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Vibrio vulnificus ATCC29307 균주에서 분비되는 단백질분해효소에 의한 procaspase-3의 활성화에 관한 연구

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Activation of procaspase-3 by a broad specificity extracellular protease from *Vibrio vulnificus* sp. stain ATCC29307

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ABSTRACT

Activation of procaspase-3 by a broad specificity extracellular protease from *Vibrio vulnificus* sp. strain ATCC29307

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Vibrio vulnificus (*V. vulnificus*) is a causative agent of serious food-borne diseases in humans related to the consumption of raw seafood. It secretes a metalloprotease that is associated with skin lesions and serious hemorrhage complications. The metalloprotease (vEP) from *V. vulnificus* sp. strain ATCC29307 has previously been shown to have prothrombin activation activity. vEP also exhibits broad substrate specificity, enabling it to cleave a numbers of plasma proteins that are associated with blood coagulation, as well as those not associated with blood coagulation such as BSA and γ-globulin. In this study, the ability of vEP to activate another zymogen, procaspase-3(D3A), was investigated. Procaspase-3 has been chosen for this study because it is an

important enzyme that mediates the final step of the apoptosis cascade. A procaspase-3 mutant (D3A) in which the native cleavage sites essential for activation have been abolished was used as a substrate for vEP. Procaspase-3(D3A) exhibited some activity towards the caspase-3-specific chromgenic substrate, Ac-DEVD-*p*NA. After being activated by vEP through proteolytic cleavage, its activity increased approximately 3 fold. Similar to the activation of prothrombin by vEP whereby the formation of thrombin is transient, the formation of mature enzyme from procaspase-3(D3A) by cleavage with vEP was also transient, with the activity increasing with time and then decreasing upon further incubation in the presence of vEP. Western blot analysis of the time-dependent activation of procapase-3(D3A) by vEP showed a band corresponding to the size of the large subunit of mature caspase-3 when monoclonal antibody raised against caspase-3 large subunit. The activated enzyme displayed similar substrate specificity to native caspase-3 in that it cleaved poly(ADP-ribose) polymerase (PARP) in a cell-free system prepared from NIH3T3 cells and was sensitive to Ac-DEVD-CHO, a highly specific caspase-3 inhibitor. Taken together, the results obtained from this study suggest that vEP could act as a procaspase-3(D3A) activator.

I. INTRODUCTION

Vibrio vulnificus (*V. vulnificus*) is an opportunistic pathogen that causes wound infection and septicemia (Janda *et al*., 1988). Infection in human hosts, which was first illustrated in 1976, characteristically displays three discernible syndromes: primary bacteremia, wound infection and gastrointestinal illness. Mortality rate was up to 55% in septic patients, with most dying within 48 hr with fulminate course after admission. Among those who had wound infections, the mortality rate was as high as 25%.

Several factors have been proposed to play a role in the virulence of *V. vunificus*, and these include metalloprotease, hemolysin, cytolysin, and polysaccharide capsules. *V. vulnificus* is known to secret a 45 kDa zinc-dependent metalloprotease that has many biological functions (Miyoshi *et al*., 2003; Chang *et al*., 2005). These functions include induction of hemorrhagic reactions through specific degradation of type IV collagen in the vascular base membrane when injected intradermally into the dorsal skin (Miyoshi *et al.,* 2001), degradation of a variety of host proteins and enhancement of vascular permeability through the generation of inflammatory mediators (Miyoshi *et al*., 2003), activation of prothrombin and cleavage of fibrinogen and fibrin (Chang *et al*., 2005). These functions include induction of hemorrhagic reactions through disorganization of the basement membrane layer after injection into the dorsal skin, a variety of host proteins and enhancement of vascular permeability through the generation of inflammatory mediators (Miyoshi *et al.,* 2003*),* Injection of the protease

into animal produced some of the pathology that are associated with *Vibrio* infection (Miyoshi *et al*., 1993 and 2003).

The metalloprotease (vEP) from *V. vulnificus* sp. strain ATCC29307 has recently been shown to activate prothrombin, in which various fragments including a functional thrombin are generated from prothrombin by vEP. The N-terminal sequencing analysis revealed that vEP cleaved Asp^{207}-Phe^{208} and Thr^{272}-Ala^{273} bonds. The cleavage site of Thr^{272}-Ala^{273} was just one amino acid away from the cleavage site recognized by FaXa, Arg^{271}-Thr^{272}. However, the of activation of prothrombin by vEP was transient, with further cleavage of the activated-enzyme, leading to a loss of thrombin activity. Since vEP is a broad specificity protease, it may also activate other kinds of zymogens to produce active enzymes. However, the cleavage of plasminogen cleavage by vEP did not yield active plasmin.

Our interest in the activation of zymogen by vEP prompted us to look at zymogens whereby the activation is a highly specific process. Recently, a mutant form of procaspase-3, referred to as procaspase-3(D3A), has been characterized in the laboratory of Prof. I-S. Park at Department of Bio-Materials, Chosun University. Caspase-3 is a member of cysteine proteases that specifically cleave carboxyl side of an aspartic acid residue. It is synthesized as a 32 kDa precursor (procaspase-3) and is converted into a mature enzyme consisting of two large (p17) and two small (p12) subunits during apoptosis. Caspase-3 is a crucial enzyme is the apoptosis cascade (Fig. 1). During apoptosis, procaspase-3 is activated to caspase-3 by caspase-8. Caspase-3 then mediate the proteolysis of a various of cellular proteins eventually leading to cell death. One of the

characteristic events of apoptosis is the proteolytic cleavage of poly(ADP-ribose) polymerase (PARP), a nuclear enzyme involved in DNA repair, DNA stability, and transcriptional regulation. Caspases, in particular caspase-3 and -7, cleave the 116 kDa form of PARP at the DEVD site to generate 85 kDa and a 24 kDa fragments (Kaufmann *et al*., 1993). The conversion of procaspase-3 to caspase-3 involves the cleavage of the zymogen at the $E^{25}SMD^{\downarrow}S$ yielding a 20 kDa and a 12 kDa (p12) fragments (Han *et al*., 1997). The 20 kDa peptide undergoes further cleavage at the site, $I^{172}ETD^{\downarrow}S$, generating of a 17 kDa fragment. The active mature enzyme is a tetrameric complex consisting of two p17 and two p12 subunits. The cleavage sites associated with activation of procaspase-3 has been abolished in the mutant procaspase-3(D3A) by through Ala substitution at residues 9, 28 and 175 (Fig.2). This mutant is therefore incapable of being activated. However, it still has some activity toward the caspase-3-specific peptide substrate, Ac-DEVD-*p*NA, exhibiting similar binding affinity to that of the mature enzyme (Karki *et al*., 2005). The catalytic efficiency is greatly reduced (about 200 folds) compared to that of the mature enzyme. The inability of procaspase-3(D3A) to undergo activation makes it an ideal zymogen to investigate the possible activation of this enzyme by vEP.

In this study, the activation of a procaspase-3(D3A) by vEP to yield active caspase-3 was investigated. The activated enzyme was characterized with respect to substrate specificity and sensitivity to inhibitor. The cleavage sites with in procaspase-3(D3A) recognized by vEP were also determined and the implication of this *in vitro* activation of a procaspase by vEP was studied.

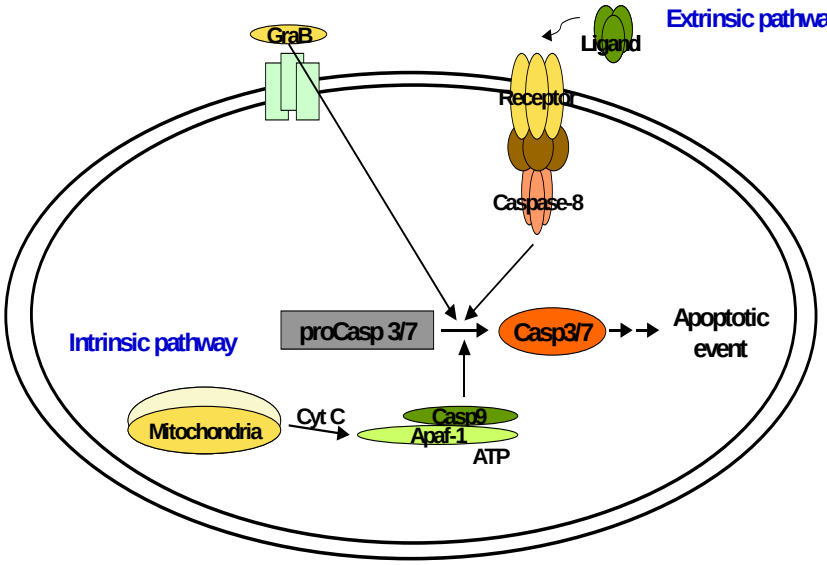

Fig. 1. Apoptosis pathway. Currently there exist three recognized points at which apical proteases are activated to initiate apoptosis. The extrinsic pathways include delivery of grandzyme B to the cells as well of receptor ligation. Following TNFR-1 or Fas receptor ligation, the initiator caspase-8 is activated by adapter-mediated recruitment to the receptor's cytosolic face (Muzio *et al.,* 1998). Alternatively in the intrinsic pathway, the initiator caspase-9 is activated following release of mitochondrial components to form the Apaf complex (Nijhawan *et al.,* 1997). Both activated initiators converge on the proteolytic activation of caspase-3. In death receptor triggered apoptosis the main pathway is direct activation of procaspase-3 by caspase-8 (Stennicke *et al.,* 1998). The important of the mitochondrial pathway in death receptor triggered apoptosis is unknown, but apparently subordinate to the dominant, direct pathway in most cell types (Kuida *et al.,* 1998).

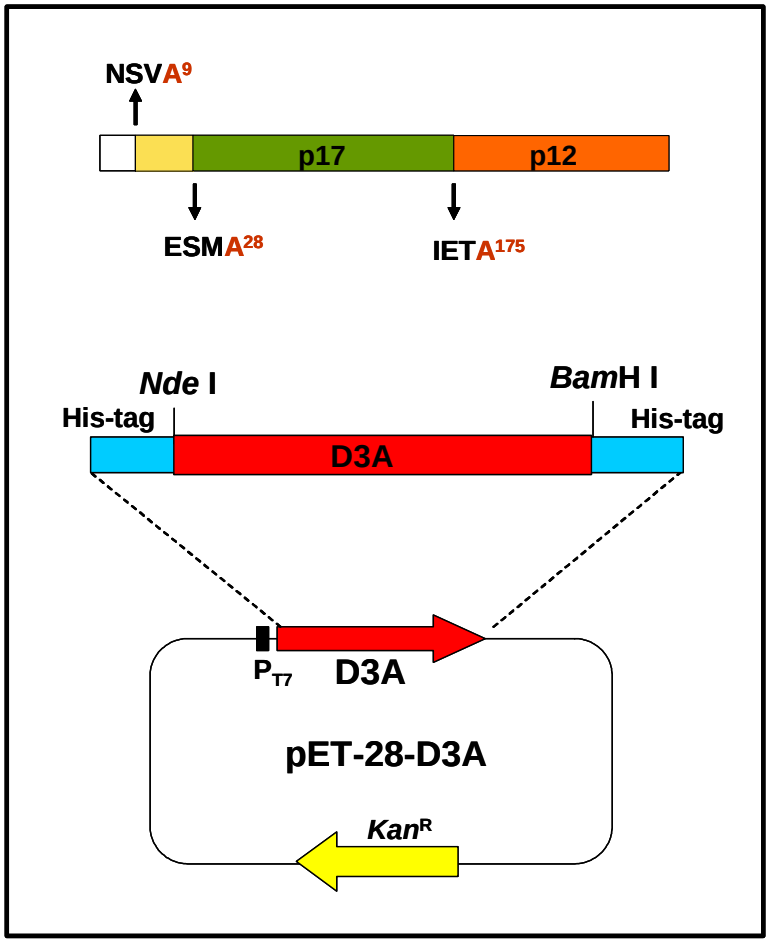

Fig. 2. Physical map of the procaspase-3(D3A) mutant construct.

II. MATERIALS AND METHODS

II-1. Materials

Hiprep 16/10 Q FF column, Superdex 75 10/300 GL columns and PD-10 column were purchased form Amersham Pharmacia Biotech Co. (Uppsala, Sweden). Protein assay kit and Polyvinylidene difluoride (PVDF) membrane were from Bio-Rad (Richmond, Calif). The caspase-3 specific chromogenic substrate, Ac-Asp-Glu-Val-Asp-*p*NA (Ac-DEVD-*p*NA) was obtained from BACHEM (Switzerland). The caspase-3 specific inhibitor, Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) was a gift from Prof. I-S. Park's lab (Department of Bio-materials, Chosun University, Korea). Mouse polyclonal antibody against poly(ADP-ribose) polymerase (PARP) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit and anti-mouse peroxidase-conjugated immunoglobulin G (IgG) was obtained from Jackson ImmunoPesearch (West Grove, PA). Rabbit monoclonal antibody raised against caspase-3 was a kind gift from Prof. H-S. Chun (Department of Bio-Science, Chosun University, Korea). NTA-chelating agarose CL-6B was obtained from Peptron (Nalgene), Phenylmethylsulfonyl fluoride (PMSF), azocasein, ammonium sulfate, bovine serum albumin (BSA), protein weight markers, trizma base, and other chemicals used were obtained from Sigma Co. (USA).

II-2. Purification of vEP from *V. vulnificus*

vEP was purified from *V. vulnificus* sp. strain ATCC29307. Cells were grown in LB medium containing 0.5% NaCl at 37°C to an A_{600} of about 2.6. The culture was centrifuged at 10,000 Xg at 4°C for 30 min to collect the supernatant. Ammonium sulfate was added to the supernatant to give 70% saturation. The resulting precipitate was collected by centrifugation at 16,000 Xg at 4°C for 40 min. The pellet was dissolved in 25 mM Tris-HCl (pH 7.5) and desalted on PD-10 columns (Amersham Biosciences) equilibrated with the same buffer. The desalted sample was applied to a HiPrep 16/10 Q FF column (Amersham Biosciences) pre-equilibrated with the same buffer at room temperature. After washing with 10 column volumes of equilibration buffer, the column was eluted with a linear gradient of NaCl from 0 to 0.5 M in the same buffer. Fractions were assayed for protease activity using azocasein as a substrate, and those fractions which contained major protease activities were pooled, concentrated by ultra-filtration using an Amicon YM10 membrane (Millipore, Billerica, MA), and then further fractionated on a Superdex 75 100/300 GL gel filtration column (Amersham Biosciences) in 50 mM Tris-HCl (pH 7.5)/0.15 M NaCl. Fractions with major protease activities were pooled, concentrated, and used as the purified enzyme and stored in small aliquots at -20°C.

II-3. Expression and purification of procaspase-3(D3A)

The procaspase-3(D3A) mutant construct was obtained from Prof. I-S. Park (Department of Bio-materials, Chosun University, Gwangju, Korea), in which the aspartate residues at 9, 28 and 175 were substituted with alanine (Fig. 2A) to prevent auto-processing of the zymogen. The enzyme contained a His tag at the N- and C-termini. The recombinant vector pET-28-D3A was transformed into *E.coli* BL21 (DE3). Cells harbouring the pET-28-D3A construct were grown in LB medium at 37^{o}C to an A_{600} of about 0.6, and then IPTG was added to the culture to a final concentration of 0.2 mM. The culture was incubated at 25^{o}C for overnight. The cells were harvested by centrifugation at 10,000 Xg at 4^{o}C for 30 min. The pellet was resuspended in binding buffer (25 mM Tris-HCl (pH 8.0)/0.5 M NaCl/5 mM imidazole) and then passed through a French Press (ThermoSpectronic) two times followed by sonication. The lysate was centrifuged at 16,000 Xg at 4^{o}C for 40 min and the supernatant was filtered through a 0.45 μm filter and then applied to a Ni-NTA column (15 cm) pre-equilibrated in binding buffer. The column was washed with 100 ml of binding buffer, followed by 50 ml of wash buffer (binding buffer containing 60 mM imidazole). Bound proteins were eluted with 25 mM Tris-HCl (pH 8.0)/0.5 M NaCl/300 mM imidazole. Fractions were collected at 1 ml per fraction, and analyzed by 15% SDS-PAGE. Fractions containing major amount of procaspase-3(D3A) protein were pooled, concentrated by ultra-filtration using an Amicon YM10 membrane (Millipore, Billerica, MA), and stored in small aliquots at -70^{o}C.

II-4. Activation of procaspase-3(D3A) by vEP

Activation of procaspase-3(D3A) was performed by incubating the zymogen (0.16 - 0.58 mg/ml) with 0 - 8 μg/ml vEP in 20 mM Tris-HCl (pH 7.5) for 10 min at room temperature. The reaction was terminated by addition of 1 mM 1,10-phenanthroline (a potent inhibitor for vEP) and 20 μl aliquots were taken and assayed for activity toward the caspase-3 specific chromogenic substrate, Ac-DEVD-*p*NA, in a 100 μl reaction volume consisting of 20 mM Hepes (pH 7.5), 1 mM EDTA, 1 mM EGTA, 20 mM NaCl, 10 mM DTT and 1 mM 1,10-phenanthroline and the 0.4 mM Ac-DEVD-*p*NA. The release of *p*NA was continuously monitored by measuring the absorbance at 405 nm at 37^{o}C using a 96-well plate reader (Molecular Device). For assay with the caspase-3 inhibitor, Ac-DEVD-CHO, 20 μg of procaspase-3(D3A) was activated with 80 ng vEP in total reaction volume of 40 μl for 10 min at room temperature and then stopped by addition of 1 μl of 40 mM 1,10-phenanthroline. Twenty μl aliquots were taken and assayed for caspase-3 activity as described above in absence of presence of various concentration of the inhibitor.

II-5. Cleavage of poly(ADP-ribose) polymerase (PARP)

NIH3T3 cells from two 10- cm dishes were harvested and washed twice with PBS and then resuspended in 100 μl of lysis buffer (10 mM Tris-HCl (pH 7.5),

0.5% Triton X-100, 5 mM EDTA, 1 mM PMSF) and incubated in ice on a shaker for 15 min. Ten μl of the clear lysate (150 mg proteins) was incubated with state concentration of procaspase-3(D3A) for 30 min or with vEP-activated procaspase-3(D3A) for 5 to 60 min at room temperature, and the reaction stopped by addition of 1 mM 1,10-phenanthroline followed by addition of 20 μl of 2X SDS-PAGE sample buffer. The samples were heated at 100^{o}C for 1 min and then subjected to SDS-PAGE followed by western blot analysis using antibody against PARP.

II-6. SDS-PAGE and Western blot

SDS-PAGE was performed according to the method of Laemmli (Laemmli, 1970). Samples to be analyzed were mixed with an equal volume of 2X SDS-PAGE sample buffer, heated at 100^{o}C for 1 min, and then loaded onto the gel. After electrophoresis, protein bands were visualized by staining the gel with Coomassie blue. For Western blot analysis, protein samples were subjected to SDS-PAGE and then transferred to a PVDF membrane. The membrane was blocked at room temperature for 2 hr with blocking solution (Tris-buffered saline [TBS] containing 5% skim milk and 0.2% Tween 20) and then incubated with rabbit monoclonal antibody against caspase-3 (1:5000 dilution) or mouse polyclonal antibody against PARP (1:500 dilution) for 1 hr. The membrane was washed 4 times in TBS plus 0.2% Tween 20, followed by incubation with peroxidase-conjugated anti-rabbit (for anti-caspase antibody) or anti-mouse (for

anti-PARP antibody) IgG antibody for 1hr. The blot was washed 4 times in TBS plus 0.2% Tween 20, followed by detection with ECL western blotting detection reagents (Amersham Biosciences).

II-7. Protein assay

Protein concentrations were determined with Bradford reagent (Sigma) according to the manufacturer's instructions.

II-8. N-Terminal sequencing

Protein samples were subjected to electrophoresis on 15% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to PVDF membrane in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer (pH 11) containing 10% methanol. The blot was stained with Coomassie blue, followed by destaining. Target bands were excised from the blot and subjected to N-terminal sequencing using an Applied Biosystem Precise Sequencer (Applied Biosystem) as described elsewhere.

III. RESULTS AND DISCUSSION

III-1. Purification of vEP from *V. vulnificus*

Approximately 0.5 mg of vEP was obtained from 2 liters of culture supernatant. The purified vEP had a specific activity of 9,110 U/mg of protein, which represents approximately a four-fold increase over the culture supernatant. Table. 1 summarizes the results of purification. The purified enzyme eluted as a single peak on Superdex 75 100/300 GL gel filtration column (Fig.3). However, on SDS-PAGE, it exhibited two bands, a minor band and a major band with apparent molecular masses of about 48 kDa and 36 kDa, respectively (Fig. 3).

III-2. Expression and purification of procaspase-3(D3A)

The purified procaspase-3(D3A) consisted mainly of the 32 kDa zymogen on SDS-PAGE with minor contaminating bands of about 14 kDa and much fainter bands between the 20 kDa and 14 kDa regions (Fig. 4). It was assumed that both bands could be a product of proteolysis by *E. coli* proteases during expression or purification.

III-3. Activation of procaspase-3(D3A) by vEP

Cleavage of procaspase-3(D3A) by vEP yielded various fragments, with a 25 kDa fragment being the most abundant (Fig. 5A). The corresponding 17 kDa and 12 kDa fragments which were absent in the case of none-cleaved zymogen were present at low amount. Western blot detected a band of approximately 20 kDa for the non-activated procaspase-3(D3A), and therefore this fragment appeared to be caspase-3 protein, derived probably from the cleavage of the zymogen during expression or purification. The presence of this band was not observed by Bose and Coworkers (2003) in their study of the same procaspase-3 mutant. This might possibly be due to the different amount of loading of the protein, which is 1 μg in this case, compared to 8 μg in Fig. 5. When procaspase-3(D3A) was cleaved with vEP, the 20 kDa band disappeared and instead two prominent bands appeared at around the 17 kDa region, one is having the same size an the large subunit of mature caspase-3 while the other is smaller. There was no detection of the small subunit as the antibody was raised only to the large subunit of caspase-3 (Fig. 5B).

Procaspase-3(D3A) exhibited some activity toward Ac-DEVD-*p*NA, but the activity clearly increased upon cleavage by vEP, and this increase was dependent on the concentration of zymogen but appeared to reach a plateau at 0.5 mg/ml zymogen (Fig.6A). The increase in activity toward Ac-DEVD-*p*NA also increased with increasing concentration of vEP used in the reaction, but the activity decreased at vEP concentration above 2 μg/ml (Fig. 6B).

The decrease in activity at higher concentration of vEP was probably due to

additional cleavage of the mature caspase-3 by vEP. Using 0.5 mg/ml procaspase-3(D3A) and 2 μg/ml vEP, the activation of procaspase-3(D3A) over time was investigated. Maximal activation appeared to occur at 10 min, and beyond this time point, the activity decreased rapidly (Fig. 7A). Such a decrease in activity could also be the result of additional cleavage of the active caspase-3 through prolonged incubation with vEP. SDS-PAGE showed the presence a fragment with apparent molecular mass of about 17 kDa (Fig. 7B) that positively correlated with enzyme activity toward Ac-DEVD-*p*NA. This is further confirmed by Western blot analysis (Fig. 7C). The drop in activity with prolonged activation correlated with the gradual loss of the 17 kDa band on Western blot, and at the same time, the increase in intensity of various bands at around the 14 kDa region. Since the anti-caspase-3 antibody does not detect the small subunit of caspase-3, there in bands must be derived from the 17 kDa band. Therefore, the loss of enzyme activity was a consequence of further cleavage of the active enzyme by vEP.

Furthermore, with the activation of procaspase-3(D3A) by vEP, the loss of intact zymogen with time did not result in a corresponding increase of the mature enzyme. This suggests that cleavage of the zymogen to yield active caspase-3 is either not a specific event or that the active caspase-3 is highly susceptible to further cleavage by vEP, whereby the rate of cleavage is faster than its production from the zymogen. An interesting feature to note is that the formation of the 25 kDa band from the cleavage of the zymogen by vEP was rather stable during the course of 60 min of activation time. It has been known

that expression of caspase zymogens in *E .coli* could lead to their activation, a process that is thought to be the consequence of intrinsic proteolytic activity residing in the caspase zymogens. This auto-processing of the zymogens to active forms has been dubbed the induced-proximity model, which states that caspase zymogens are auto-processed once they are brought into proximity with each other (Salvesen and Dixit, 1999). In the case of wild-type caspases, it is therefore possible to obtain either processed active caspase or unprocessed zymogen from the same construct depending on the expression conditions. Short induction times (< 30 min) yield unprocessed zymogens, but longer ones (> 3 hours) yield fully processed enzymes. Using an uncleavable procaspase-3 mutant such as D3A (D9A/D28A/D175A), the problem associated with auto-processing by the recombinant zymogen could be avoided, therefore making it possible to study the activation of the enzyme by another protease. It has been reported that the unprocessed procaspase-3 is catalytically competent but the catalytic efficiency is about 130 to 200 folds lower than its mature form despite having similar K_M values (Bose *et al*., 2003; Karki *et al*., 2005). Attempts have been made to obtain kinetic properties for the vEP-activated procaspase-3(D3A). However, it was not possible to determine such properties as K_M and k_{cat} values with Ac-DEVD-*p*NA as a substrate since the activity could not reach saturation at the highest possible concentration of substrate used. This may due to the different processing of the zymogen by vEP as opposed to that obtained with auto-processing.

Activation of procaspse-3 by other proteases has previously been reported

for enzymes such cytotoxic T cell serine protease granzyme B (Darmon *et al*., 1995) or other interleukin-1 b-converting enzyme (ICE) like proteases (Liu *et al*., 1996). Zhou et al., (1997) studied the activation of procaspase-7 by various serine proteases covering a cross-section of the specificity repertoire of proteolysis and showed that the cleavage of the zymogen occurred within the linker region between the large and small subunits converting a single-chain zymogen to two-chain forms (Zhou and Salvesen, 1997). vEP is a broad specificity protease that can cleave at the carboxyl side of Asp, Thr and Tyr, with the surrounding amino acid sequence playing an important part in the cleavage sites (Chang *et al*., 2005).

From those cleavage results, it is clear that vEP cleaves at multiple sites within procaspase-3(D3A), and the rate of cleavage for each site might be different. However, the cleavage point did need not happen at the highly conserved Asp residue that directs cleavage specificity *in vivo*, as evident in this study. Thus, the formation of active caspase-3 could only result from the cleavage at sites that are near the native cleavage sites of $ESMD^{28}$ and $IETD^{175}$. Since the presence of the 17 kDa band disappeared with longer activation time, cleavage at other sites within this peptide is likely to be the cause of its loss. Assuming that cleavage of the zymogen by vEP does yield both p17 and p12 subunits, then the loss of caspase-3 activity is caused either by further cleavage of p17 or p12 subunits or both. The loss of p12 subunit could not be detected with the monoclonal anti-caspase-3 antibody used in this study. Cleavage of the zymogen by vEP within the region corresponding to that

of p12 is also obvious due to the presence of the 25 kDa band, which corresponds to a C-terminally truncated from of the zymogen. This can represents to be a N-terminally-truncated fragment, because N-terminal sequence analysis was not performed yet. Thus whether cleavage of the zymogen by vEP will produce an active enzyme with a p17 and p12 like species or a catalytically less competent truncated intermediate form depends on where the cleavage occurs.

In order to determine in the cleavage sites of procaspase-3(D3A) by vEP occurs, three fragments of the cleavage products representing both the 17 kDa and 12 kDa regions were subjected to N-terminal sequencing. Two of the larger fragments with apparent molecular masses of 17 and 18 kDa were found to have the same sequence I^{20}IHGS, whereas the smaller fragment of apparent molecular mass of 14 kDa has the sequence, M^{61}TSRS. The sequences obtained from these fragments showed that vEP cleaved the peptide bonds between K^{19}-$I^{20}$and also G^{60}-M^{61} bonds within the zymogen. Unfortunately, the small subunit-like fragment could not be identified since what appeared to be the small subunit turned out to be still largely composed of the large subunit domain (Fig. 8).

Ni^{2+} and Cu^{2+} which are inhibitor of vEP did not selectively inhibit the degradation of 17 kDa fragment, but rather, inhibited the cleavage of the zymogen (Fig. 9A). The lack of caspase-3 activity generated in the presence of Ni^{2+} and Cu^{2+} (Fig. 9B) further confirmed the action of vEP and shows that the proteolysis of the zymogen by vEP could be a key factor in yielding an increase

in caspase-3 activity as detected by the release of *p*NA group from Ac-DEVD-*p*NA. As with native caspase-3, vEP-activated procaspase-3 was also sensitive to the inhibitor Ac-DEVD-CHO (Fig. 10). The sensitivity appears to be lower than that reported for caspase-3 (Karki *et al*., 2005).

III-4. Cleavage of poly(ADP-ribose) polymerase (PARP)

Addition of recombinant procaspase-3 to a cytosolic extract prepared from NIH3T3 cells resulted in the cleavage of PARP. However, if the zymogen was first treated with vEP, and then added to the cytosolic extract, more cleavage of PARP was observed under the same incubation time. This confirms the ability of the activated caspase-3 to cleave PARP. In the presence of 1,10-phenanthroline, vEP has no proteolytic activity (Chang *et al*.,2005). Treatment of the cytosolic extract with vEP in the absence of inhibitor resulted in cleavage of this protein to fragments substantially smaller than 85 kDa (Fig. 11). The unexpectedly high level of PARP cleavage in the case of non-activated procaspase-3(D3A) could result from some form, of activation of the zymogen by proteases present in the extract of NIH3T3 cells. Such a problem could be better clarified by using purified PARP protein instead of cell extract. Cleavage of PARP has become a standard way to identify the presence of caspase-3 and its activity. vEP-activated procaspase-3(D3A) therefore still preserves the same specificity despite the possibility of being processed a different cleavage sites. It appears that as long as the processed enzyme could still retain the correct

active conformation, it can retain the substrate specificity of the enzyme that is processed by precise cleavage at the correct Asp residues. This is a significant event that emphasizes the versatility of a protease that has broad specificity whereby random cleavage of zymogen could lead to active enzyme that could further amplify a particular reaction cascade *in vivo*. However, there is currently no evidence to suggest that activation of procaspase-3(D3A) or other caspase by vEP actually takes place within the cell. As vEP could cause cell death to cultured mammalian cells such as NIH3T3 cells, the programed cell death pathway *in vivo* through by vEP-driven procaspase-3(D3A) activation remains to be investigated.

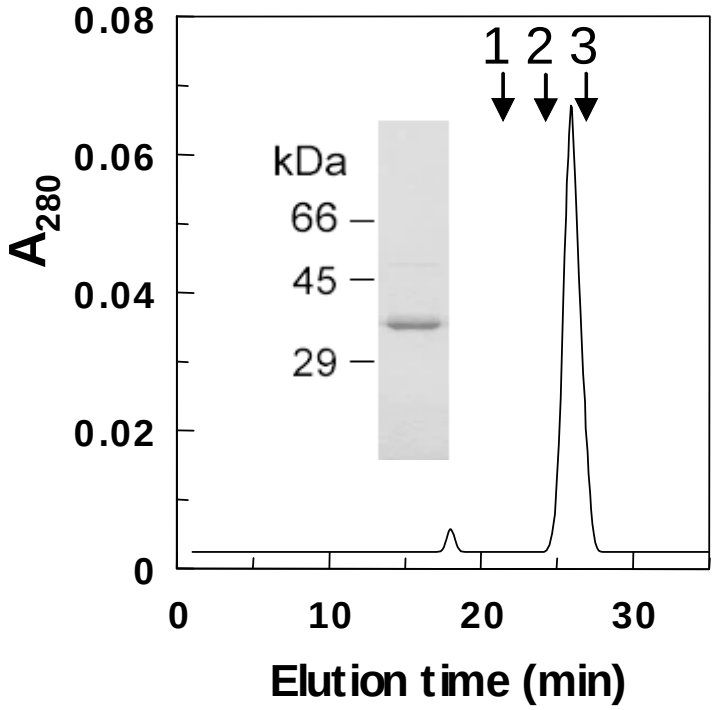

Fig. 3. SDS-PAGE analysis of purified vEP. vEP resolved from the Superdex 75 100/300 GL column was electrophoresed on 10% gel under reducing conditions. Arrows indicate positions of molecular markers: 1, 66 kDa; 2, 29 kDa; 3, 12.4 kDa.

Table 1. Summary of the purification of vEP

Fraction	Total Protein (mg)	Total Activity (U)[a]	Specific Activity (U/mg)	Yield (%)
Supernatant	38	88,900	2,340	100
$(NH_4)_2SO_4$	21	62,200	3,960	70
HiPrep Q	1.2	8,100	6,750	9.1
Superdex 75	0.56	5,100	9,110	5.7

[a], One unit of enzyme activity is defined as the amount of enzyme that catalyzes the proteolysis of 1 μg of azocasein per min at 37°C.

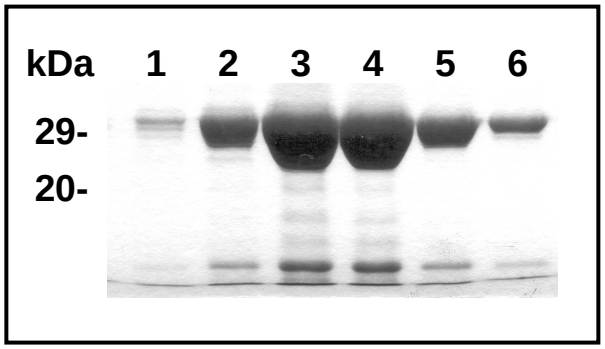

Fig. 4. SDS-PAGE analysis of purified procaspase-3(D3A). Fractions eluted from the Ni-NTA column were electrophoresed on 12% gel under reducing condition. Lanes 1 to 6 indicate fractions with major procaspase-3(D3A) protein.

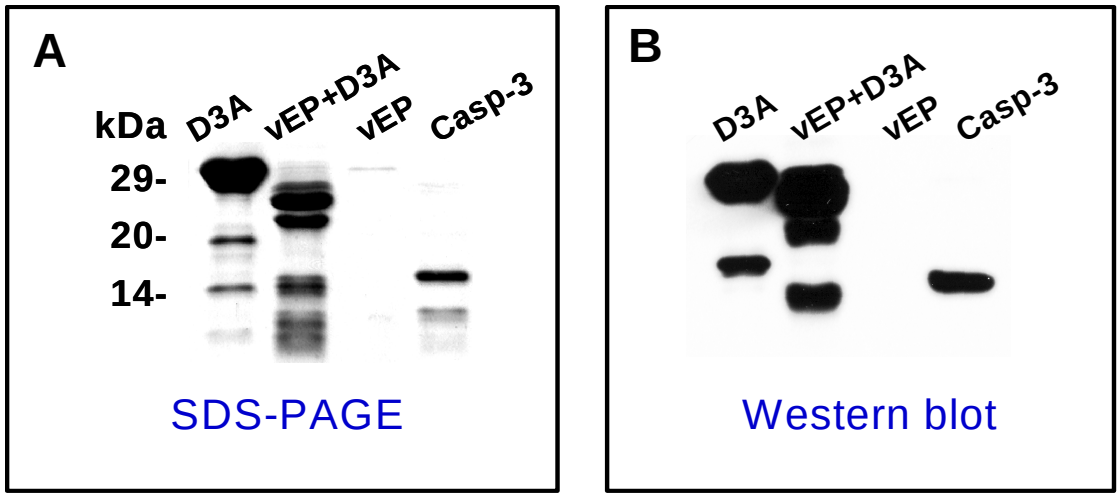

Fig.5. Activation of procaspase-3(D3A) by vEP. Purified procaspase-3(D3A) was cleaved with vEP as described in material and method and then subjected to SDS-PAGE (A) or western blot analysis with monoclonal antibody against the large subunit of caspase-3 (B). Casp-3, caspase-3; D3A, procaspase-3(D3A).

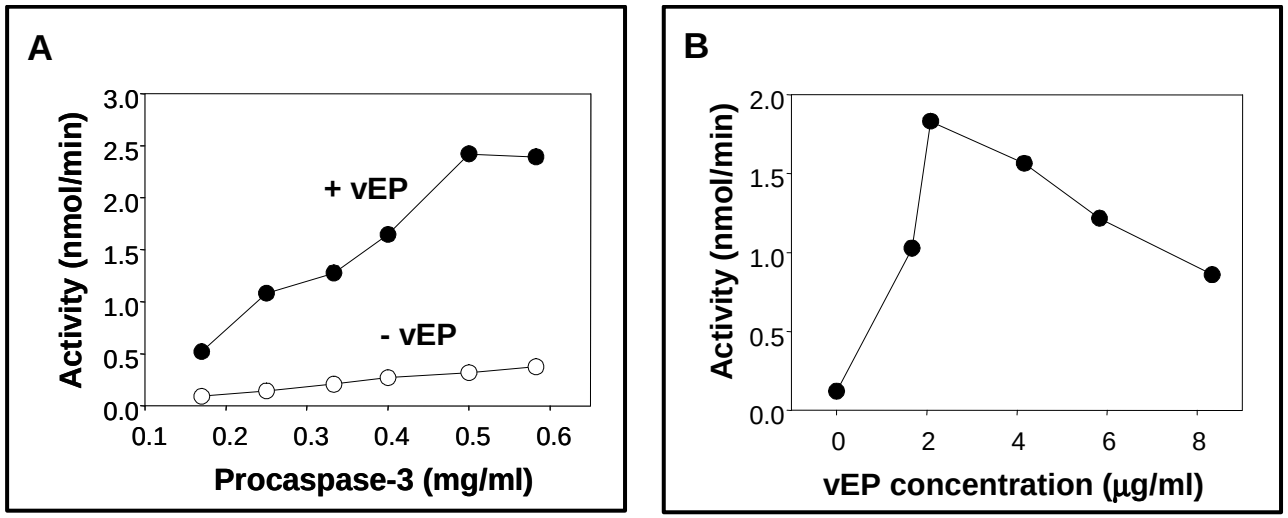

Fig. 6. Effect of zymogen and vEP concentration on the activation of procaspase-3(D3A). (A) Concentration effect of procaspase-3(D3A). vEP was used at a concentration of 2 μg/ml. (B) Concentration effect of vEP. Procaspase-3(D3A) was used a concentration of 0.5 mg/ml.

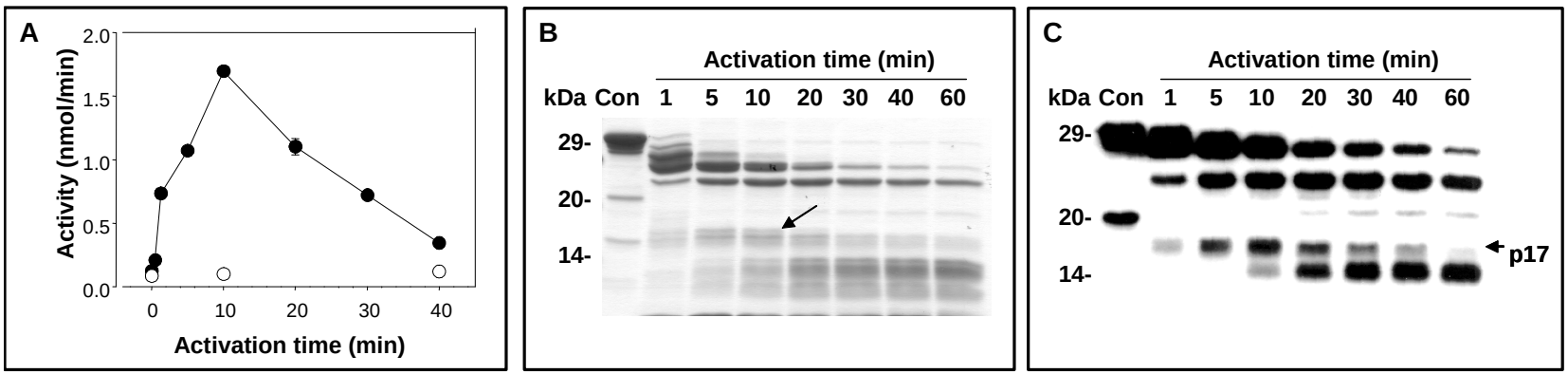

Fig.7. Time-dependent activation of procaspase-3(D3A) by vEP. Procaspase-3(D3A) activated by vEP for different time intervals were analyzed by activity assay toward Ac-DEVD-*p*NA (A), SDS-PAGE (B) or western blot analysis (C). Symbols: ○, procaspase-3(D3A); ●, vEP-activated procaspase-3(D3A). The arrow in panel B shows the fragment that is correlated with the activity profile and western blot result.

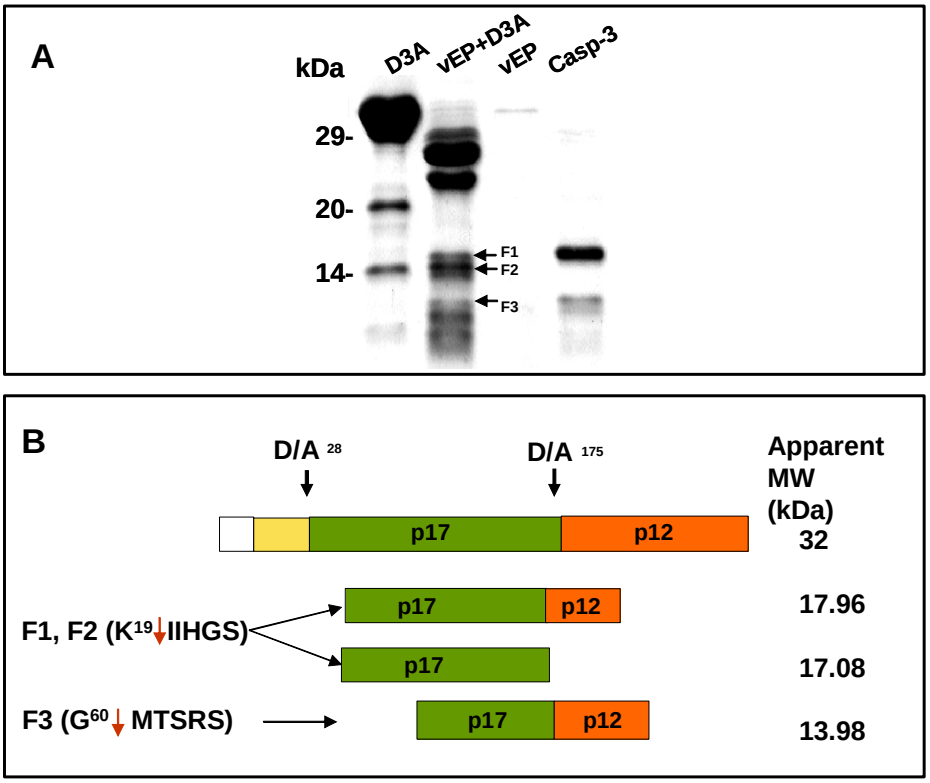

Fig. 8. Determination of vEP cleavage sites within procaspase-3(D3A). (A) SDS-PAGE of none activated and vEP-activated procapase-3(D3A). Fragments derived from the cleavage of procapase-3(D3A) by vEP as indicated by arrows were subjected to N-terminal sequencing. (B) The cleavage sites as obtained from N-terminal sequencing are shown together with a diagramtic representation of the fragment sizes as estimated from SDS-PAGE in panel A.

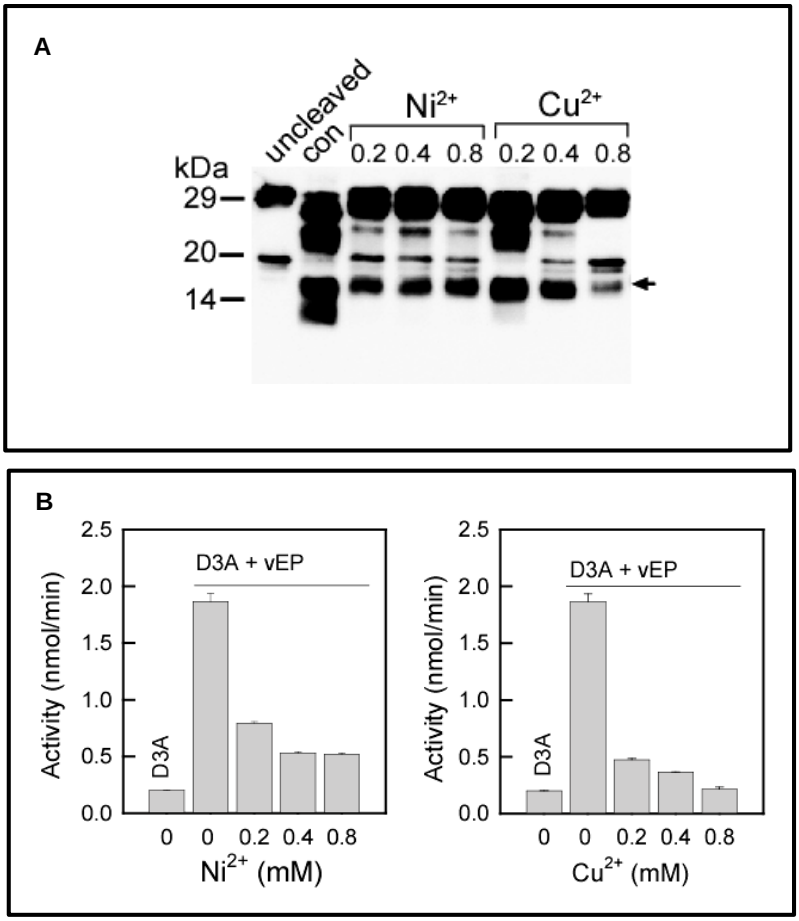

Fig. 9. Effect of Ni^{2+} and Cu^{2+} on the activation of procaspase-3(D3A). Procaspase-3(D3A) was activated with vEP in the absence or presence of different concentration of Ni^{2+} or Cu^{2+} and then subjected to activity assay with Ac-DEVD-*p*NA as substrate (A) or western blot analysis using polyclonal antibody directed against both the large and small subunits of caspase-3 (B).

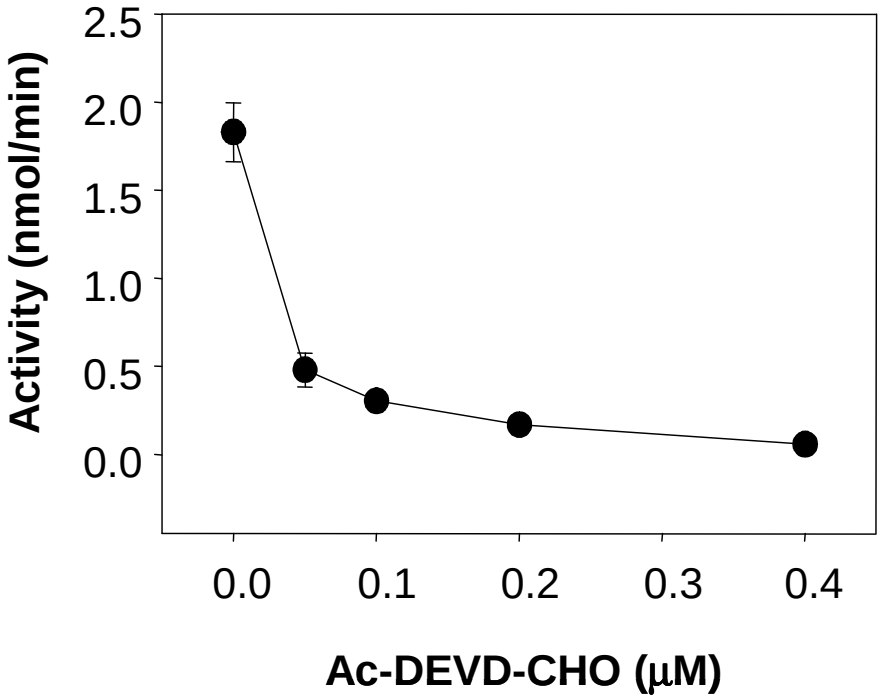

Fig. 10. Inhibition of vEP-activated procaspase-3(D3A) by caspase-3-specific inhibitor. vEP-activated procaspase-3(D3A) was assayed for activity towards Ac-DEVD-*p*NA without or with different concentrations of the inhibitor, Ac-DEVD-CHO.

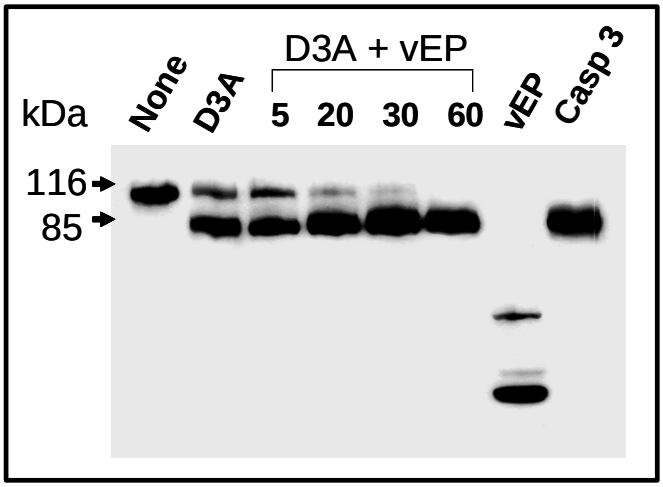

Fig. 11. Cleavage of poly(ADP-ribose) polymerase (PARP) by vEP-activated procaspase-3(D3A). Cleavage of PARP in cell extract of NIH3T3 was conducted at room temperature with procasepase-3(D3A) for 30 min, or with vEP-activated procaspase-3(D3A) for 5, 20, 30 and 60 min, or with vEP or mature wild-type caspase-3 only for 60 min. Uncleaved and cleaved forms of PARP were detected by western blot using polyclonal antibody raised against PARP. Casp.3, caspase 3.

Ⅳ. 적요

Vibrio vulnificus ATCC29307 균주에서 분비되는 단백질분해효소에 의한 procaspase-3의 활성화에 관한 연구

金 孝 永

朝 鮮 大 學 校 生 物 新 素 材 學 科

단백질분해효소는 상처의 치유, 세포 이동, 수정란 착상, 배의 발생 등 세포의 환경 변화에 관여하며 염증, 감염, 혈액 응고 등 생체의 항상성 유지에 매우 중요한 역할을 수행한다. 따라서 단백질분해효소의 기능과 세포내 역할 규명 연구는 단백질분해효소들과 관계있는 질병들의 치료에 매우 중요하다. 본 연구에서는 *Vibrio vulnificus* ATCC29307 균주로부터 단백질분해효소를 세포 배양액의 ammonium sulfate 침전을 통해 농축시킨 후, 이온교환 크로마토그래피 (HiPrep Q) gel filtration (Superdex 75) 크로마토그래피를 수행하여 정제하였다. 정제된 단백질 분해효소를 vEP라고 명명하였으며, 이 단백질분해효소는 다양한 생물학적인 활성을 지니고 있음을 규명하였다. vEP 프로트롬빈을 활성화시키며, 피브린을 가수분해하고, 세포외기질 단백질인 엘라스틴과 콜라겐을 파괴하는 특이성 등을 지니고 있음을 선행연구로부터 확인하였다. 따라서 본 연구에서는 vEP가 예정세포사에 밀접하게 관여하는 caspase-3의 전구체인 procaspase-3를 활성화시키는지를 규명하고자 하였다. Procaspase-3와 같은 전구체는 자가절단하는 활성을 지니고 있어, *E. coli*에서 전구체 형태로 분리하기가 어렵다. 따라서 본 연구에서는 활성상태의 caspase-3로 전환되는데 필수적 절단자리인 9, 28, 175위치의 Asp (D)잔기를 Ala

(A)로 치환한 procaspase-3 돌연변이체를 사용하였으며, 이것을 procaspase-3(D3A)라고 명명하였다. Procaspase-3(D3A)는 caspase-3의 특이적인 기질인 Ac-DEVD-*p*NA에 대해 약간의 활성을 지니고 있었으나, vEP에 의해 절단될 때 3배 이상의 높은 활성을 보였다. vEP가 프로트롬빈을 일시적으로 트롬빈 형태로 활성화시키는 것과 유사하게 vEP에 의한 procaspase-3(D3A)의 활성화도 일시적인 것이었다. Caspase-3 특이적인 기질 Ac-DEVD-*p*NA를 이용한 효소활성 분석과 caspase-3의 large subunit에 대한 항체를 사용한 Weatern blot 분석으로 확인한 결과, 사용한 vEP 농도 범위에서 procaspase-3(D3A) 활성화는 처리시간 10분에서 최고수준을 보였다.

생체내에서 caspase-3가 poly(ADP-ribose) polymerase (PARP)를 활성화시키는 것처럼 vEP에 의해 활성된 procaspase-3(D3A)로부터 생성된 caspase-3도 PARP를 활성화시킴을 western blot 분석을 통해 규명하였다. vEP에 의해 생성된 caspase-3는 caspase-3의 저해제인 Ac-DEVD-CHO에 의해 활성이 저해되었다. N-말단 아미노산 서열분석을 수행한 결과, vEP는 procaspase-3(D3A)의 치환부위의 안과 밖 즉, K^{19}-I^{20}, G^{60}-M^{61} 사이의 펩티드 결합을 절단하여 기능적인 caspase-3를 생성함을 확인할 수 있었다. 이와 같은 연구결과는 비브리오균과 같은 세균에서 분비되는 vEP와 같은 단백질 분해효소에 의해 예정세포사과정의 주요한 인자인 비활성 procaspase-3가 활성화되어 예정세포사가 유도될 수 있음을 제시하는 것이다.

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