide that is bound to the resin was successfully cleaved with trifluoroacetic acid (TFA). Further, the synthetic peptide mixture along with other contaminants were loaded on to PRP3 reverse-phase HPLC column, and the impurities were removed using a linear gradient of water:acetonitrile. Purity of the peptide was confirmed by MALDI-TOF MS analysis.

2.2. Construction of novel heparin-binding affinity fusion tag (HB-tag)

The nucleotide sequence corresponding to HB tag was constructed by GeneArt, Life Technologies Inc. using the *Escherichia coli* codon optimized gene synthesis facility. A thrombin cleavage site (-LVPRGS-) was added at the C-terminus of the affinity tag to allow the cleavage of the target protein from the tag. The amino acid sequence of the recombinant HB peptide is shown below: MASKAQKAQAKQWKQAQKAQKAQAKQAKQAKQW.

2.3. Measurement of heparin-binding affinity of HB-peptide using isothermal titration calorimetry

All isothermal titration calorimetric experiments were performed using iTC200 (MicroCal Inc., Northampton, MA) at 25 °C. The lyophilized HB-peptide, cleaved from its target protein, was dissolved in 10 mM sodium phosphate buffer containing 100 mM NaCl at pH 7.2, resulting in a final concentration of 200 μM HB-peptide, determined by absorbance spectroscopy at 280 nm. Low-molecular-weight (~3000 Da) heparin was dissolved in the same buffer to achieve a concentration of 2 mM. All samples were degassed under vacuum and equilibrated prior to the experiment. Heparin solution inside the syringe was titrated into the cell containing HB-peptide. Titration curves were analyzed using Origin Software supplied by MicroCal Inc. The raw ITC curves were best fitted to a one-binding-site model.

2.4. Cloning of C2A, S100A13, and CAlb3 into HB vector

The target proteins chosen (C2A domain (MW ~ 16 kDa) of mouse synaptotagmin-1 (C2A domain); mouse S100A13 (MW ~ 12 kDa) and the C-terminal domain of Alb3 (MW ~ 14 kDa) (CAlb3) from *Arabdopsis thaliana*), to demonstrate the usefulness of the HB-tag, are not known to be heparin-binding proteins and do not have significant affinity to bind to heparin-Sepharose resin. Two expression vectors pET28a and pET22b from Novagen Inc. were used to generate the constructs consisting of the HB affinity tag. C2A gene was cloned into pET28a-HB and the genes S100A13 and CAlb3 were cloned into pET22b-HB vectors.

2.5. Overexpression and purification of recombinant HB-fusion proteins

The recombinant pET-28a-HB-C2A fusion construct was used to transform the BL21* *E. coli* expression host, whereas the pET-22b-HB fusion constructs were used to transform the BL21 (DE3) cells for S100A13 and Rosetta DE3 cells for CAlb3. Resulting transformants were grown in Luria-Bertani (LB) or Terrific (TB) medium containing kanamycin (100 μg/ml) for HB-C2A or ampicillin

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