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硕士学位论文

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重组人胶原绑定骨形态发生蛋白 2 在大肠杆菌中的表达、纯化与复性

Expression, purification and renaturation of recombinant human collagen-binding bone morphogenetic protein-2 from *Escherichia coli*

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中文摘要

重组人胶原绑定骨形态发生蛋白2在大肠杆菌中的表达、纯化与复性

骨形态发生蛋白2（bone morphogenetic protein-2，BMP2）作为骨形态发生蛋白（bone morphogenetic protein，BMPs）家族中的一员，可作用于骨修复和骨再生。为控制其在体内的释放，为解决BMP2在体内半衰期短、易随血液流失等缺点，对天然BMP2进行改造，增加了一个胶原绑定结构域（collagen-binding domain, CBD）。原核表达系统具有成本低、产量高、操作简单、遗传背景清楚等特点，广泛应用于重组蛋白的制备。本研究利用大肠杆菌（*Escherichiacoli*，*E.coli*）表达系统制备携带有CBD的人源BMP2。并对重组蛋白的构建、表达、纯化、复性以及活性检测进行了研究。

方法：本研究构建含有GST纯化标签的重组人源BMP2(recombinanthuman BMP2， rhBMP2)重组质粒 pGEX-6P-1/BMP2，转化入大肠杆菌 BL21 菌株内,18 ℃条件下添加诱导剂异丙基硫代半乳糖苷（isopropyl-β-d-thiogalactoside，IPTG）持续震荡表达，加入IPTG浓度分别为0.1、0.2、0.5和1.0 mmol/L，诱导时间为 2 h、4 h 以及 16 h；同时构建含有 6×His 纯化标签的 rhBMP2、CBD-BMP2的重组载体pET21b/BMP2、pET21b/CBD-BMP2。转化入大肠杆菌BL21菌株内，于37 ℃、IPTG浓度为1 mmol/L条件下持续震荡表达。采用镍离子亲和层析柱进行纯化，选用15% SDS-PAGE对三种融合蛋白的表达情况进行分析。采用透析复性的方法，在不同透析外液中进行复性，依据复性前蛋白质浓度不同以及是否添加左旋精氨酸进行分组，低温4 ℃条件下缓慢降低变性剂尿素的浓度对BMP2单体进行复性，采用15%SDS-PAGE分析重组蛋白的复性效果。利用MC-3T3-E1细胞对CBD-BMP2的生物活性进行表征，复苏后细胞37 ℃孵育24 h后按每孔1×10^4个细胞接种于24孔板中，分别加入重组蛋白CBD-BMP2（或商品化BMP2），将复性后CBD-BMP2配制4个浓度梯度（25 ng/mL、50 ng/mL、75 ng/mL、100 ng/mL），以相应浓度的商品化BMP2作为对照对照系列，继续培养 3 d，通过检测 3 d 后细胞中碱性磷酸酶的活性对重组

BMP2 的成骨诱导能力进行鉴定。将 CBD-BMP2 与胶原支架绑定，37 ℃释放 6 h、12 h、24 h、3 d、5 d、7 d 和 8 d 后取出胶原支架，分别检测蛋白释放量并绘制释放曲线。比较了不同的释放时间对检测结果的影响，从而选择最佳的释放时间。

结果：采用传统的 GST 标签表达 BMP2 过程中，IPTG 浓度对蛋白表达量影响较小，诱导表达 4 h 时 BMP2 表达量较好且呈可溶性表达，诱导时间的延长，目的蛋白呈现包涵体表达，与 6×His 标签蛋白相比，表达量显著减少；改用 6×His 标签蛋白表达过程，重组蛋白以包涵体形式大量表达，采用 8 mol/L 尿素对包涵体进行溶解，结果显示包涵体溶解率较高达到，经过镍柱亲和层析纯化，目的蛋白单体存在于洗脱液 B 中，BMP2、CBD-BMP2 单体相对分子质量均约为 13000；对 BMP2、CBD-BMP2 进行复性尝试中复性前重组蛋白单体浓度在小于 100 μg/mL、左旋精氨酸存在的条件下复性效果良好，SDS-PAGE 分析显示重组蛋白单链成功复性为二聚体结构，相对分子质量均约为 26000。体外实验表明, CBD-BMP2 对 MC-3T3-E1 细胞表现出良好的成骨诱导能力,但成骨诱导活性略低于商品化的 BMP2; 与不携带有 CBD 的商品化 BMP2 进行蛋白释放比较，携带有 CBD 的重组蛋白具有良好的缓释效果。

结论：传统的 GST 标签重组 BMP2 蛋白可溶性表达过程中表达了量较低，不符合进一步实验要求；本实验改用 6×His 标签表达，重组蛋白以包涵体形式大量表达，并利用优化的透析复性法对 BMP2、CBD-BMP2 进行复性；比较了携带有胶原绑定结构域的蛋白质的生物活性检测的实验方法，确定了最佳的检测方法，对 CBD-BMP2 生物活性进行检测并显示复性成功，并与商品化 BMP2 相比与胶原支架结合后具有缓慢释放的作用。

关键词：

胶原绑定结构域；骨形态发生蛋白 2；大肠杆菌；包涵体；复性

Abstract

Expression, purification and renaturation of recombinant human collagen-binding bone morphogenetic protein-2 from *Escherichia coli*

Bone morphogenetic protein-2(BMP2), as a member of BMPs, plays an important role in bone regeneration and repair, to control its distribution in vivo and solve the problem about its short half-life and diffusion with the blood, we reformed the natural protein by addinga collagen-binding domain(CBD). *Escherichiacoli* has several merits, such as the low cost, high-yield, simply manipulation, clear heredity background and so on, it has been used to prepare the recombinant protein commonly. In this article, recombinant human bone BMP2 with CBD was prepared from *Escherichia coli*. Construction expression, purification, refolding and activity assay of the recombinant protein were studied.

Method: The recombinant vector pGEX-6P-1/BMP2, containing GST tag and recombinant human BMP2(rhBMP2)gene segment was construct and transformed into *E.coli* BL21, the expression of recombinant protein was induced by using isopropyl β-D-thiogalactopyranoside (IPTG) and stirred at 18 ℃. IPTG concentrations were 0.1, 0.2, 0.5 and 1.0 mmol/L, Induction time were 2 h, 4 h and overnight. Another two recombinant vector pET21b/BMP2, containing 6×His tag and BMP2 gene segment, and recombinant vector pET21b/CBD-BMP2, containing 6×His tag and CBD-BMP2 gene segment, were construct and transformed into *E.coli* BL21. The expression of recombinant protein was induced using IPTG(1 mmol) and stirred at 37 ℃, nickel chelate chromatography was used to purify the recombinant monomer. The expression of three recombinant protein were detected by SDS-PAGE method; Dialysis refolding method using different refolding buffers was selected to refold the BMP2 monomer, groups were divided based on different

protein concentration before refolding and whether to add L-arginine, the concentration of denaturant was reduced slowly to refold the BMP2 monomer at 4℃;the expression was detected by 15% SDS-PAGE method; The biological activities of CBD-BMP2 was characterized by MC-3T3-E1 cells, incubated the resuscitated MC-3T3-E1 cells at 37 ℃ for 24 h, then inoculated the MC-3T3-E1 cells at 24-hole-board,and each hole has 1×10^4 cells, added recombinant protein CBD-BMP2 or commercial BMP2 respectively, added 4 different concentrations of refolded BMP2（25 ng/mL、50 ng/mL、75 ng/mL and 100 ng/mL）to incubate with cells for 3 d, the corresponding concentration commercial BMP2 were standard controls, examined the ALP activity of MC-3T3-E1 after incubating for estimating the osteoinducation ability of recombinant BMP2. Then we linked the CBD-BMP2 to the collagen scaffolds and continued incubating at 37 ℃, at various time points(6 h、12 h、24 h、3 d、5 d、7 d and 8 d), the scaffolds was removed and the released protein amount was determined to make the release cure. Compared the effects of different release times on the testing results in order to selete the optimum release time.

Results: During the expression progress of BMP2 containing GST purification tag, the concentration of IPTG had less influence on expression quantity, the yield of BMP2 was considerable and the BMP2 showed solubility when induced for 4h, extended the induced time, recombinant protein expressed as inclusion bodies, the expression quantity was much less than that of recombinant protein containing of 6×His purification tag. The recombinant BMP2 containing of 6×His purification tag abundantly expresses as inclusion bodies, we used 8M urea to dissolve the inclusion bodies, the results showed a higher rate of dissolution, after purification using nickel chelate chromatography, the purified recombinant protein BMP2 and CBD-BMP2 existed in elution buffer B with relative molecular mass about 13000; the refolding of BMP2 and CBD-BMP2 performed considerable results when the concentration of recombinant before refolding was lower than 100 μg/mL and the refolding buffer contained L-Arginine, the SDS-PAGE results indicated that the monomer was

successfully refolded into dimer with relative molecular mass about 28000. In vitro, the CBD-BMP2 manifested a good but the osteogenic ability of CBD-BMP2 was slightly lower than that of commercial BMP2, the recombinant protein with CBD showed a flat release curve comparing the commercial BMP2 without CBD.

Conclusions: recombinant BMP2 consists of GST purification tag expresses a small amount of soluble protein, do not meet the requirements of further experiments; so we apply 6×His purification tag to replace the GST purification tag, recombinant protein abundantly expresses as inclusion bodies, the BMP2 and CBD-BMP2 are refolded by using optimized dilution method， the bioactivity testing experiment schemes of the protein with CBD are compared and determine the best detection method, we use this method to test the biological activity of CBD-BMP2 and prove its succeed in refolding. Comparing with the commercial BMP2, the CBD-BMP2 has a slow release after combined with collagen scaffold.

Key words:

collagen binding domain，bone morphogenetic protein 2，*Escherichiacoli*，inclusion body，renaturation

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