Fast establishment and application of caspase-3 inhibitor detection system

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Abstract: Our objective to study the expression and activity of the apoptosis protease Caspase-3 in E. coli BL21 (DE3) Plyss. The cDNA of Caspase-3 was amplified by PCR and inserted into the cell of K562, it was then cloned to prokaryotic expression vector pET-21b, after which Caspase-3 was induced by IPTG. The Protein induced was identified by SDS-PAGE and Western blot. After induced by IPTG for 5 hours, the concentration of Caspase-3 reached the highest level. Finally active Caspase-3 can be induced within E. coli BL21 (DE3) Plyss, further research can be done to chose the Caspase-3 inhibitors.

Introduction

Caspases play important roles in apoptosis and cell signaling. Caspase-3 is the core of the execution phase of apoptosis [1] . Caspase-3 has become attractive target in the treatment of many disease such as Alzheimer [2]，Parkinson's [3], myocardial infarction [4] and cancer [5]. Thus, key apoptosis factors serve as attractive molecular targets for designing specific pharmaceuticals for apoptosis diseases [6]. So Caspase-3 is identified as a drug-screening target. Caspase inhibitors have anti-cancer and other pharmalogical potentials. It has been proven that Caspase-3 substrate selectivity for the peptide sequence Asp-Glu-Val-Asp (DEVD) [7]. Our strategy was to establish Caspase-3 inhibitor rapid screening platform and Ac-DEVE-CHO [8-10] can be used as a control.

Materials and methods

Plasmid construction.

Human Caspase-3 full-length cDNA was amplified by the cell of K562. cDNA was used as a template for PCR amplification of the region in the open reading frame encoding the mature human Caspase-3. PCR amplification was performed using 25 ng of template DNA, 0.1 ng of the appropriate primers [5'- GCGAATTCATGGAGAACACTGAAAAC -3' (5'-primer) and 5'-GCGCGGCCGCTTAGTGATAAAAATAGAG-3'(3'-primer)], and 1U of Pfu turbo DNA polymerase (Stratagene) in a final reaction volume of 20 μL.The reaction mixture was subjected to amplification for 35 cycles (94 C, 0.5 min;55 C, 0.5 min; 72 C, 1.5 min) using a thermal cycler. The result of Caspase-3 PCR is shown in **Fig.1**. After purification, PCR products were cleaved with EcoRI and NotI and ligated between the corresponding restriction sites of the vector pET-21b .This vector has a His-tag coding sequence and can produce a C-terminal 6 His-tag fusion protein.The

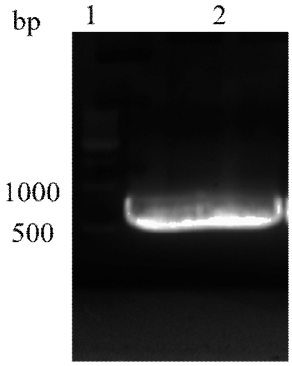

Fig.1

Fig.1: lane 1:Maeker
lane 2:caspase-3

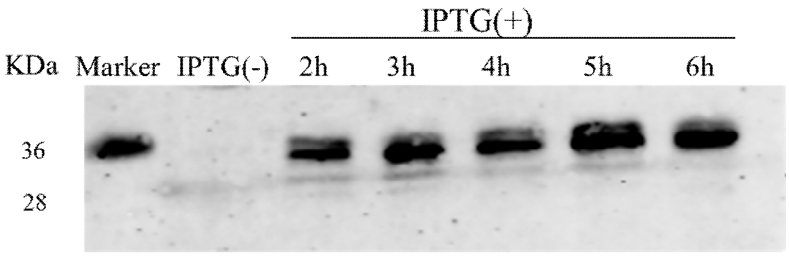

Fig.2

Fig.2: 1 mM IPTG induced the expression of Caspase-3 increased with increasing time and the maximum expression at 5 h.

double digestion products of fragment Caspase-3 and vector connection at 16 ˚C overnight。Then the ligation product was transformed into competent in E.col top 10 Screening of positive transformants were used to induce the expression of Caspase-3 protein and then to study the function and activity of other aspects.

Expression of the recombinant human Caspase-3

To express the recombinant human Caspase-3, E. coli BL21(DE3) Plyss cells were grown in LB medium (1 % bactotryptone,0.5 % yeast extract, 0.5 % NaCl) containing 100 μg/mL ampicillin at 37 ˚C to OD600 nm = 0.6-0.8.Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM and cells were further incubated for 1h-5h at 16 ˚C. The result of SDS-PAGE analysis was used to determine the optimal conditions for the expression of Caspase-3.

Purification of caspase-3.

Single colonies were used to inoculate a 5ml LB medium, containing the appropriate antibiotic and were then grown at 37 ˚C overnight. This culture was used to inoculate 50 ml of LB medium (added in 50 μL Ampicillin) at 37 ˚C to OD 600 nm = 0.6. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM and cells were further incubated for 5h at 16˚C. The inclusion bodies of Caspase-3 were prepared by cell lysis in a buffer containing 20 mM Tris–HCl (pH 8.0), 500 mM NaCl. Cells were broken using an noise isolating tamber (Ningbo Scienfz Biotechnology Co, LTD). The protein were supernatant by centrifugation at 5000×g for 5 min at 4 ˚C and loaded on a Ni2+-nitrilotriacetic acid at 4 ˚C over night. The Caspase-3 was eluted using a buffer containing 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 150mM imidazole. Fractions containing the Caspase-3 proteins were concentrated by Dialysis at 4 ˚C overnight ($Na_2HPO_4 \cdot 12H_2O$ 16.692g,$Na_2H_2PO_4 \cdot 2H_2O$ 0.53g,NaCl 5.844g,Glycerin100ml and water 900 ml). And then Caspase-3 were supernatant by centrifugation at 5000×g for 5 min at 4 ˚C.

caspase-3 activity assay

For determination of inhibitor potency against the purified enzyme, inhibitors were titrated against recombinant Caspase-3 and the enzyme activity was measured by Caspase-3 activity assay kits（Tianjin Sungene Biotech Co.,Ltd）.The Amplite™ Fluorimetric Caspase-3 Assay Kit uses Ac-DEVD-AMC as a fluorogenic indicator for Caspase-3 activity. AMC peptides are almost non-fluorescent. Cleavage of AMC peptides by Caspase-3 generates strongly fluorescent AMC that is fluorimetrically monitored at 450 nm with excitation of 350 nm. It can be used to continuously measure the activities of Caspase-3 in cell extracts and purified enzyme preparations with a fluorescence microplate reader or fluorometer. The Caspase-3 activity assay kits containing Component A: the fluorogenic tetrapeptide substrate Ac-DEVD-AMC（10 mM）,Component B:Assay buffer:（20 mM Hepes buffer with 4 mM EDTA）,C:1M DTT. The Caspase-3 activity

assay using a 384-well (Corning 3573) . Each well contained 15 μL working solution (the component A 、B and C (v/v/v) （2:100:1）、15 μL enzyme 、0.5 μL Compound). After incubated, the solution was determined immediately (excitation at 350 nm, emission at 450 nm).

Results and discussion

Expression and purification Caspase-3

The optimum conditions of the expression human caspase-3 is : 1 mM IPTG induced 5h at 16˚C.After IPTG induction, Caspase-3 was expressed as a major protein at 31 kDa in the total protein of E. coli, as shown in **Fig. 2**. The size of expressed Caspase-3 was in good agreement with the theoretical molecular weight from the amino acid sequence of the lec-EGF domains and 6 His-tag. The protein was expressed mainly as soluble protein. After sonication Caspase-3 protein sample was purified by single step Ni^{2+} affinity chromatography with the purification tags. The result of SDS-PAGE analysis for the purification of Caspase-3 is shown in **Fig. 3**. The molecular weight of the soluble protein was evaluated as about 31 kDa.

Fig.3:lane 1:OD600 nm=0.6 lane 2:IPTG induction 5h

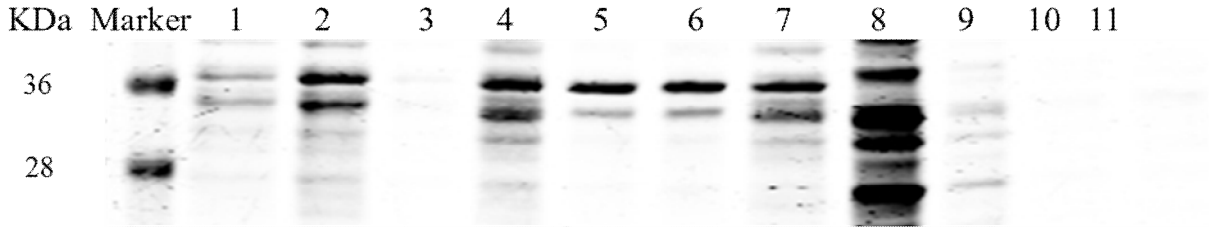

Fig.3

lane 3:centrifugal cell supernatant lane 4:After cell sonication

lane 5:supernatant after sonication lane 6: After sonication precipitate

lane 7:the supernatant after centrifugation combined beads

lane 8:first elution lane 9:second elution

lane 10:the third elution lane 11:beads after elution

Caspase-3 inhibition assay.

To determine the in vitro inhibitory potencies of Ac-DEVE-CHO toward Caspase-3, the recombinant enzyme was preincubated on ice in 20 mM HEPES,0.1 M NaCl,10% sucrose,0.1% CHAPS and 2 mM EDTA(pH 7.4) and the final concentration of the Caspase-3 is 5 ng/ml. The increasing concentrations of Ac-DEVE-CHO's final concentration is 0.1 nM,0.3 nM,1 nM,3 nM,10 Nm,30 nM wereadded . The percentage of cleavage was evaluated and compared with 100% relative activity,and the IC50 value was determined accordingly. By calculating the IC50 value of the Ac-DEVE-CHO is 2.28±0.05.

Conclusion

In this study, simplified and efficient Caspase-3 assay was established. Our work has shown that the Ac-DEVE-CHO has strong Caspase-3 inhibitory activity. In the next study, Ac-DEVE-CHO can be used as a Caspase-3 inhibitor screening test control. This study may provide valuable information for development of inhibiting apoptosis with more potent activities.

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