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Production of MBP–HepA fusion protein in recombinant *Escherichia coli* by optimization of culture medium

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Abstract

Enhanced production of MBP (maltose-binding protein)-heparinase I (HepA) fusion protein in recombinant *Escherichia coli* was achieved by the optimization of the M9-based culture medium. First, by using the modified expression vector capable of releasing the C-terminus of HepA free to improve the specific activity of MBP–HepA, characteristics of the purified fusion protein were analyzed, which were similar to those of the native HepA except for the decreased affinity towards the substrate. M9-based culture medium was subsequently optimized for the enzyme production by orthogonal experimental design in shake flasks. Three major components were examined, namely glucose, yeast extract and calcium ion. The recombinant *E. coli* was further cultivated in a fermentor. As a result, total activity of HepA reached 20,650 IU l^{-1} in the optimized medium by a fed-batch mode in the 5-l fermentor. This study indicated that effective production of MBP–HepA by the present system would facilitate the large scale preparation of low molecular weight heparin (LMWH), which is a useful anticoagulant drug.

Keywords: Fusion protein; HepA; MBP; Orthogonal experimental design; Recombinant *Escherichia coli*; Vector modification

1. Introduction

Heparin is a complex glycosaminoglycan composed of alternate sequences of uronic acid (usually L-iduronic acid) and D-glucosamine linked by (1,4) bonds, with sulphate groups in the glucosamine ring. Heparinase I (HepA) is one of the bacterial polysaccharide lyases that degrade heparin and heparan sulfate [1]. The mechanism for HepA depolymerization of heparin has been studied by Ernst et al. [2], showing that HepA mainly starts with an exolytic processive mechanism for depolymerization from the non-reducing end and then cleaves the same molecule processively [2]. HepA is an important enzyme in the production of low molecular weight heparin (LMWH) as an anticoagulant drug [3]. However, the application of HepA in large scale production of LMWH has been hindered by the high cost of the enzyme due to the low productivity by the original heparinases producer of *Flavobacterium heparinum*, as well as the difficulties in the separation of HepA from heparinase II and III which are simultaneously produced [4]. Although the HepA gene has been cloned, it has been shown that its expression in *E. coli* is prone to aggregate into insoluble inclusion bodies, which is also difficult for refolding [5–7].

In order to establish an enzymatic method for the production of LMWH, economical and effective production of heparinase I and the subsequent enzyme separation and the enzymatic reaction process should be taken into account. From the viewpoint of process integration, protein fusion strategy, normally by using a fusion tag with affinity to some chemicals, is a useful approach for enzyme production, separation and immobilization to reduce the cost of the whole bioprocess. In our previous study, functional expression of HepA in *E. coli* has been achieved by fusion to maltose-binding protein (MBP) [8]. MBP is an ideal fusion partner not only because it can effectively enhance the solubility of attached proteins [9], but also because it can simplify the purification of the fusion protein by the effective affinity purification process [8]. Our recent study has also shown that the purified MBP–HepA fusion protein can degrade heparin effectively to produce LMWHs with the weight average molecular weight (M_w) less than 3000 and narrow polydispersity by controlling the reaction time,

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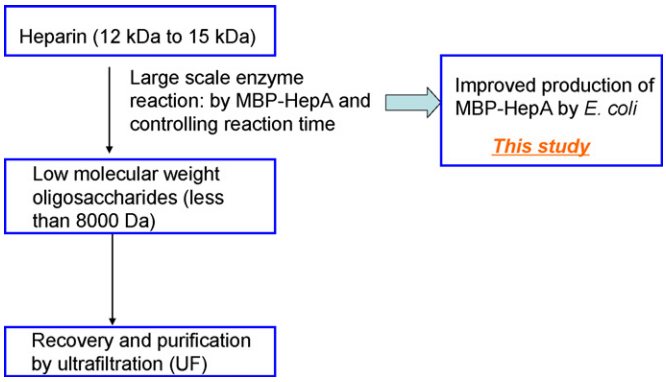

Scheme 1. Flow of enzymatic degradation of heparin to produce LMWHs.

implying that an enzymatic ultrafiltration (UF) membrane bioreactor with cut-off molecular weight of less than 10,000 will be possible for the separation of LMWHs [10]. In addition, benefiting from its affinity to maltose, MBP has the potential application in the oriented immobilization of MBP-fused proteins on maltose-coating particles [11], which can possibly enable the effective production of LMWHs. Incidentally, the UF membrane bioreactor can also help for the filtration of the *E. coli*-originated pyrogens, which need to be removed from LMWHs.

The schematic flow for the enzymatic preparation of LMWHs from heparin is shown in Scheme 1. As mentioned above, since the major obstacle for industrial enzymatic depolymerization of heparin is due to the high cost of the *F. heparinum*-originated HepA, effective production of MBP–HepA with high activity in recombinant *E. coli* system will contribute to the development of the enzymatic process. However, the activity of the MBP–HepA by recombinant *E. coli* constructed in our previous study [8] is still much lower (218 U l^{-1}) than the enzymatic activity of CBD-HepA, which was produced and refolded from the recombinant *E. coli* (10,500 IU l^{-1}) [7]. Thus, further study for the practical production of MBP–HepA with high activity should be performed for the purpose of the enzymatic preparation of LMWHs. The aim of this study was to establish the production system of MBP–HepA with high activity in recombinant *E. coli*. Since two putative calcium binding regions in C terminus of HepA have been identified (CB1 Glu^{207}-Ala^{219} and CB2 Thr^{373}-Arg^{384}) and have been suggested to play a prominent role in calcium activation of the enzyme [12], an improved expression vector (pMHS) was constructed by releasing the C-terminus of HepA free from the MBP–HepA fusion protein. Based on the examination of the enzymatic kinetics of MBP–HepA, optimization of the basal M9 medium with carbon sources, nitrogen sources and additional calcium ion was carried out to improve the enzyme productivity by the recombinant *E. coli* TB1 [pMHS]. As a result, the modified M9 medium with additional yeast extract and calcium ion supplemented with glucose was found to be effective for both the cell growth and enzyme production. Total enzyme activity was dramatically increased to 20,650 IU l^{-1} by growing the recombinant *E. coli* in a 5-l fermentor by a fed-batch mode.

2. Materials and methods

2.1. Bacterial strains, expression vectors and media

A modified expression vector of pMHS was constructed as follows to produce MBP–HepA fusion protein. *HepA* was amplified from the plasmid of pMHL [8] (with forward primer 5′-GCCTGGATCCCAGCAAAAAAAATCCGGTAAC-3′ and reverse primer 5′-CTTAAAGCTTTTACTATCTGGCAGTTTC-GCTGTAC-3′) and inserted into plasmid pMAL-c2x (New England Biolabs) to generate pMHS. The underline indicated the two consecutive stop codons. Vector pMHS was different from pMHL in that only MBP–HepA fusion protein could be expressed, where the C-terminus of HepA was made free by the insertion of the stop codons. *E. coli* TB1 was purchased from New England Biolabs (NEB). Recombinant *E. coli* cells containing the expression vectors were grown in either Luria Bertani (LB) medium or modified M9 medium (see below) supplemented with 100 μg ml^{-1} ampicillin.

2.2. Optimization of culture medium

Preliminary results showed that higher productivity of MBP–HepA was obtained in M9-based medium ($Na_2HPO_4 \cdot 12H_2O$ 17.1 g l^{-1}, KH_2PO_4 3.0 g l^{-1}, NaCl 0.5 g l^{-1}, NH_4Cl 1.0 g l^{-1} and 0.1 mM $MgSO_4$) supplemented with glucose than in LB medium. Thus, effects of the carbon and nitrogen sources added into the basal M9 medium on the enzyme production were examined by using 200 ml shake flasks in duplicate. Different carbon sources (glycerol, maltose, lactose, sucrose and glucose) were tested for the enzyme production and yeast extract was added as additional nitrogen source.

The method for optimization of the M9-based medium was carried out by the approaches as reported elsewhere [13]. First, *one-at-a-time* method was used. One factor at three to five different levels was tested without changing the other components in the basal M9 medium. Then the significant factors of the medium were further tested by *orthogonal array* [13]. Three major factors with three levels were examined by *orthogonal array* including glucose (1, 4 and 8 g l^{-1}), yeast extract (10, 12.5 and 15 g l^{-1}) and calcium ion (0.1, 0.3 and 0.5 mM). The 3×3 orthogonal table was employed and gave nine different combinations as shown in Table 1.

The modified M9 medium (M9 basal salts with glucose and yeast extract) after *orthogonal array* was further tested by *one-at-a-time* again with higher concentrations of glucose (up to 20 g l^{-1}) and calcium (up to 5 mM). The optimized M9-based medium obtained from the above experiments (named as M9YGC, consisting of M9 basal salts, 12.5 g l^{-1} yeast extract, 12 g l^{-1} glucose and 1.0 mM $CaCl_2$) was used for the cell cultivation and the production of MBP–HepA unless otherwise stated. In the statistic analysis, F value is used to estimate the importance of the three significant factors. The larger the F value, the more significant the factor was.