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技术与方法

重组人bFGF的原核表达及功能分析

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摘要 为研究重组人碱性成纤维细胞生长因子(human basic fibroblast growth factor, bFGF)的原核表达及纯化条件, 并分析其功能, 该研究根据*bFGF*基因序列, 优化密码子, 构建pET-28a原核表达载体, 经IPTG诱导, SDS-PAGE电泳分析验证, 采用Ni柱进行分离纯化目的蛋白bFGF。培养NIH3T3细胞、HEK293细胞及CHO细胞, 并进行CCK-8实验检测蛋白活性。结果显示, 成功构建原核表达载体。经电泳分析, 在1 mmol/L IPTG诱导条件下, 成功表达目的蛋白bFGF, 表达量约占菌体蛋白量32%, 蛋白纯度约为96%。活性检测结果显示, ED_{50}分别为5.97 ng/mL、4.21 ng/mL、6.71 ng/mL, 可以有效促进NIH3T3细胞、HEK293细胞及CHO细胞增殖。该研究得出, 经原核表达系统成功表达人bFGF蛋白且其活性较高。

关键词 大肠杆菌; 重组人bFGF; 密码子优化; 表达; 纯化

Prokaryotic Expression and Functional Analysis of Recombinant Human bFGF

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Abstract In the present study, we studied the prokaryotic expression and purification of recombinant human basic fibroblast growth factor (bFGF), and analyzed its functions. Firstly, according to the *bFGF* gene sequence, the codon was optimized and the prokaryotic expression vector of pET-28a was constructed. And protein expression was induced by IPTG. Secondly, The expression of bFGF was determined by SDS-PAGE electrophoresis and confirmed by SDS-PAGE. Finally, NIH3T3 cells, HEK293 cells and CHO cells were cultured and CCK-8 assay was performed to detect protein activity. The results showed that the prokaryotic expression vector was successfully constructed. The target protein bFGF was successfully expressed under the induction of 1 mmol/L IPTG. The expression amount was about 32% of the bacterial protein and the protein purity was about 96%. The results of the activity test showed that the ED50 was 5.97 ng/mL, 4.21 ng/mL, and 6.71 ng/mL, which could effectively promote the proliferation of NIH3T3 cells, HEK293 cells, and CHO cells. This study demonstrates that the human

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bFGF protein was successfully expressed by prokaryotic expression system and its activity was high.

Keywords *E.coli*; bFGF; codon optimization; expression; purification

碱性成纤维细胞生长因子(human basic fibroblast growth factor, bFGF)又叫肝素结合生长因子(heparin binding growth factor), 分子量为18 kDa左右, 为单链阳离子多肽, 主要来源于内皮细胞, 是成纤维细胞生长因子家族中的一员[1-6]。bFGF是由垂体和下丘脑分泌的能促进中胚层和神经外胚层细胞分裂及血管形成的促进细胞生长的多肽多功能生长因子[7-8], 在体内具有强的神经原效应, 在中枢神经系统功能发挥重要作用[9-10]。在体外, bFGF在创伤愈合及肢体再生中能够修复损害的内皮细胞, 促进新血管形成, 在生物学作用中有组织重建功能, 在创面的保护和恢复方面具有明显优势[11-15]。bFGF与酸性成纤维细胞生长因子相比, 对pH敏感度较低、蛋白活性较高、促进细胞生长的能力更强[16]。外源性bFGF蛋白通过激活ERK-MAPK路径在细胞中发挥作用[17] 。由于大肠杆菌(*Escherichia coli*, *E.coli*)表达异源重组蛋白具有省时、易培养、生长迅速、成功率高、低成本及高水平等优点, 成为最常用的原核表达系统[18-27]。人*bFGF*基因早已被克隆, 并有重组bFGF的原核表达的文献报道, 但均存在表达水平较低、生物活性不高等问题[28-30]。本研究通过密码子优化等方法, 在*E.coli*表达了bFGF蛋白, 并进行了纯化和功能分析。据文献报道, NIH3T3细胞是检测bFGF蛋白活性的常用靶细胞[31-33]。本研究中, 利用NIH3T3细胞、HEK293细胞及CHO细胞三种细胞测定了所表达的bFGF蛋白的体外活性。

1 材料与方法

1.1 载体和细胞株

pET-28a载体购自通用生物系统(安徽)有限公司; HEK293细胞株购自北京协和细胞资源中心; NIH3T3细胞株及CHO细胞由本室保存。

1.2 工具酶和试剂

质粒提取试剂盒购自北京康为世纪生物科技有限公司; His标签蛋白纯化试剂盒购自上海碧云天生物技术有限公司; 蛋白marker购自上海碧云天生物技术有限公司; 胎牛血清购自赛默飞世尔科技(中国)有限公司; DMEM基础培养基由本室提供; 硫酸卡那霉素购自上海鼎国生物技术有限公司。

1.3 重组载体的构建和鉴定

将*bFGF*序列进行密码子优化, 优化后的序列交由安徽通用生物公司合成。将目的基因和质粒pET-28a分别用*Xba* I和*Xho* I进行双酶切, 琼脂糖凝胶电泳, 胶回收*bFGF*基因片段及pET-28a载体DNA片段, 利用T4连接酶将目的基因与载体相连接, 构建成重组人pET-28a-bFGF质粒, 经酶切及测序鉴定载体的正确性。

1.4 目的基因在*E.coli*中的诱导表达

取少量含*bFGF*的重组质粒穿刺菌, 在卡那霉素抗性培养基中扩大培养, 提取质粒后转化BL21感受态细胞, 过夜培养后挑取生长良好的单克隆菌落过夜活化, 取500 μL菌液加入到含50 mL LK培养基的中培养至D_{600}值为0.5~0.6时加入终浓度1 mmol/L IPTG, 诱导7~8 h后取1 mL样品离心。弃上清后, 于SDS电泳检测。剩余菌液离心弃上清, 加入1 mL PBS缓冲液重悬沉淀, 超声破碎处理后离心取上清于SDS-PAGE电泳检测。

1.5 蛋白纯化及电泳检测

按照His-tag蛋白纯化试剂盒说明书进行重组蛋白的纯化, 取10 μL样品于15%的SDS-PAGE电泳检测, 检测目的蛋白分子量和纯度。将产物做成冻干粉蛋白, 储存于–80 °C冰箱备用。

1.6 bFGF生物活性检测

NIH3T3细胞、HEK293细胞和CHO细胞分别在含10%胎牛血清的DMEM高糖培养基培养3代, 当细胞密度达到85%时, 用0.25%的胰酶进行消化传代并计数。取传代细胞铺于96孔板, 计数为$1×10^4$/孔。用含1%胎牛血清的DMEM高糖培养基维持培养, 计量为100 μL/孔。按照CCK-8试剂盒说明书的具体步骤进行检测。

1.7 统计学处理

使用SPSS 18.0软件(SPSS Inc., Chicago, IL, USA)分析所有的实验数据, 数据表示为平均值±标准差。所有实验重复三次。P<0.05为差异有统计学意义。

2 结果

2.1 密码子优化

由于不同宿主细胞存在密码子偏爱性, 为提高蛋白表达量, 根据*E.coli*的密码子偏爱性进行了密码

```
Query  23   TGCCGGCACTGCCGGAAGATGGCGGCAGCGGTGCCTTTCCGCCGGGTCATTTTAAAGATC  82
Sbjct  2    TGCCAGCTTTGCCGGAAGATGGTGGTAGCGGTGCATTTCCGCCGGGCCACTTCAAGGACC  61

Query  83   CGAAACGCCTGTATTTGTAAAAATGGCGGCTTTTTCCTGCGCATTCATCCGGATGGTCGCG  142
Sbjct  62   CGAAACGTCTGTACTGTAAAAACGGGGGCTTCTTCCTGCGCATCCACCCCGACGGCCGAG  121

Query  143  TTGATGGCGTTCGTGAAAAAGTGATCCGCATATTAAGCTGCAGCTGCAGGCCGAAGAAC  202
Sbjct  122  TTGACGGGGTCCGGGAGAAGAGCGACCCTCATATAAAGCTACAACTTCAAGCAGAAGAGA  181

Query  203  GCGGTGTTGTTAGC-ATTAAGGGTGTGTGCGCCAATCGCTATCTGGCCATGAAAGAAGAT  261
Sbjct  182  GAGGAGTTG-TGTCTATCAAAGGAGTGTGTGCTAACCGTTACCTGGCTATGAAGGAAGAT  240

Query  262  GGCCGCCTGCTGGCAAGCAAATGTGTGACCGATGAATGTTTCTTTTTCGAACGCCTGGAA  321
Sbjct  241  GGAAGATTACTGGCTTCTAAATGTGTTACGGATGAGTGTTTCTTTTTTGAACGATTGGAA  300

Query  322  AGCAATAATTATAATACCTATCGTAGCCGTAAGTATACCAGTTGGTATGTGGCACTGAAA  381
Sbjct  301  TCTAATAACTACAATACTTACCGGTCAAGGAAATACACCAGTTGGTATGTGGCACTGAAA  360

Query  382  CGTACCGGCCAGTATAAACTGGGCAGTAAAACCGGTCCGGGCCAGAAAGCAATTCTGTTT  441
Sbjct  361  CGTACTGGTCAATATAAACTGGGTTCCAAAACGGGTCCTGGTCAGAAAGCTATCCTGTTC  420

Query  442  CTGCCGATG  450
Sbjct  421  CTGCCAATG  429
```

图1 *bFGF*密码子优化图谱

Fig.1 *bFGF* codon optimization map

1 2 3 bp

4 000

1 500

600

1: 未酶切质粒; 2: *Xba* I-*Xho* I双酶切质粒; 3: DNA marker。

Lane 1: un-digested plasmid; 2: *Xba* I-*Xho* I digestion plasmid; 3: DNA marker.

图2 重组质粒酶切结果

Fig.2 Recombinant plasmid digestion result

子优化, 优化后的*bFGF*基因序列比对结果见图1, 粗体代表优化的碱基。

2.2 载体构建

将构建好的含*bFGF*的载体进行*Xba* I和*Xho* I双酶切鉴定, 琼脂糖凝胶电泳结果显示在600 bp左右条带大小与预期一致(图2)。

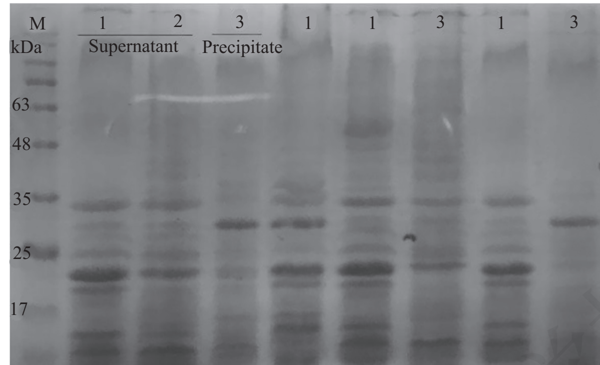
2.3 重组bFGF在*E.coli*中的表达

构建的重组表达载体转化感受态细胞BL21, 摇菌活化, IPTG诱导蛋白表达, 超声破碎菌体沉淀, 将产物离心后的上清进行SDS-PAGE检测, 结果显示, 目的条带大小约20.9 kDa, 1 mmol/L IPTG诱导表达量高于0.5 mmol/L(图3)。同时将蛋白条带进

行灰度扫描分析, 测得原核细胞*E.coli* BL21中bFGF蛋白最高表达量约为32%。将1 mmol/L IPTG诱导的样品超声破碎后的菌体沉淀溶于适量PBS缓冲溶液进行电泳检测, 结果显示没有目的条带, 说明所表达的bFGF蛋白为可溶性蛋白, 不存在包涵体中(Lane 1-3, 图3)。

2.4 重组bFGF的纯化

对表达产物进行His标签蛋白纯化, 进行4次洗脱, 通过SDS-PAGE电泳分别检测, 发现在20 kDa有特异蛋白条带, 与bFGF分子量大小一致(图4)。对电泳结果进行灰度扫描分析, 最终结果显示bFGF原核表达纯度高达约96%。

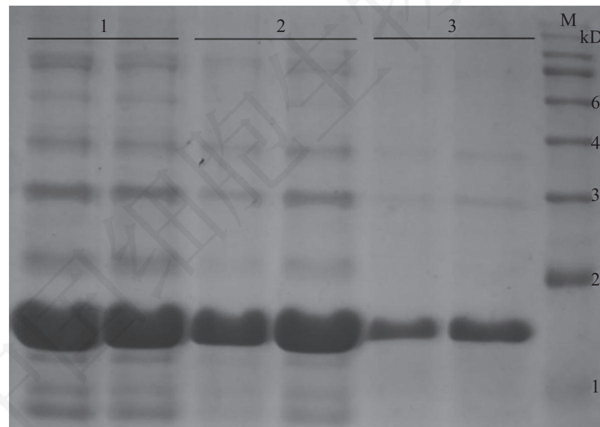


1: 1 mmol/L; 2: 0.5 mmol/L; 3: 超声破碎后菌体沉淀。

1: 1 mmol/L; 2: 0.5 mmol/L; 3: bacterial precipitate after ultrasonication.

图3 bFGF在*E.coli*不同IPTG浓度表达结果

Fig.3 Expression of recombinant bFGF under different IPTG concentration in *E.coli*



1: 两次; 2: 三次; 3: 四次。

1: twice; 2: three times; 3: four times.

图4 不同洗脱次数表达产物纯化结果

Fig.4 Purification results of expression products with different elution times

2.5 重组bFGF蛋白活性

用NIH3T3细胞、HEK293细胞和CHO细胞将纯化后的bFGF用CCK-8法检测其生物学活性, 结果显示, bFGF对细胞增殖有明显促进作用, 并且在10 ng/mL浓度下效果最显著。与不加bFGF相比, 加入10 ng/mL的bFGF增殖最高分别达2.01倍、4.12倍、2.40倍。半数有效量(median effective dose, ED_{50})分别为5.97 ng/mL、4.21 ng/mL、6.71 ng/mL(图5, $P<0.05$, 差异有统计学意义)。

3 讨论

研究发现, 不同的生物甚至同种生物不同的蛋白质编码基因, 对密码子的使用频率并不相同, 具有一定的偏爱性, 进行密码子优化可以在一定程度上提高转基因的表达水平。因此我们根据*E.coli*的密码子偏爱性对*bFGF*基因进行了优化。在不改变后续氨基酸序列的序列前提下, 将*bFGF*基因重新设计, 对个别氨基酸残基进行突变以获得新的结构类似物从而有利于bFGF进行过表达, 目的蛋白表达量

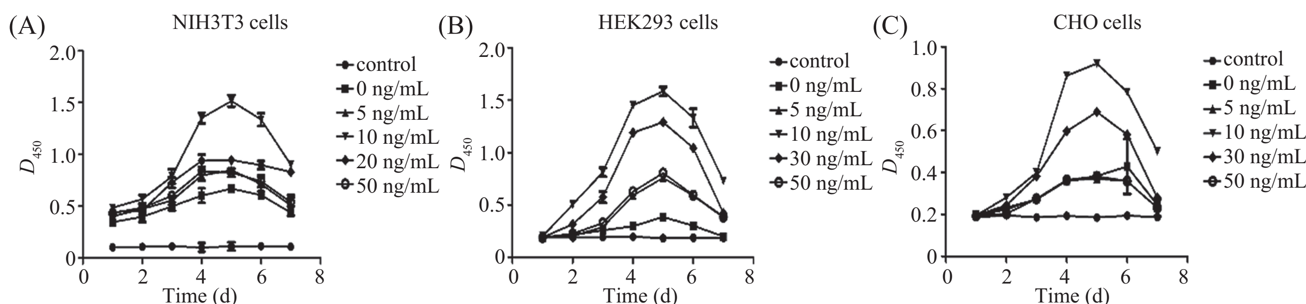


图5 重组bFGF促进细胞分裂活性

Fig.5 Recombinant bFGF promotes cell division activity

高至32%, 同时有效提高了外源蛋白在*E.coli*中表达的稳定性。本实验中在BL21中使用pET-28a表达系统来表达bFGF, 该表达载体常用来有效表达原核细胞的重组蛋白。一方面, pET-28a载体带有一个N-端的His/Thrombin/T7蛋白标签, 在T7噬菌体聚合酶的作用下, 启动蛋白表达, 同时蛋白表达将被T7终止子序列的作用下终止蛋白翻译[34]。另一方面, pET-28a载体有允许表达高水平重组蛋白的强大启动子, 随着pET-hbFGF载体转化入BL21(DE3)细胞, 用IPTG诱导*E.coli*表达T7RNA聚合酶后, *bFGF*基因随即与T7启动子结合从而进行大量转录翻译[35]。由于该载体同时含有一个可选择的C-端His标签, 因此表达出bFGF之后利用该标签对蛋白进行纯化。

本试验使用His标签对目的蛋白进行分离纯化。一方面组氨酸在高浓度的变性剂溶解后能和金属离子螯合亲和层析与杂质分离, 另一方面His标签分子量小, 既不影响蛋白功能, 又不形成二聚体。SDS-PAGE结果显示, IPTG浓度为1 mmol/L时蛋白表达量较高, 为32%, 而在0.5 mmol/L时表达量较低, 为25%。说明IPTG浓度对蛋白表达量也有较大影响。IPTG浓度过低导致诱导不充分, 过高则会对细胞产生一定毒性, 形成包涵体过多, 可溶性蛋白减少。

由于NIH3T3细胞富含bFGF受体, 因此常用于检测重组蛋白bFGF的生物活性。本实验检测结果显示, 纯化后最终蛋白表达量约为96%, 在*E.coli*成功表达人bFGF并对其进行了活性分析, 为大规模生产重组人源化bFGF奠定了基础。

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