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Caspase-3-facilitated Stoichiometric Cleavage of a Large Recombinant Polyprotein

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In this study, it is reported that a large polyprotein can be stoichiometrically cleaved by the use of caspase-3-dependent proteolysis. Previously, it has been shown that the proteolytic IETD motif was partially processed when treated with caspase-3, while the DEVD motif was completely cleaved. The cleavage efficiency of the DEVD-based substrate was approximately 2.0 times higher than that of the IETD substrate, in response to caspase-3. Based on this, 3 protein genes of interest were genetically linked to each other by adding two proteolytic cleavage sequences, DEVD and IETD, for caspase-3. Particularly, glutathione-S transferase (GST), maltose binding protein (MBP), and red fluorescent protein (RFP) were chosen as model proteins due to the variation in their size. The expressed polyprotein was purified by immobilized metal ion affinity chromatography (IMAC) via a hexa-histidine tag at the C-terminal end, showing 93 kDa of a chimeric GST:MBP:RFP fusion protein. In response to caspase-3, cleavage products, such as MBP:RFP (68 kDa), MBP (42 kDa), RFP (26 kDa), and GST (25 kDa), were separated from a large precursor GST:MBP:RFP (93 kDa) via SDS-PAGE. The results obtained from this study indicate that a multi-protein can be stoichiometrically produced from a large polyprotein by using proteolytic recognition motifs, such as DEVD and IETD tetra-peptides, for caspase-3.

Key words : Caspase-3, DEVD, IETD, polyprotein, proteolytic cleavage

Introduction

A polyprotein consists of several domains which are generated by proteolytic cleavage of large precussor polypeptide. The synthesis of polyprotein is related with the concept of "genetic economy", since functional gene products are separated from a single polypeptide which has beneficial effects on time and energy consumption [9, 10, 13]. Indeed, such a polyprotein processing mode is the strategy employed by many animal and plant viruses for gene expression [1, 2]. Previously, it has been shown that proteolytic IETD motif was partially processed when treated with caspase-3, while DEVD motif was completely cleaved [6-8]. The cleavage efficiency of DEVD-based substrate was approximately 2.0 times higher than that of IETD-based substrate in response to caspase-3 [8]. In the current study, for the purpose of caspase-3-facilitated stoichiometric cleavage of a large polyprotein, a recombinant polyprotein consisting of 3 different proteins, which were designed to be detached by DEVD and IETD tetrapeptides, was constructed. Particularly, glutathione-S transferase (GST), maltose binding protein (MBP), and red fluorescent protein (RFP) were used as model proteins, because they exhibit marked difference in size to each other, facilitating an easy separation based on molecular weight. Following treatment with caspase-3, four cleavage subunits such as MBP:RFP (68 kDa), MBP (42 kDa), RFP (26 kDa), and GST (25 kDa) were generated from a single polypeptide GST:MBP:RFP (93 kDa). These results showed that multi-protein can be stoichiometrically produced from a single recombinant polyprotein associated with the proteolytic recognition motifs, DEVD and IETD, showing cleavage preference for caspase-3.

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Materials and Methods

Strains, vectors, and enzymes

E. coli strain DH5 was utilized as a host for subcloning and *E. coli* BL21 (DE3) (Novagen, WI, USA) was used for

gene expression. The *E. coli* strain was grown in LB medium at 37°C and 50 mg/ml of ampicillin was added for the plasmid-harboring strains. pBluescript SK^+ (Stratagene, CA, USA) was utilized as a vector for the subcloning and amplification of the chimeric gene, and pET-21a (Novagen, WI, USA) for expression. The restriction enzymes and modifying enzymes were obtained from Boehringer-Mannheim (Mannheim, Germany) and utilized in accordance with the supplier's recommendations. Vector DNA was prepared using a QIAEX II gel extraction kit (Qiagen, Hilden, Germany).

Gene cloning, expression, and purification of recombinant polyprotein

In order to clone the chimeric GST:MBP:RFP harboring DEVD and IETD motifs, the full-length genes encoding for MBP and RFP were amplified with the 5′ primers (CG GGA TCC GAT GAG GTG GAT GGA GAA TTC ATG AAA ACT GAA GAA GG, CCC AAG CTT ATG GTG CGC TCC TCC AAG) and the 3′ primers (CCC AAG CTT TCC ATC GGT CTC GAT GTC GAC AGT CTG CGC GTC TTT CAG, CCG CTC GAG CAG GAA CAG GTG GTG GCG), respectively, via the polymerase chain reaction (PCR). The 5′and 3′ termini were designed to harbor the BglII-BamHI, EcoRI-SalI, HindIII-XhoI restriction enzyme cleavage sites, respectively. The PCR product was then purified using a DNA purification kit (Qiagen), and digested with the corresponding restriction enzyme sites. The resultant DNA fragment was ligated to each other, and inserted into the GST-fused pET-21a plasmid to generate the in-frame fusion of pGST:MBP:RFP chimeric gene, verified via DNA sequencing, and then transformed into *Escherichia coli* BL21 (DE3) for the expression of the recombinant protein. The transformed cells were grown at 37℃ with shaking to an OD600 of 0.6. The cells were induced with 1 mM isopropyl-2-D-thiogalactopyranoside (IPTG) (GibcoBRL, MD, USA), and grown for an additional 4 hr. The cells were then harvested after 10 minutes of centrifugation at 6,000 g at 4°C. In order to purify the GST:MBP:RFP:6His protein, one liter of crude cell lysate was loaded onto a IDA-miniexcellose affinity column (Bioprogen Co., Daejeon, Korea) and washed three times with 10 ml of equilibration buffer (50 mM phosphate, 0.5 N NaCl, pH 8.0), respectively. The recombinant proteins were then eluted with 5 ml of 0.5 M imidazole in the same buffer (50 mM phosphate, 0.5 N NaCl, pH 8.0). In order to generate the pHis×6:Caspase-3 fusion gene, caspase-3 gene was amplified with the 5′ primer (CG GGA TCC ATG GAG AAC ACT GAA AAC) and the 3′ primer (CG GAA TTC GTG ATA AAA ATA GAG TTC) using PCR, then cloned into the pET-23a plasmid using the BamHI/EcoRI sites. The cysteine residue at position 163 was mutated by PCR-based sitedirected mutagenesis using the 5′ primer (TTC ATT ATT CAG GCC AGC CGT GGT ACA GAA CTG) and 3′ primer (CAG TTC TGT ACC ACG GCT GGC CTG AAT AAT GAA). The recombinant wild and mutant caspase-3 proteins were expressed in *E. coli* and isolated as described [11].

SDS-PAGE analysis

One hundred micrograms of the purified recombinant GST:MBP:RFP protein were incubated for the indicated times with 15 U of caspase-3 in 20 mM PBS buffer (pH 7.4) at 4°C. The resultant protein solutions were resolved on 10% SDS gel. The gel was stained with Coomassie's Brilliant Blue R250. Protein concentrations were determined by the Bradford method, using bovine serum albumin as a standard. For the quantification of SDS-PAGE data, the band densitometry analysis was performed with Image J software.

Results and Discussion

Research concept of stoichiometric cleavage of a large recombinant polyprotein

Polyprotein cleavage is the strategy that many viruses exploit for facilitating viral replication from their minimum genetic information [3]. Viral polyprotein must be processed by protease to produce functional cleavage products from a single precussor polypeptide. This work began with the idea that the feature of polyprotein cleavage can be genetically mimicked by inserting the cleavage sites between the target protein genes, and the mode of polyprotein processing can be used to obtain multi-protein from a single recombinant polyprotein. In this study, caspase-3 as a protease responsible for the cleavage was chosen, because its recognition tetrapeptides are well extensively studied [12]. Therefore, the proteolytic cleavage of recombinant polyprotein used in this study is caspase-3-dependent. This kind of caspase-3-dependent proteolysis in *Drosophila* olfactory neurons can also cause changes in animal behavior during the normal aging process [5]. Based on the previous study showing that DEVD and IETD motifs exhibit quite a different cleavage efficiency for caspase-3 [7], it was assumed that more combinations of recombinant proteins can be produced in the presence of the proteolytic DEVD and IETD motifs

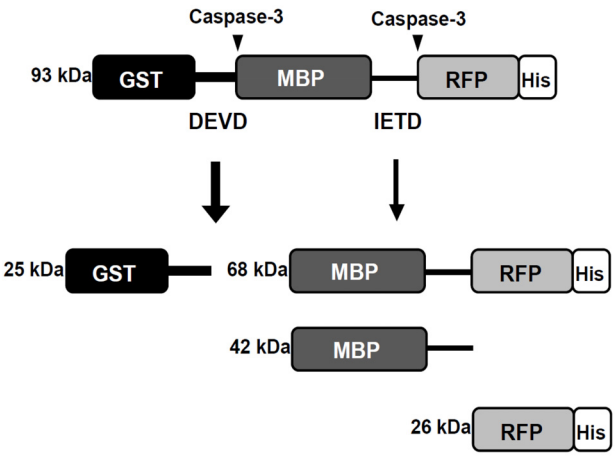

Fig. 1. Schematic diagram of the proteolytic processing of GST:MBP:RFP polyprotein for muti-protein isolation. The recombinant GST:MBP:RFP polyprotein is processed by caspase-3. Caspase-3 completely (~100%) proteolyzes DEVD motif, while the IETD site is partially (~50%) cleaved by caspase-3. Based on this notion, four cleavage products will be obtained from the precussor molecule.

due to the cleavage preference for caspase-3 (Fig. 1). To evaluate the performance of this protocol, the recombinant GST:MBP:RFP as a model polyprotein was genetically modified by inserting DEVD between GST and MBP, and IETD between MBP and RFP respectively, following the gene cloning technique as described in "Materials and Methods". It is thought that the GST:MBP:RFP precussor may be possibly cleaved into four fragments by caspase-3, due to their difference in proteolytic efficiency between DEVD and IETD (Fig. 1).

Expression and purification of the recombinant GST:MBP:RFP polyprotein

To Purify the recombinant GST:MBP:RFP polyprotein, a chimeric plasmid pGST:MBP:RFP was constructed. Two proteolytic sequences, DEVE and IETD, were placed between GST and MBP, and between MBP and RFP, respectively (Fig. 2A). After IPTG induction, an extra protein band with an approximate molecular weight of 93 kDa, which is consistent with the expected mass of GST:MBP:RFP predicted using ExPASy Proteomics tools (www.expasy.org), was found only in the *E. coli* cultures harboring pGST:MBP:RFP plasmid, and most of the expressed target protein has appeared in the soluble fraction, and has seldom existed in inclusion bodies (data not shown). In order to obtain chimeric GST:MBP:RFP precussor molecule, the recombinant GST:MBP:RFP polyprotein was purified using by metal chelate affinity chroma-

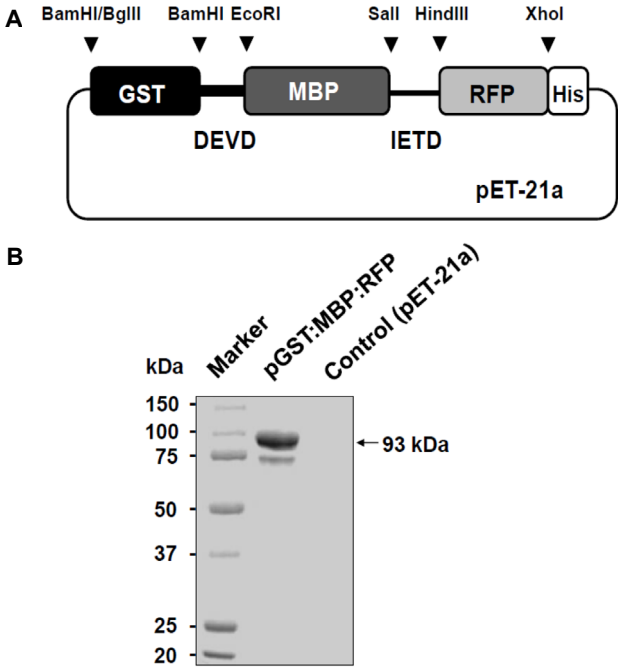

Fig. 2. Gene construction and purification of recombinant chimeric protein. (A) Construction of the pET 21a-GST:MBP:RFP vector. The chimeric GST:MBP:RFP polyprotein was combined together by two types of caspase-3-dependent proteolytic motifs, DEVD and IETD. Arrow heads indicate the restriction enzyme cleavage sites. (B) Purification of GST:MBP:RFP (93 kDa) as a large precussor polypeptide for muti-protein isolation. The purified protein was visualized by Coomassie staining on SDS-PAGE. *E. coli* cells were transformed with an empty vector pET-21a as a control.

tography performed in IDA Excellose column charged with Ni^{2+} ions. One hundred microliters of the cell lysate were loaded onto an IDA Excellose affinity column (Bioprogen Co., Korea), and the column was washed three times with 100 ml of equilibration buffer (50 mM Tris-HCl, 0.5 M NaCl, pH 8.0). The recombinant protein was eluted with 200 ml of 0.5 M imidazole in the equilibration buffer, and dialyzed in dialysis buffer (50 mM Tris-HCl, pH 8.0) for 48 hr at 4°C. As shown in Fig. 2B, the large recombinant protein of GST:MBP:RFP (93 kDa) was clearly purified, but not in the control plasmid pET-21a sample. The purified protein was further used as a precussor protein for separating cleavage products from a single polyprotein. Separation of muti-protein from a single recombinant polyprotein. Following the purification of GST:MBP:RFP, the recombinant polyprotein was treated with caspase-3 at the concentration of 15 U (50 ul of 300 U ml-1), since the increased level in caspase-3 activity has shown to be saturated at this concentration [7].

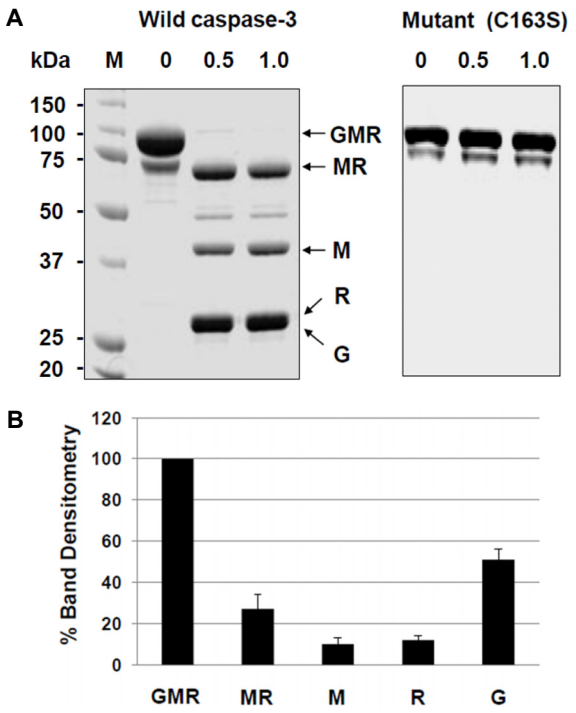

Fig. 3. SDS-PAGE separation of caspase-3-dependent proteolytic cleavage products from a large GST:MBP:RFP polyprotein. (A) Cleavage of GST:MBP:RFP polyprotein in response to caspase-3. The purified protein was incubated with active caspase-3 or mutant caspase-3 (C163S) for the indicated times ranging from 0 to 1 h, and then analyzed by SDSPAGE. (B) The band densitometry analysis for quantifying SDS-PAGE data. The experiment was repeated three times. GMR, GST:MBP:RFP; MR, MBP:RFP; M, MBP; R, RFP; G, GST.

Caspase-3 has a general biochemical property that its catalytic cysteine is important for its proteolytic activity [4]. To verify whether the mutation of this active site blocks the proteolytic processing of DEVD- and IETD-bearing polyprotein, cysteine at position 163 of caspase-3 was substituted into serine (C163S) by site directed mutagenesis technique. After expression and purification of mutant caspase-3, the GST:MBP:RFP polyprotein was also treated with mutant caspase-3. The GST:MBP:RFP polyprotein treated with active caspase-3 or mutant caspase-3 (C163S) was analyzed by 10% SDS-PAGE, and visualized via Coomassie staining. As shown in Fig. 3A, the GST:MBP:RFP polyprotein was fully processed in 30 minutes after treatment with active caspase-3. The resulting cleavage products including MBP:RFP (68 kDa), MBP (42 kDa), RFP (26 kDa), and GST (25 kDa) were successfully separated via SDS-PAGE as indicated by the fragmented products, while no cleavage of GST:MBP:RFP was observed in response to mutant caspase-3, verifying the critical role of 163 cysteine of caspase-3 in its proteolytic activity. The finding obtained from this study shows the advantages of the polyprotein-based muti-protein separation such as simplicity and rapidity of use. For the quantification of SDSPAGE data, the band densitometry analysis was performed with Image J software. As the amount of GST:MBP:RFP precussor polyprotein without caspase-3 was set at 100%, the relative level of MBP:RFP, MBP, RFP, and GST was approximately 27%, 10%, 12%, and 51%, respectively (Fig. 3A and 3B). Considering that approximately 50% of IETD substrates were partially cleaved in comparison with DEVD substrates, the expected relative amounts of the processed products come to 25.0% for MBP:RFP, 12.5% for MBP, 12.5% for RFP, and 50.0% for GST. Therefore, our results are in good agreement with the theoretical expectation. In order to make this technique usable, there remain two issues, the removal of caspase-3 from the protein solution and the purification of the cleaved muti-protein, to be resolved. It is possibly assumed that caspase-3 can be eliminated by the antibody affinity chromatography. Also, a variety of anion- and cation-exchange chromatography columns with high efficiency are available for isolating subpolypeptide fragments. Ion exchange chromatography is commonly used as a first purification step of proteins. The method of ion exchange can be also combined with gel filtration and affinity chromatography in a single column, allowing for the effective purification of target proteins varying in molecular weight as well as the value of the isoelectric point (pI). This work showed the possibility of muti-protein production, based on caspase-3-dependent cleavage fragments from a single polyprotein in denaturing SDS-PAGE separation. Thus, further studies are required for the removal of protease used, and the successful isolation of proteolytic cleavage products as a biologically active form.

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초록 : 카스파제-3 효소를 이용한 폴리-단백질의 정량적 프로세싱 분석

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선행연구에서 카스파제-3 효소가 DEVD 기질을 완전히 절단하는 반면 IETD 기질은 DEVD의 약 50%정도만을 부분적으로 분해한다는 사실을 보고하였다. 본 연구에서는 정제된 폴리-단백질이 카스파제-3 단백질 분해효소의 기질에 따른 차별적인 분해활성에 의해 프로세싱 되는 양상을 분석하였다. 모델 단백질로서 GST, MBP, RFP 세 종류의 단백질을 DEVD 및 IETD 펩타이드로 연결시킨 폴리-단백질을 제작하였으며, 폴리-단백질은C-말단에 6개의 히스티딘 태그가 결합되도록 클로닝 되었다. IMAC 크로마토그래피를 이용하여 분리 및 정제된 재조합 단백질은 SDS-PAGE 분석을 통해 분자량이 약 93 kDa으로 나타났으며, 카스파제-3 효소의 처리에 의해 각각 MBP:RFP, MBP 그리고 GST 3종류의 단백질 절편으로 절단 및 분리되었다. 이 연구를 통해 단백질 분해효소와 기질 간의 반응성의 차이를 이용하여 폴리-단백질을 정량적으로 프로세싱 할 수 있다는 예를 보여주었다.