Chapter 140
Expression of Recombinant Human Bone Morphogenetic Protein 2 in Insect Cells, Purification and Activity Analysis

Wen Chu, Shenheng Luo, Lihong Xu, Minhui Long and Aipo Diao

Abstract The aim was to produce recombinant human bone morphogenetic protein 2 (BMP2) using baculovirus-insect cell protein expression system. The recombinant protein was purified by high affinity Ni-charged resin, then the bio-activity of recombinant protein was detected by alkaline phosphatase assay, and the result showed that the recombinant BMP2 stimulated alkaline phosphatase activity in MC3T3-E1 cells, which lay the foundation for further study and application of recombinant BMP2.

Keywords Human bone morphogenetic protein 2 · Baculovirus-insect cells protein expression system · High5 · Alkaline phosphatase

140.1 Introduction

Bone morphogenetic proteins (BMPs) belong to the members of the transforming growth factor TGF-β super genes family [1], people have already separated BMP from many animals' bone matrix like cows, pigs, sheeps, mice, rabbits and people [2], there are 21 members in BMP family [3], they have the ability to induce mesenchymal cells and osteoprogenitor cells to differentiate into chondrocytes and bone cells, which can induce new bone formation except BMP1 [4–6]. Along with the wide application of genetic engineering and deeper research of the bone induction and BMP biological characteristics, BMPs had been proved to be the most important differentiation factors [7] for normal embryonic period bone [8] and adult bone repair [9–11]. This group of proteins have important value no

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matter for the basic biological research, or for the clinical application. So they had been the hotspot in the field of bone since they found [12].

In this experiment, the Sf9 insect cells were cotransfected with plasmids of pBAC-2CP-BMP2 and baculovirus flashBAC DNA to produce recombinant baculovirus, containing the BMP2 mature peptide encoded gene. High5 insect cells were infected with recombinant baculovirus to express BMP2 recombinant protein mature peptides efficiently, the expressed BMP2 protein was purified by high affinity Ni-charged resin, the bioactivity of recombinant protein was detected by alkaline phosphatase assay and the result showed that the recombinant BMP2 could stimulate alkaline phosphatase activity in MC3T3-E1 cells.

140.2 Materials and Methods

140.2.1 Materials

Restriction enzyme BamHI, EcoRI and T4 DNA ligase, pfu and Taq DNA polymerase were bought from Fermentas company; DNA purification and Miniprep plasmid extraction kit were bought from Tiangen company; Insect cell expression vector pBAC-2CP was from Novagen; Insect cells culture medium, DMEM medium and bovine serum were bought from GIBCO company; Escherichia Coli TOP10 competent cells, Sf9 insect cells, High5 insects cells, MC3T3-E1 mice fibroblast cells were preserved in the laboratory.

140.2.2 Methods

140.2.2.1 Insect Cell Culture

Insect cells were grown at 28 °C without CO_2 in a monolayer are split 1:5–1:8 every 3–4 days when confluence was between 85 and 95 %. To initiate shake culture from monolayer cultures, cells were dislodged from flask by pipetting medium over cells, and diluted with pre-warmed 28 °C medium to final concentration of 0.75×10^6 cells/ml [13]. Sf9 or High5 insect cells were grown and maintained in suspension culture in a temperature controlled orbital shaker operating at 150 rpm.

140.2.2.2 Production of Recombinant Baculovirus

To produce recombinant baculovirus, insect cells were co-transfected with transfer plasmid DNA and flashBAC DNA in 35 mm culture dish, cells were grown at

28 °C without CO_2 for 6 days. Homologous recombination between these two molecules yielded a baculovirus genome with the promoter and target sequence from the transfer plasmid located between the ORF1629 and the lef2 loci. The recombination also restored the function of the essential viral ORF1629, enabling the recombinant baculovirus to replicate and produce a population of recombinant viruses, which were released into the medium [14]. Six days after, the recombinant baculovirus in the supernatant was collected.

140.2.2.3 Amplification of Recombinant Virus

1 ml recombinant viruses were added to 10 cm sf9 insect cells culture dish, cells were grown at 28 °C without CO_2. Six days later, the supernatant was collected. Prepared 100 ml suspension culture of sf9 cells at an appropriate cell density, cells should be infected at a low multiplicity of infection, appropriate recombinant virus seed stock harvested from 10 cm sf9 insect cells culture dish was added to suspension culture cells. Incubated with shaking until cells were well infected (usually 6 days). When cells appeared to be well infected with viruses, cell culture medium was collected by centrifugation at 3,000 rpm for 4 min at 4 °C. Stored supernatant (recombinant virus stock) in dark at 4 °C and took out a small amount for PCR test.

140.2.2.4 BMP2 Protein Expression

Prepared 500 ml suspension culture of High5 cells at an appropriate cell density, appropriate recombinant virus seed stock harvested from 100 ml sf9 insect cells culture dish was added to suspension culture High5 cells, cells were grown at 28 °C for 3 days, cells and culture medium were collected at different time points after infection (24, 48 and 72 h) and protein expression were evaluated by SDS-PAGE analysis.

140.2.2.5 Protein Purification

The recombinant protein was purified using high affinity Ni-charged resin (GenScript). Cell pellet was resuspended in 50 mM sodium phosphate buffer containing 8 M urea, after centrifugation the supernatant wase incubated with Ni-charged resin for 1 h at 4 °C. The Ni-charged resin was washed with buffer containing gradient concentration of urea (6 M, 4 M, 2 M, 1 M, 0 M) and 20 mmol imidazole. Purified protein was eluted with 0.5 M imidazole and dialysed in PBS. The purified protein was analysed by SDS-PAGE.

140.2.2.6 Activity Assays of BMP2 Protein

The bioactivity of recombinant BMP2 protein was detected by alkaline phosphatase assay. MC3T3-E1 cells were cultured in 96-well with the density of the cells at 10^5 cells/ml, 100 μl each well, cells were grown at 37 °C for 1 day. Different concentration protein samples and standard samples were added, parallel and controls were set, then cells were grown at 37 °C for further 5 days. Five days later, the medium was removed, 100 μl 1 % Triton X-100 solution was added and placed at 4 °C for 1 h, then repeated freeze-thaw three times. 50 μl alkaline phosphatase reaction liquid substrates (1 mmol/L PNPP, 0.1 mol/L MgCl2) were added, placed at 37 °C for 1 h then 1.25 mol/L NaOH was added to terminate the reaction. Enzyme standard instrument was used to determine the spectrophotometry at 405 nm wavelength.

140.3 Results and Discussion

140.3.1 Production and Amplification of Recombinant Baculovirus

The DNA fragment encoded human BMP2 mature peptide was amplified by PCR, and inserted into insect cell expression vector pBAC-2CP to produce recombinant plasmid pBAC-2CP-BMP2, and indentified by DNA sequencing. Sf9 cells were co-transfected with plasmid pBAC-2CP-BMP2 and flashBAC DNA in 35 mm culture dish to produce recombinant baculovirus containing the BMP2 mature peptide encoded gene. To indentify the recombinant baculovirus, the virus DNA was extracted by proteinase K and indentified by PCR. Figure 140.1 showed that the BMP2 mature peptide encoded DNA fragment was successfully inserted into the virus genome to produce recombinant baculovirus.

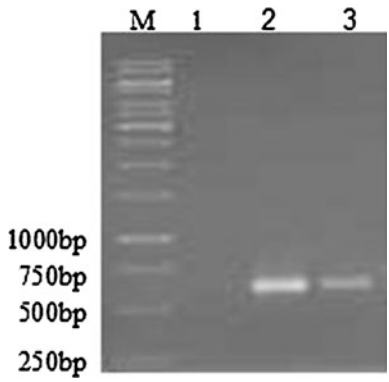

Fig. 140.1 Identification of recombinant virus by PCR. *M* DNA marker, *1* negative control (without template), *2* positive control (pBAC-2CP-BMP2 plasmid), *3* pBAC-2CP-BMP2 virus DNA

140.3.2 BMP2 Protein Expression

Appropriate recombinant virus seed stock harvested from 100 ml sf9 insect cells culture dish was added to 500 ml suspension culture High5 cells, when the cell density reach 2×10^6 cells/ml, placed at room temperature for 1 h, then 200 ml fresh medium was added, cells were grown at 28 °C for 3 days. Samples were taken everyday and the protein expression was evaluate by SDS-PAGE analysis. The result showed that recombinant BMP2 started to express 2 days after infection, with the highest expression level at the third day (Fig. 140.2).

140.3.3 Protein Purification

High5 cells were harvested 3 days after infection and resuspended in 50 mM sodium phosphate buffer containing 8 M urea. The recombinant protein was

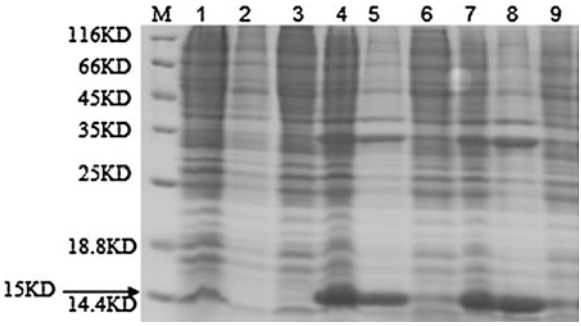

Fig. 140.2 Expression assays of BMP2 protein. *M* protein marker (14/18/25/35/45/66/116 kDA), *1* Bmp2 (1 day)-total, *2* Bmp2 (1 day)-pellet, *3* Bmp2 (1 day)-supernatant, *4* Bmp2 (2 days)-total, *5* Bmp2 (2 days)-pellet, *6* Bmp2 (2 days)-supernatant, *7* Bmp2 (3 days)-total, *8* Bmp2 (3 days)-pellet, *9* Bmp2 (3 days)-supernatant

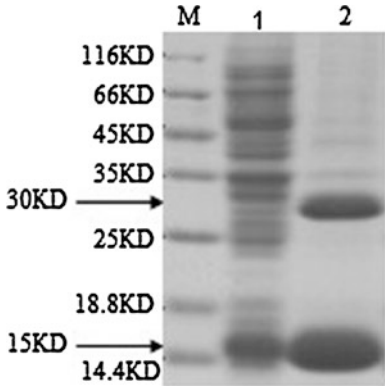

Fig. 140.3 Purification assays of BMP2 protein. *M* protein marker, *1* BMP2 total, *2* BMP2 purified

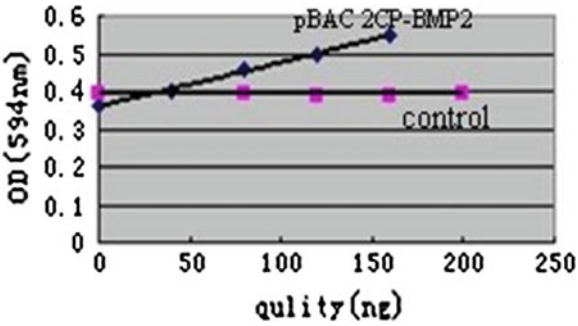

Fig. 140.4 Activity assays of BMP2 protein

refolded and purified using high affinity Ni-charged resin. The purified protein contains ~15 kDa BMP2 protein monomer and ~30 kDa BMP2 protein dimer (Fig. 140.3).

140.3.4 Activity Assays of BMP2 Protein

The differentiation of osteoblasts and the activity of intracellular alkaline phosphatase can be stimulated by BMP2 [15]. The bioactivity of recombinant BMP2 protein was detected by alkaline phosphatase assay using MC3T3-E1 cells. The activity of intracellular alkaline phosphatase increased obviously with the increasing concentration of recombinant BMP2 protein, but not for the control using recombinant protein His-interferon α (Fig. 140.4).

140.4 Conclusion

Bone morphogenetic proteins (BMPs) are a group of growth factors, originally discovered by their ability to induce the formation of bone and cartilage. The natural BMP2 protein was separated and purified in 1988, which plays a key role in osteoblast differentiation [16]. Recombinant human BMP2 protein has been produced using genetic-engineering technology, however, because recombinant BMP2 had lower activity to induce ossify, it still not widely used in clinic [17].

In this experiment, baculovirus-insect cells protein expression system was used to express recombinant human BMP2 protein. Baculovirus expression system is an eukaryotic protein expression system widely used in recent years [18–20], it has some advantages including: high protein expression level; expressed protein has proper modification (glycosylation) and easy to operate. We successfully produced recombinant human BMP2 using baculovirus-insect cell protein expression system, and the recombinant BMP2-stimulated alkaline phosphatase activity in MC3T3-E1 cells, which lay the foundation for further study and application of recombinant BMP2.

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