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Biochemical study on activation mechanism of caspases-3, -4, -9 and Bid cleavage by caspase-2

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Caspases-3, -4, -9 활성화와

caspase-2 에 의한 Bid 절단의 생화학적 연구

2008 년 2 월 25 일

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ABSTRACT

Biochemical study on activation mechanism of caspases-3, -4, -9 and Bid cleavage by caspase-2

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Caspases, an evolutionarily conserved family of cysteine proteases, are the key mediators of apoptotic cell death program. The knowledge on the activation mechanism of these proteases provides the basis to understand the regulation of whole apoptotic machinery. In this study, the biochemical mechanism of activation of caspases-3, -4 and -9 was elucidated in terms of representative members of effector, inflammatory and initiator groups of caspases, respectively. During the comparative kinetic study between pro- and mature form of caspase-3, the enzymes showed identical K_M values but procaspase-3 had ~ 200 times lower k_{cat} than its mature form. Both of the enzymes also exhibited similar optimum pH values. However, in $1/K_M$ vs. pH study, a residue with pK_a of 6.89±0.13 was detected only for caspase-3 and V_{max} vs. pH kinetic results were consistent with the existence of a residue with

pK_a of 6.21±0.06 in procaspase-3 mutant (Asp9, Asp28 and Asp175/Ala). In diethylpyrocarbonate inactivation assays, a residue with pK_a of 6.61±0.05 was determined for caspase-3, whereas a residue with pK_a of 6.01±0.05 was assigned for procaspae-3 with iodoacetamide. These results indicate that the pK_a values of catalytic cysteine and histidine residues are changed during the activation process of caspase-3. With citrate, a well-known kosmotrope to enhance the monomer-dimer transition, caspase-4 was activated ~40 times that was comparable with that of caspase-9 (~75 fold increments). The activation reaction was mainly bimolecular ($n = 1.67 \pm 0.04$) for monomeric caspase-4. The interdomain cleavage was responsible to activate caspase-4 more than 100-fold, again comparable with that of effector caspases. These results suggest that caspase-4 shows a novel activation mechanism of the synergism between dimerization and proteolysis. In this study, the constitutively dimeric forms of procaspase-9 were constructed by connecting the termini of two caspase-9 molecules. The dimers could be overexpressed and purified from *Escherichia coli*. The recombinant dimers showed two-fold increment in specific activity over their respective monomer that was lower than expected if dimerization was the only mechanism to activate caspase-9. The failure to gain the substantial catalytic advancements by the dimers suggests a different scenario of dimerization between the constructed dimers and Apaf-1 activated caspase-9. On analyzing the influence of biochemical parameters on caspase-2 activity, Bid, a substrate of caspase-8, was cleaved about one-fourth less efficiently by caspase-2 and it also cleaved procaspase-7 much weakly but not procaspase-3. In contrast to the optimal pH

determined for its synthetic substrate, caspase-2 cleaved Bid and procaspase-7 much more efficiently at lower pH, while caspase-8 was more active at pH ≥7.0. Unlike the tolerance to 0-150 mM NaCl in hydrolyzing synthetic substrates, both caspases were sensitive towards higher concentration of salt in cleaving proteins. These observations suggest that the activity of caspase-2 for its physiological substrates is largely influenced by pH which might be a possible reason behind the inconsistency to observe the cleavage of its established *in vitro* substrates including Bid during caspase-2-induced apoptosis.

I. INTRODUCTION

I-1. Apoptosis

Programmed cell death or apoptosis is a physiological process of cellular autodestruction that plays critical roles in development, maintenance of homeostasis and host defense in multicellular organisms [1]. Dysregulation of this process is implicated in various diseases ranging from cancer and autoimmune disorders to neurodegenerative diseases and ischemic injuries [2]. Cells undergoing apoptosis exhibit a series of characteristic morphological changes, including plasma membrane blebbing, cell body shrinkage, and formation of membrane-bound apoptotic bodies [3]. Apoptosis is also accompanied by certain biochemical changes, notably the appearance of discrete DNA fragments in the form of ladder on conventional gel electrophoresis, and limited cleavage of various cellular proteins [4]. The core component of this machinery is a proteolytic system involving a family of proteases known as caspases.

I-2. Caspases

The name caspase derives from cysteine-dependent aspartate specific protease [5] and the term denotes two characteristic features of these proteases: they are cysteine proteases as the catalysis is governed by a

critical conserved Cys side chain of the enzyme, and they have a stringent specificity for cleaving the peptide bond C-terminal to Asp residue [3, 6]. The use of a Cys side chain as a nucleophile during peptide bond hydrolysis is common to several protease families but the primary specificity for Asp is very rare among proteases [7]. Of the currently known human and mouse proteases, only the caspase activator granzyme B, a serine protease, shares this specificity [8]. The critical involvement of caspases in apoptosis was first documented in 1993, when CED3 was found to play a central role in the programmed cell death in nematode worm *Caenorhabditis elegans* [9]. But the first known member of the caspase family was caspase-1, initially known as interleukin-1β-converting enzyme (ICE), an enzyme required for the maturation of interleukin-1β [10]. Since then, in the subsequent years, 11 caspases have been described in human, 10 in mouse, 4 in chicken, 4 in zebra fish, 7 in *Drosophila melanogaster* and 4 in *Caenorhabditis elegans* [11].

I-3. Members of the caspase family

The structure and primary function have been taken as two major criteria to classify caspases. On the functional basis, caspases are grouped into two main streams, distinguishing between inflammatory and apoptotic caspases (Fig. 1A). Caspases-1, -4, -5, and -11 fall into the category of inflammatory caspases since they are implicated in executing the innate immune response by activating specific pro-inflammatory cytokines in response to sensitizing pathogen-derived signals [12, 13]. The remaining family members are

primarily involved in apoptotic signaling pathways. These apoptotic caspases can be further divided into 'initiators' (caspases-2, -8, -9, -10, -12) and 'effectors' (caspases-3, -6, -7, -14) on the basis of their sequence of activation during apoptosis [14]. Upon activation by an initiator caspase, effector caspases are immediate 'executioners' of the apoptotic program, cleaving certain cellular substrates to cause demolition of the cell [15].

The second method of caspases classification utilizes the length of their prodomain and divides into two groups: those that have a relatively long prodomain and those containing a short prodomain. Caspases-1, -2, -4, -5, -8, -9, -10, -11 and -12 belong to the former category [12]. The long prodomain encompasses structural motifs including caspase-recruitment domain (CARD) or death effector domain (DED). These motifs enable caspases to associate with other proteins via homotypic interaction mechanisms [13]. Caspases-3, -6, -7 and -14 fall into the latter category. These caspases bear short prodomains and are activated upon proteolytic cleavage by other caspases [6].

I-4. Structure and activation of caspases

Almost all healthy cells contain several caspases as inactive precursors, often called caspase zymogens. Although caspase zymogens contain a small amount of catalytic activity, they are kept in check by a variety of regulatory molecules [16]. A caspase is never constitutively active unless an upstream signal triggers the conversion of the latent conformation to the active one [17]. Upon receiving an apoptotic signal, the caspase zymogens undergo

proteolytic processing at specific Asp residues to generate active enzymes comprising large (17-20 kDa) and small (10-12 kDa) subunits [18]. All caspases contain an active-site pentapeptide of general structure QACXG (where X is R, Q or G). The amino acids Cys-285 and His-237 involved in catalysis in caspase-1 are conserved in all the other caspases [7]. Although there is the absolute requirement for an Asp in the P_1 position in their optimal tetrapeptide substrates, the P_2-P_4 residues are not well conserved, suggesting that they may determine the substrate specificities of the different caspases [19]. The cleavage of the zymogen is not always an obligatory requirement for caspase activation, but all activated caspases can be detected as cleaved fragments in apoptotic cells [12]. Mature caspase is a tetramer (homodimer of the p20 and p10 heterodimers), with the two adjacent small subunits surrounded by two large subunits [20].

There are two well characterized apoptotic pathways in mammalian cells, classified on the basis of origin of death stimulus: the extrinsic (death receptor) pathway and intrinsic (mitochondrial) pathway (Fig. 1B). The extrinsic pathway is initiated by the interaction of death receptors like Fas, situated at the cell membrane, with their specific ligands. Upon ligation, the adaptor molecule FADD (Fas-associated protein with death domain) recruits caspase-8 zymogens by virtue of homophilic interaction with their N-terminal DEDs and within this death-inducing signaling complex (DISC), procaspase-8 is processed to generate active caspase-8 that can cleave downstream signaling proteins like procaspase-3 [21]. On the other hand, the intrinsic cell death pathway is triggered by cellular stress signals such as DNA damage

which generally leads to mitochondrial damage and subsequent release of cytochrome *c* [22]. This protein associates with Apaf-1, caspase-9 and dATP to form a mutiprotein complex called apoptosome. The complex acts as a holoenzyme, inducing the activation of procaspase-3 and -7 [23]. A cross-talk exists between the two apoptotic pathways. Caspase-8 cleaves Bid, whose c-terminal fragment acts on the mitochondria to release cytochrome c [24]. Conversely, caspase-3 and other downstream caspases can process procaspase-8 and -9 [25]. The requirement for this activation is not clear, but it may amplify the apoptotic signal.

I-5. Activation mechanism of caspases

The onset of apoptosis requires a cascade of sequential activation of initiator and effector caspases. The caspase cascade ensures quick generation of large amounts of active caspases to irreversibly damage cells upon apoptosis induction. At the same time, the deadly outcome of this cascade demands the process to be tightly regulated. The activation of effector caspases is executed by an initiator caspase through proteolytic cleavage after a specific internal Asp residue to separate the large and small subunits of mature caspase [3]. As a consequence of the intrachain cleavage, the catalytic activity of an effector caspase is enhanced by several orders of magnitude [26]. Once activated, the effector caspases are responsible for the proteolytic degradation of a broad spectrum of cellular targets that ultimately lead to cell death. As there are no any upstream proteases to cleave initiator

caspases, their activation and regulation is quite complex [13]. Although an initiator caspase undergoes an autocatalytic intrachain cleavage, this cleavage appears to have only modest effect on its activity [27]. In fact, the activation of initiator caspases is facilitated by specific adaptor protein complexes. Such complexes include PIDDosome, DISC and apoptosome for caspase-2, -8 and -9, respectively [28]. Recent studies have revealed that human inflammatory caspases (-1 and -5) also assemble in the interleukin-1β-activator complex, the inflammasome [29].

I-6. Caspase-3

Caspase-3 is the main downstream effector caspase responsible for the cleavage of majority of the cellular substrates during apoptosis. Its activation is initiated by trans-cleavage by caspase-8 or -9 followed by autocleavage [20]. The first cleavage at Asp175 in the intersubunit linker is sufficient to allow for full activity of the enzyme. The protein is then cleaved at Asp9 and Asp28 so that the prodomain is removed [3]. Unlike initiator caspases, caspase-3 is known to exist constitutively as a homodimer, *in vitro*, as well as *in vivo* and both before and after the intrachain activation cleavage [30]. In this context, an interesting question is why large pool of capsase-3 (~ 100 nM) found in the cell is not active [31].

Caspase-3 shares 55% sequence homology with caspase-7 and crystallographic structure of procaspase-7 has shown that the intrachain cleavage results in the conformational changes in active site [32, 33]. The

steric hindrance imposed by the interdomain linker in the zymogen is incompatible with substrate binding and catalysis, and the cleavage in the linker is essential to facilitate rearrangement of essential loops in the active site [32]. Further, the catalytic competency of caspase-3 was demonstrated to be under strict regulatory self-control by an Asp-Asp-Asp (Asp179-Asp181) tripeptide contained within the enzyme itself [34]. This regulatory tripeptide termed as “safety-catch” accounts for procaspase-3 resistance to autolytic maturation as well as resistance to proteolytic activation by upstream proteases such as caspase-9 and granzyme B [34]. Later, a study reported that the uncleavable procaspase-3 mutant had lower k_{cat}, higher optimal pH and same K_M as compared to that of mature caspase-3 [35]. The dimerization of caspase-3 molecules through their protease domains was shown to be required for their processing by initiator caspases and the subsequent auto processing could take place through cleavage between the dimeric intermediates [36]. The prodomain of caspase-3 was described as an intramolecular chaperone that facilitates both dimerization and active site formation in addition to stabilizing the native structure [37].

I-7. Caspase-4

A subgroup of caspase family of inflammatory caspases (-1, -4, -5, -11, and -12) play important role during cytokine maturation and inflammation but their regulation is not well understood as much as the initiator and effector caspases [38]. Caspases–4 and -5 are closely related to caspase-1 with 53%

and 51% identities, respectively and exhibit similar inhibitor preference for CrmA and p35 and could also cleave procaspase-3 *in vitro* [39]. Caspase-4 expression, while lower than that of caspase-1, generally shows a similar tissue distribution, being found in most tissues examined with the exception of brain [40]. Caspase-4 and -5 have different substrate specificities from that of caspase-1 and cleave pro-interleukin-1β much poorer [41].

Recent studies have revealed that proximity-induced activation may apply to initiator caspases involved in inflammation as caspases-1 and -5 seem to assemble in the interleukin-1β activator complex called the inflammasome [42]. But the knowledge is clearly deficient regarding substrates, expression and role of caspase-4 although it is closely related (77% sequence identity) to caspase-5 [3, 38]. Further, caspase-4 also shares 48% sequence homology with caspase-12 and some reports suggested that caspase-12 is predominantly localized in endoplasmic reticulum (ER), undergoes specific cleavage by ER stress and it can also play an important role in the pathogenesis of Alzheimer's disease [43]. In human caspase-12, the polymorphisms resulted from the gene interruption by frame shift, premature stop codon and amino acid substitution in the critical site for caspase activity has shown to drastically alter its function and thus ruling out its any functional significance [44]. Later, caspase-4 was shown to play a key role in ER stress- and amyloid beta-induced cell death, suggesting a possible functional substitution of mouse caspase-12 in human system [45, 46]. However, a controversy has arisen after a report that both caspase-4 and -12 are not required during ER stress-induced apoptosis [47].

I-8. Caspase-9

In human intrinsic apoptotic pathway, caspase-9 functions as an apical protease in response to cytotoxic stress, genome damage [48]. Caspase-9 exists predominantly as a monomer at normal physiological concentration and its activation is associated with dimerization facilitated by the interaction between its prodomain and adaptor protein Apaf-1 [28]. Although extensive studies have already established that proteolytic processing is largely meaningless in caspase-9 activation and dimerization is the sole activating event but the exact mechanism by which caspase-9 gains its catalytic competency after oligomerization still goes on continuous debate [49]. Among the two contrasting hypothesis proposed, the induced proximity model states that the initiator caspases autoprocess themselves when brought into close proximity of each other [26]. On the contrary, the allosteric regulation or induced conformation model insists the conformational change of caspase-9 active site upon binding to the apoptosome [27].

The apoptosome is a multiprotein complex comprised of Apaf-1, cytochrome *c* and caspase-9 in a 1:1:1 molar ratio [50]. The apoptosome is assembled when seven Apaf-1:cytochrome *c* heterodimers oligomerize to form a symmetrical “wheel” and procaspase-9 molecules become associated noncovalently to Apaf-1 via caspase-9 CARD/Apaf-1 CARD heterophilic interaction [51]. Binding of procaspase-9 to Apaf-1 is important for two reasons. First, it increases the intrinsic catalytic activity of the caspase-9 (~ 10^3 fold) protease leading to autolytic cleavage of procaspase-9 at Asp315 to

yield a large (p35) and a small (p12) subunit [52]. And second, cleavage exposes a neo-epitope comprising the NH_2- terminal four amino acids (ATPF) of the small subunit that has been shown to be both necessary and sufficient for binding to BIR3 domain of X-linked inhibitor-of-apoptosis protein (XIAP), leading to inhibition of caspase-9 [53].

I-9. Bid, as a substrate of caspase-2

Caspase-2 was the second identified mammalian caspase and remains one of the best conserved across species, but little is known about its mechanisms of activation, target substrates and potential roles in apoptosis [54]. Caspase-2 is also unique among its family members in that it has the properties of both initiators and effectors caspases. It shares sequence homology with initiator caspases, especially caspases-1, -4, -5, and -9 and its prodomain is most closely related in structure to that of caspase-9, both having a CARD in common [3]. Its activation mechanism is also shown to be similar to that of other initiator caspases [55], dimerization being the initial event during the activation, and PIDDosome, an analogous to Apaf-1 apoptosome, has been described as the activation complex for caspase-2 [55]. On the other hand, the cleavage specificity of caspase-2 resembles with effector caspase-3 and -7, although optimal caspase-2 substrates and inhibitors should contain pentapeptide sequences- VDVAD [56]. Unlike other initiator caspases, caspase-2 neither processes nor activates any effector caspases directly [57] but a study has reported that caspase-2 can activate

procaspase-7 [58].

Caspase-2 has recently received considerable attention, as several groups have sought to define its biological role in apoptosis signaling. Overexpressing caspase-2 provoked the release of pro-apoptotic molecules including cytochrome *c* from mitochondria [59], whilst diminished caspase-2 expression or a peptide caspase-2 inhibitor blocked etoposide-induced cytochrome *c* release from mitochondria [60]. This suggests that caspase-2 may function upstream of mitochondrial changes associated with stress-induced apoptosis. This could be recapitulated *in vitro* [60] and has been proposed to occur via direct caspase-2 mediated permeabilization of mitochondrial membranes [61].

A very limited number of substrates have been assigned to caspase-2 that includes its own precursor procaspase-2, Golgin, Bid and αII spectrin [57, 62, 63]. However, there exists a controversy regarding Bid as good substrate for caspase-2 as that for caspase-8 due to various inconsistent reports. Bid has been shown to be cleaved by caspase-2 at the same site by caspase-8 [57] and the cleavage was found to be an essential step in caspase-2-induced apoptosis [64]. On the contrary, some studies have proposed that caspase-2 induced mitochondrial outer membrane permeabilization independently of Bid cleavage and other cytosolic factors [57] and in one case, even independently of catalytic activity of caspase-2 [65]. Despite these controversies on the requirement of Bid cleavage and other cytosolic factors, the unparalleled ability of caspase-2 to engage the mitochondrial apoptotic pathway by permeabilizing the outer mitochondrial membrane and/or by breaching the

association of cytochrome *c* with the inner mitochondrial membrane has now been well appreciated [66].

I-10. Outline of the thesis

The major part of this thesis deals with the biochemical study on activation mechanism of caspases-3, -4, and -9. These three caspases were selected as representative members of the effector, inflammatory and initiator groups of the caspase family. The remaining portion of the thesis presents the biochemical analysis of caspase-2 mediated Bid cleavage and some related studies with the prospects for future research. The rationale behind these studies is summarized below.

1. A comprehensive comparative kinetic study was carried out between procaspase-3 and mature caspase-3 to explain the activation mechanism of this enzyme with biochemical details. For this purpose, an uncleavable procaspase-3 mutant (Asp9, Asp28 and Asp175/Ala) was employed as the surrogate for natural procaspase-3.
2. The activation mechanism for initiator and effector caspases has been described to be distinctly different, dimerization for the former and intrachain cleavage for the latter. This notion was examined by checking the ability of caspases to be activated with a well-known dimerization agent, citrate. In addition, the biochemical mechanism of caspase-4 activation was also elucidated.
3. The constitutively dimeric wild type and non-cleavable mutant forms of

caspase-9 were constructed in accord with the crystallographic structure of active caspase-9. The comparison of catalytic activity of the dimers with their respective monomers to cleave natural as well as synthetic substrates was performed to explore the important features of caspase-9 activation.

4. The optimal biochemical conditions for caspase-2 were determined and the efficiency of caspase-2 to cleave two of the reported protein substrates- Bid and procaspase-7 was compared with that of Caspase-8. The influence of major biochemical parameters (pH and salt) on the cleavage was also investigated.
5. The role of N-peptide of caspase-7 in the activity and activation of the enzyme was checked. For this purpose, full length and ΔN caspase-7 enzymes were purified and catalytic parameters were compared. The catalytic cysteine mutant forms of both caspase-7 were also purified to check any difference in their affinity for caspase-9 cleavage. Finally, several forms of hybrid dimeric caspase-9 mutant proteins were generated to analyze the expression, activity and citrate activation.

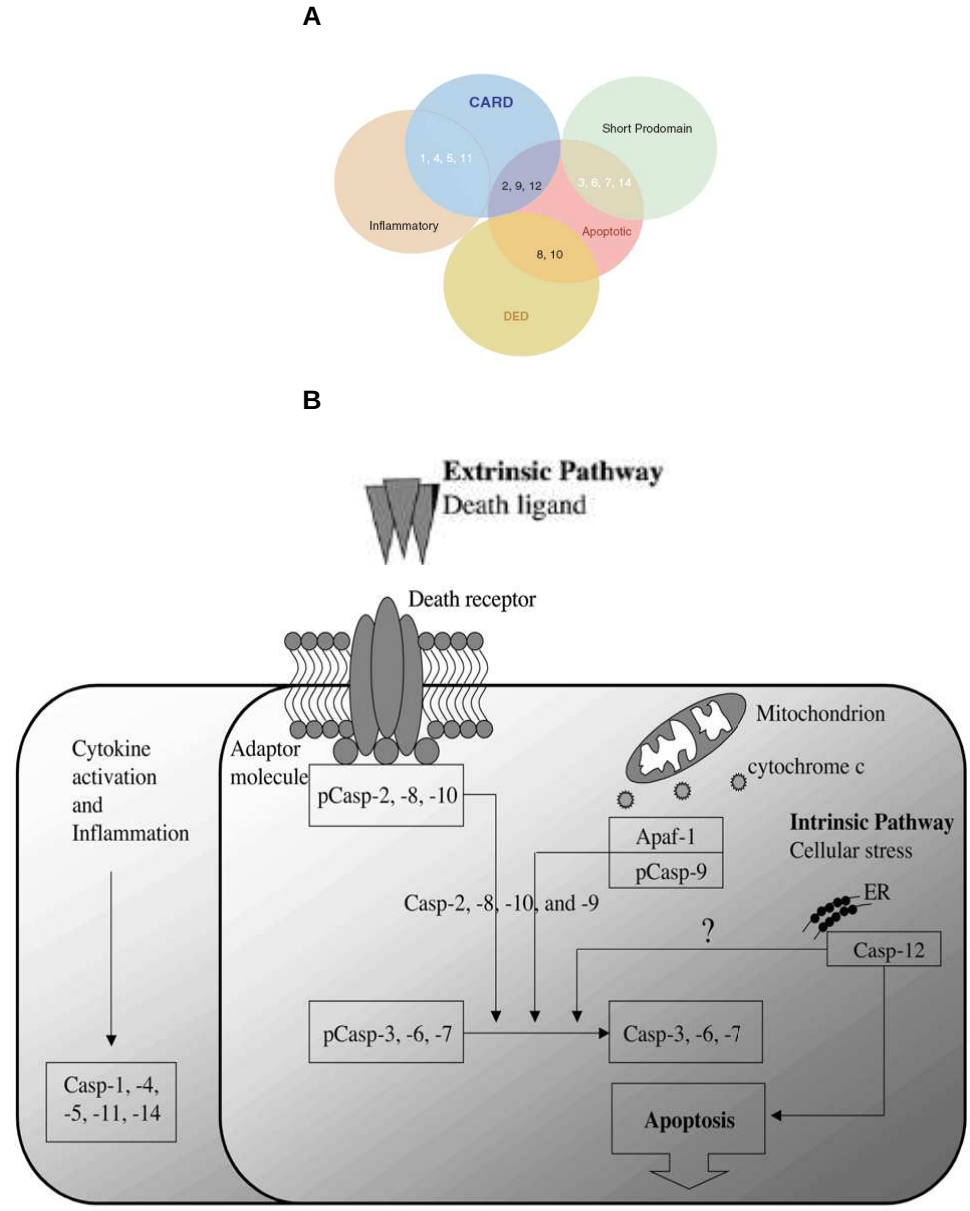

Fig. 1. Classification of caspases and framework of apoptosis [13, 67]. **(A)** Caspases are classified on the basis of their prodomain structure or primary function. **(B)** Two major apoptotic pathways are shown. p, procaspase zymogen.

II. MATERIALS AND METHODS

II-1. Materials

Sodium chloride, diethylpyrocarbonate (DEPC), iodoacetamide (IAM), imidazole, EDTA, EGTA, $MgCl_2$, and 2-(Cyclohexylamino) ethanesulfonic acid (CHES), were purchased from Sigma (St. Louis, USA). Sodium citrate, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), 2'-(N-morpholino)ethanesulfonic acid (MES), Tris[hydroxymethyl]aminomethane (Tris) were from USB (Cleveland, USA), Dithiothoureitol (DTT) was from Amresco (Ohio, USA), all caspase substrates and inhibitors were from AG Scientific Inc. (San Diego,USA), and Ni^{2+}-NTA, Hitrap Q columns were from Amersham Biosciences (Piscataway, USA). Caspase-9 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Welgene (Daegu, Korea). Polyclonal antibody against Bid was generated by injecting mice with purified recombinant Bid. All other chemicals were from Sigma (St. Louis, USA) unless specified.

II-2. Plasmid construction

The cDNA coding for human wild type caspase-3 was cloned in bacterial expression vector pET28b (Novagen) using *NdeI* and *BamHI* sites. The procaspase-3 mutant (D9A/D28A/D175A) was prepared in three successive steps by *DpnI* mediated site directed mutagenesis by PCR (New England BioLabs Inc.). The human caspases -2, -4, -6, -7, -8 and -9 were cloned in bacterial expression pET vectors (Novagen) and to generate the various mutant versions of these caspases point mutations were carried out.

To prepare the dimeric forms of caspase-9, *NdeI* site within caspase-9 was removed by point mutation and following PCR amplification the new molecule was inserted ahead of already cloned caspase-9 using *NdeI* and *BamHI* site in frame. All the primers used in the study are summarized in Table 1. The mutated plasmids were confirmed by sequencing both strands.

II-3. Expression and purification of recombinant proteins

All of these recombinant caspases were expressed as either N-terminal (pET 15b) or C-terminal (pET 21b) or both termini (pET 28b) histidine tags in BL21 (DE3) pLysS *Escherichia coli* strain (Novagen) and protein induction was carried out at 30 ℃ for 4 h with 0.4 mM isopropyl-β-D-thiogalactopyranoside. Two chromatographic steps of Ni^{2+}-NTA column followed by Hitrap Q sepharose were carried out for protein purification. During each step of purification, the basis of protein fractions selection was enzyme activity corresponding to the amount and purity of proteins observed on SDS-PAGE. Bid was purified with single step of Ni^{2+}- NTA column chromatography. The purified proteins were dialyzed against 20 mM HEPES (pH 7.5), 10 mM NaCl, 1 mM EDTA, 2 mM DTT, 10% glycerol and the aliquots were saved at –80 ℃ before use.

II-4. Enzyme assay and determination of kinetic constants

The caspase activity assay was performed with the various fluorogenic substrates Ac-VDVAD-AMC (for caspase-2), Ac-DEVD-AMC (for caspase-3

Table 1. Primers used for site directed mutagenesis

Mutation	Sense Primer*	Antisense Primer
Caspase-3 D175A	5’-GGCATTGAGACAGCTAGTGGTGTTG-3’	5’-CAACACCACTAGCTGTCTCAATGCC-3’
Caspase-3 D28A	5’-GAATCAATGGCCTCTGGAATATCC-3’	5’-GGATATTCCAGAGCCCATTGATTC3’
Caspase-3 D9A	5’-GAAAACTCAGTGGCTTCAAAATCC-3	5’-GGATTTTGAAGCCACTGAGTTTTC-3’
Caspase-4 E93A	5’-CGAATATGGCGGCTGGACCAC-3’	5’-GTGGTCCAGCCGCCATATTCG-3’
Caspase-4 D104A	5’-GAATCTACAGCTGCCCTCAAG-3’	5’-CTTGAGGGCAGCTGTAGATTC-3’
Caspase-4 D270A	5’-TGGGTCAGAGCTTCTCCAGCA-3’	5’-TGCTGGAGAAGCTCTGACCCA-3
Caspase-4 D289A	5’-CTGGAGGAAGCCGCTGTTTAC-3’	5’-GTAAACAGCGGCTTCCTCCAG-3’
Caspase-6 D23A	5’-CAGAAACAGCTGCCTTCTATAAAAG-3’	5’-CTTTTATAGAAGGCAGCTGTTTCTG-3’
Caspase-6 D193A	5’-CTGAGGTGGCTGCAGCCTCCG-3’	5’-CGGAGGCTGCAGCCACCTCAG-3’
Caspase-6 D179A	5’-GATGTAGTAGCTAATCAGACAG-3’	5’-CTGTCTGATTAGCTACTACATC-3’
Caspase-9 E306A	5’-CCTGAAGACGCTTCCCCTGGC-3’	5’-GCCAGGGGAAGCGTCTTCAGG-3’
Caspase-9 D315A	5’-GTAACCCCGAGCCAGCTGCCACCCC-3’	5’-GGGGTGGCAGCTGGCTCGGGGTTAC-3’
Caspase-9 D330A	5’-GACCAGCTGGCCGCCATATCTAG-3’	5’-CTAGATATGGCGGCCAGCTGGTC-3’

*The underlined sequences refer to the incorporated mutating sequence in each case.

and -7) Ac-VEID-AMC (for caspase-6), Ac-IETD-AMC (for substrates Ac-VDVAD-AMC (for caspase-2), Ac-DEVD-AMC (for casapse-3caspase-8), Ac-LEHD-AMC (for caspase-9) and Ac-LEVD-AMC (for caspase-4). The enzyme assay was carried out at 30 ℃ by monitoring the re lease of AMC at excitation and emission wavelengths of 360 and 480 nm respectively, using micro plate spectofluoremeter (Molecular Devices, CA, USA). The caspase assay buffer was supplied with MES (pH 5.5-6.5) or HEPES (pH 6.75- 7.75) or Tris (pH 8.0-8.5) or CHES (pH 9.0-10.0), 20 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM $MgCl_2$ and 10 mM DTT. The reaction volume was 100 μl and a varying range of substrate concentrations (10-500 μM) was employed for different caspases considering their K_M values. For citrate activation, 1M citrate was included in caspase assay buffer and the enzymes were pre-activated at 30 ℃ for 30 minutes before adding the substrate to initiate the reaction. 1. To determine the basic catalytic parameters $V_{\max}, k_{cat}$ *and* K_M Michaelis-Menten equation was employed [56]:

$$v = \frac{V_{\max}[S]}{K_M + [S]} \quad (1)$$

$$V_{\max} = k_{cat}.[E]_T \quad (2)$$

Where, $v =$ initial velocity, $[S] =$ substrate concentration, $[E]_T =$ total amount of enzyme used in reaction.

2. To calculate pK_a values from the various plots of pH profiles, the following scheme and equations were used.

$$
\begin{array}{ccc}
 & E + H^+ & ES + H^+ \\
pK_{a1} & \updownarrow & pK_{as1} \updownarrow \\
 & E_H + S \leftrightarrow E_H S \rightarrow E + P & \\
pK_{a2} & \updownarrow & pK_{as2} \updownarrow \\
 & E_{HH} & E_{HH} S
\end{array}
$$

$$\log V_{\max}' = \log V_{\max} - \log\left(1 + \frac{[H^+]}{K_{as1}} + \frac{K_{as2}}{[H^+]}\right) \qquad (3)$$

$$\log \frac{V_{\max}'}{K_M'} = \log \frac{V_{\max}}{K_M} - \log\left(1 + \frac{[H^+]}{K_{a1}} + \frac{K_{a2}}{[H^+]}\right) \qquad (4)$$

At low pH and $V_{\max} = V_{\max}'$,

$$\log \frac{1}{K_M'} = \log \frac{1}{K_M} - \log\left(1 + \frac{[H^+]}{K_{a1}}\right) \qquad (5)$$

Where, pK_{a1}, pK_{a2}, pK_{as1} *and* pK_{as2} are dissociation constants. To get the parameters; regressions were performed using Sigmaplot 7.0 (Systat Software Inc. Richmond, California, USA) as indicated in the manual. The assay data and the corresponding equations were entered into the program and after 100 times iterated calculations the parameters were obtained.

II-5. Inactivation assay

The enzyme was inactivated at different pH with respective buffer containing 20 mM MES, HEPES, Tris or CHES, 20 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM $MgCl_2$ at 30 ºC and inactivated enzyme was assayed as

described above. To eliminate the excess residual inhibitors, 100 μM imidazole and 100 μM DTT was added in enzyme assay buffer for DEPC and IAM inactivation respectively. DEPC was dissolved in ethanol just before use. The pseudo-first-order loss of enzyme activity was obtained by plotting ln (v_t/v_o) vs. inactivation time and inactivation rate constant was determined as described previously [68]. The enzyme used in all inactivation assays was 2.5 μg of caspase-3 and 25 μg of procaspase-3 and after inactivation the enzyme assay was performed with 25 ng of inactivated caspase-3 and 1 μg of procaspase-3 with 30 μM DEVD-AMC. The inhibitory parameters from the inactivation assays were calculated using the following equation:

$$k_{app} = K_i[I] \quad (6)$$

$$\ln \frac{v_t}{v_o} = k_{app} t \quad (7)$$

where v_t, v_o, k_{app}, K_i and [I] are velocity at time point t, the initial velocity, apparent inactivation rate constant, inactivation constant and Inhibitor concentration, respectively. The pH dependent inactivation profile was used to determine pK_a values by applying following equation:

$$\log k_{app}' = \log k_{app} - \log\left(1 + \frac{[H^+]}{K_a}\right) \quad (8)$$

II-6. Protection assay

The enzyme (10 μM) was first incubated with DEVD-CHO (100 μM) at

30 ºC for 45 min and then the respective inhibitor (IAM or DEPC) was added and incubation was continued for 5 min more. To remove the excessive inhibitors, the reaction mixture was allowed to pass through 10 kDa centricons in three successive steps of dilution and concentration [69]. Finally the enzyme assay was performed with excess of DEVD-AMC (200 μM).

II-7. Reactivation assay

The enzyme was first inactivated completely at pH 7.0 buffer with DEPC (50 μM) and then 100 mM hydroxylamine was added [68]. The reactivation of the enzyme was checked by carrying out the enzyme activity assay at each 30 min interval for 3 h.

II-8. Spectrotometric analysis

The absorbance from 220-300 nm was monitored for both enzymes (10 μM) without DEPC and then after 30 min incubation with 200 μM DEPC [70]. To check the protection, the enzyme was first incubated with DEVD-CHO (100 μM) at pH 7.0 buffer for 30 min at 30 ℃ and the abs orbance was measured. Then 200 μM DEPC was added and the incubation was continued for more 30 min and again the absorbance was measured to check any change in spectra.

II-9. Citrate activation

The enzymes were pre-activated at 30 ºC for 30 min in caspase assay buffer supplemented with 1.0 M citrate and the reaction was initiated by adding the substrate. The readings were taken in the spectrofluoremeter as described above. To measure the concentration dependence of enzyme activity in citrate buffer, the following equation was employed [49].

$$\mathrm{A} = k[\mathrm{E}]^n \qquad (9)$$

Where n defines the number of caspase monomers from active species, $A =$ initial velocity and $[\mathrm{E}] =$ concentration of enzyme.

II-10. Size exclusion chromatography

The gel filtration of the purified proteins (80 μg) was performed on Amersham Pharmacia Superose 6 column (10/30) in 20 mM HEPES, 150 mM NaCl buffer (pH 7.5) with a flow rate of 0.5 ml min^{-1} in FPLC. The collected 0.5 ml fractions were analyzed on SDS-PAGE following Laemmli's method [71] and enzyme activity was also checked.

II-11. Chemical cross-linking of caspase-4

Caspase-4 (6 μg) was incubated in 50 μl of 50 mM HEPES pH 7.5 buffer with 1 mM DTT in presence of 0-1.0 M sodium citrate for 30 min at 30 ℃. The cross-linking reaction was initiated by adding 1 mM of

disuccinimidyl suberate (DSS) and incubated at room temperature for 45 min [49]. The reaction was stopped by precipitating the protein with 10% TCA and after washing the TCA pellet with -20 ℃ acetone; it was dissolved in SDS sample buffer (50 mM Tris pH 6.8, 10% glycerol, 2% SDS and 0.1% β-mercaptoethanol) to run in 15% reducing SDS-PAGE [71]. The gel was visualized with Coomassie staining.

II-12. Cleavage assays

For procaspase-3 cleavage assays, 0.5 μg of substrate (procaspase-3 C163S mutant) was subjected to various forms of caspase-9 at indicated concentrations and time points and the reaction was done at 30 ℃ in caspase assay buffer (pH 7.0). All the Bid cleavage reactions were carried out at respective caspase assay buffer at 30 ℃ for 1 h. The reaction was stopped by adding 6X SDS sample buffer and run on 15% reducing SDS-PAGE. The gels were visualized with Coomassie staining.

II-13. Cell extract preparation and Western blotting

Human neuroblastoma SK-N-BE(2) cells were cultured in DMEM with 10% heat-inactivated FBS, penicillin (100 units/mL) and streptomycin (100 μg/mL), in 5% CO_2 at 37 ℃. The harvested cells were washed with ice-cold phosphate-buffered saline and resuspended in cell lysis buffer (20 mM HEPES-NaOH, pH 7.0, 1 mM EDTA, 1 mM EGTA, 20 mM NaCl, 1 mM DTT, 1

mM phenylmethanesulphonylchloride, 10 μg/mL leupeptin, 5 μg/mL pepstatin A, 2 μg/mL aprotinin, 25 μg/mL ALLN) on ice for 20 min. The cell extract was obtained by centrifuging at 14,000 rpm for 20 min at 4 ℃. Equal amounts of protein samples were reacted with caspases in the caspase assay buffer of varying pH at 30 ℃ for 1 h. The samples were separ ated in SDS-PAGE and transferred to polyvinylidene difluoride membrane [72] (Bio-rad laboratories, Hercules, USA). The blots were hybridized with polyclonal Bid antibodies and then with horseradish peroxides-conjugated goat anti-mouse IgG (Santa Cruz, USA). The blots were visualized with ECL plus reagent kit (Amersham Biosciences, Piscataway, USA).

III. RESULTS AND DISCUSSION

III-1. Kinetic comparison of procaspase-3 and caspase-3

III-1-1. pH dependency of catalytic parameters

Caspases are aspartate specific cysteine proteases and their optimal pH values range from 6.5 to 7.5 [73]. To characterize procaspase-3 biochemically, the enzyme that was mutated in the three processing sites, one from interdomain linker region and two from prodomain has been used [35]. The comparison of basic catalytic parameters (Table 2) shows that procaspase-3 has much lower catalytic efficiency than its mature form with ~200-fold lower k_{cat} and k_{cat}/K_M values which was in agreement with previous reports [35]. From the plot of V_{max}/K_M (Fig. 2A) the optimal pH value of the both enzymes were found to be nearly equal which differs from the previous reports arguing the optimal pH value for procaspase-3 more than 8.0 and the pK_a values of 6.3 and 7.3 for caspase-3 and procaspase-3, respectively. However, it is possible that the shift in optimal pH value for procaspase-3 observed in other studies may be due to just the initial velocities determined at fixed substrate concentrations. Actually, the similar pattern was obtained while determining the velocity (v) with a single fixed substrate concentration and also a tendency of increasing in optimum pH with an increasing substrate

Table 2. Catalytic parameters for procaspase-3 and caspase-3 at pH 7.0

Enzymes / Parameters*	Procaspase-3	Caspase-3
K_M (μM)	13.22±0.71	10.99±1.36
k_{cat} (sec^{-1})	0.0455±0.0005	8.05±0.25
k_{cat}/K_M ($M^{-1}s^{-1}$)	3450±223	747000±11500

*The parameters were determined using equations (1) and (2).

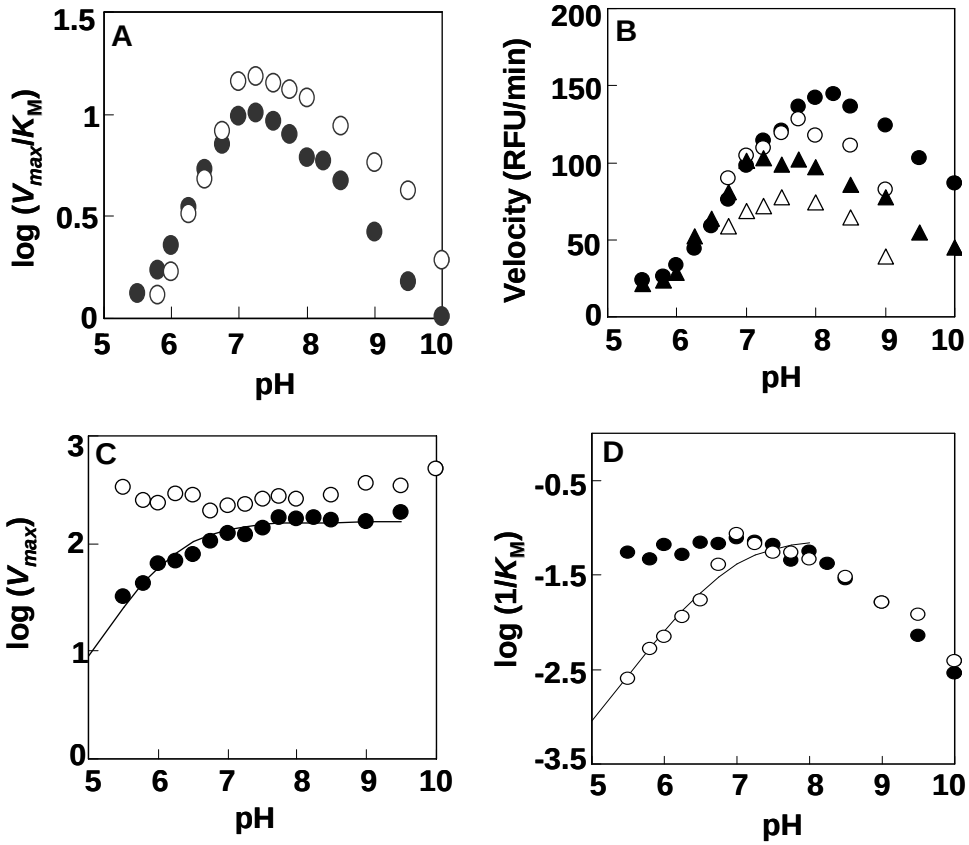

Fig. 2. Effect of pH on catalytic parameters of caspase-3 and procaspase-3. Eight ng of caspase-3 (○) and 1.1 μg of procaspase-3 (●) were used for enzyme assay with a varying appropriate range of substrate (DEVD-AMC) concentrations. (**A**) log (V_{max}/K_M) vs. pH. (**B**) Initial velocity (v) for procaspase-3 at 20 (△), 50 (▲), 70 (○) and 100 (●) μM DEVD-AMC vs. pH. (**C**) log (V_{max}) vs. pH. (**D**) log ($1/K_M$) vs. pH. The line represents the data fitted to equations (3) and (5) for C and D, respectively.

concentration was noticed (Fig. 2B). The plot in Fig. 2A did not fit well to the concentrations was noticed (Fig. 2B). The plot in Fig. 2A did not fit well to the equation (3) and unusual pattern was observed especially in the region of higher pH (data not shown). It may account for the involvement of multiple amino acids residues participating in complex ionic interactions. To analyze the effect of pH on V_{max} and K_M separately and to estimate pK_a values, the equations (2 and 4) were used (Figs. 2C and 2D). In the mature caspase-3, the pH dependent activity profile did not obey the usual bell-shaped nature and value of V_{max} was almost linear through out the pH range of 5.5-10 (Fig. 2C). In contrast, the K_M value was altered with pH (Fig. 2D), which appears to be the reason for the pH dependency of V_{max}/K_M (Fig. 2A). The results indicated that substrate binding can be affected by pH in the mature enzyme. From the plot of $1/K_M$ as a function of pH, the pK_a value was determined to be 6.81±0.13 by employing equation (5). On the contrary, procaspase-3 showed the deviation of linearity of V_{max} with lower pH (<7.5) with relatively constant K_M in the pH range, which showed the pH dependency of V_{max}/K_M should be due to the change in V_{max}. Here, pK_a was determined using equation (3) to be 6.21±0.06. Again, the unusual pattern in both cases was observed in the high pH that was ignored for the calculation. Procaspase-3 exists as monomeric form below pH 5.0 [30] that restricted us to carry out the kinetic assays above pH 5.0.

III-1-2. Crystallographic structures of caspase-7 and its proform

During the activation of procaspase-7, the reorganization of residues on active site occurs as described by Riedl *et al*. [33]. Although the structure of procaspase-3 has not been determined yet, the residues on the active site are well conserved in caspase-3 and –7 (Fig. 3A). It is notable that the catalytic dyad of caspase consisting of cysteine and histidine maintain their positions relatively well in the process (Fig. 3B). This may explain the activity of proenzyme. The two residues are also recognized as the only ionizable residues in the active site (Fig. 3A) [7]. Inactivation assays with specific inhibitors have been often performed to determine pK_a values of the ionizable residues. Hence, in the next the values were calculated with the aid of the inactivation assays and compared with those determined above.

III-1-3. Inactivation of caspases with DEPC

DEPC is widely employed as a specific histidine modifier. The pseudo-first-order loss of caspase-3 activity was observed (Fig. 4A) and the similar kinetics was observed for procaspase-3 (data not shown). There was only a little difference in degree of sensitivity toward the inhibitor for procaspase-3 and mature caspase-3 (Fig. 4B). The k_i values calculated using equation (6) were ~3.24×10^4 $M^{-1}min^{-1}$ and ~2.28×10^4 $M^{-1}min^{-1}$ at pH 7.0 for each form ofcaspase-3, respectively. To assign the modified residue, the protection assay with DEVD-CHO was carried out. The blocking of the active site with this inhibitor was able to protect the enzyme from the effect of DEPC for both caspase-3 (Fig. 5A) and procaspase-3 (data not shown). The results are in

A. Alignment of the active site residues of caspase-3 and caspase-7

Casp-3 H121 Q161 C163 L168 Y204 S205 W206 R207 N208 S209 F256
Casp-7 H144 Q184 C186 L191 Y230 S231 W232 R233 S234 P235 F282

B. Structural comparison of procaspase-7 and mature caspase-7

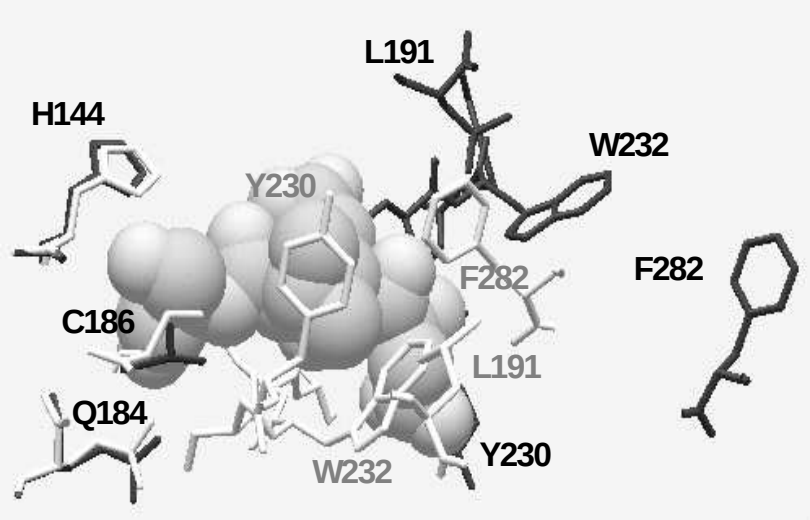

Fig. 3. Comparison of the active site residues of caspase-3 and -7 and crystallographic structures of procaspase-7 and caspase-7. (A) The residues within 8 Å from Cα of valine of the inhibitor (DEVD) in caspase-3 structure are aligned for caspase-3 and –7 [17, 74]. **(B)** The structures for those residues of procaspase-7 and caspase-7 are compared [17, 74]. The space filled model represents the inhibitor that was included during crystal structure determination. The structure of procaspase-7 is displayed in black, while that of caspase-7 is in white. The figure was drawn using Swiss-Pdb viewer [75].

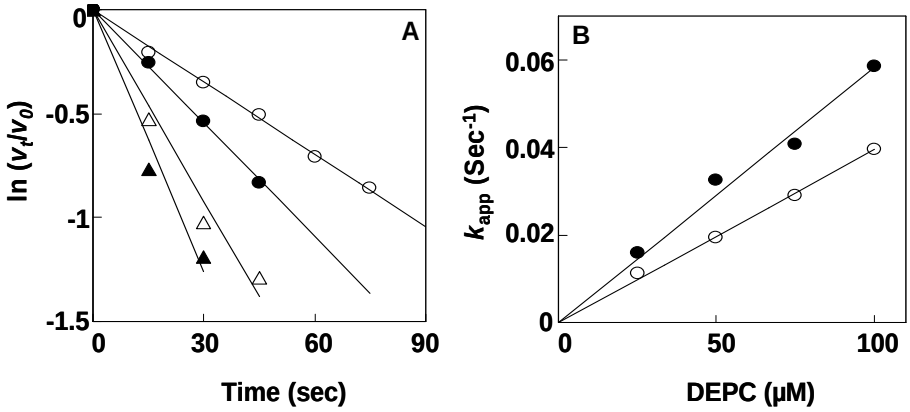

Fig. 4. DEPC inactivation of caspase-3 and procaspase-3. Inactivation was carried out with DEPC for caspase-3 and procaspase-3 and enzyme assay was performed for inactivated enzyme with DEVD-AMC. (**A**) Pseudo-first-order loss of enzyme activity with 25 (○), 50 (●), 75 (△) and 100 (▲) μM DEPC. (**B**) The apparent inactivation rate is shown as a function of inhibitor concentration. The line in (A) represents the data fitted to equation (7).

agreement with the location of residue in the active site. The incubation of the of the DEPC inactivated enzyme with hydroxylamine restored 70~80% of the enzyme activity within 30 min (Fig. 5B), confirming the histidine-selective modification rather than tyrosine [68]. Also, the inactivation of the enzyme by DEPC was followed by monitoring the absorbance at 220-300 nm. The differential spectra between the native and inactivated enzyme clearly indicated the substantial increase in absorbance at 240-244 nm which is an indicative of histidine modification (Figs. 5C and 5D) [68, 70]. The results were consistent with modification of ~15 and ~6 histidine residues per one catalytic unit of pro- and mature caspase-3 which have 21 and 20 histidine residues, respectively. The reason for this big difference between the two forms of enzyme is not known. More importantly, the blocking of the active site of the enzymes with DEVD-CHO followed by DEPC treatment resulted in decrease in absorbance at 242 nm, indicating protection of the histidine residue in the active site from the modification (Figs. 5C and 5D). The decrease indicated 1~2 residues were protected by the inhibitor. All of these data demonstrated that one of the histidine residue modified by DEPC was residing on the active site.

In the case of procaspase-3, the inactivation was rarely affected by pH and the inactivation rate was near linear at the pH range of 5.5-8 (Fig. 6), which might indicate that the residue have very low pK_a value. It corresponded to the profile obtained for this enzyme in pH dependency of its K_M values (Fig. 2D). In caspase-3, the inactivation was affected by pH especially at lower region (Fig. 6). In the both cases, the data did not fit well

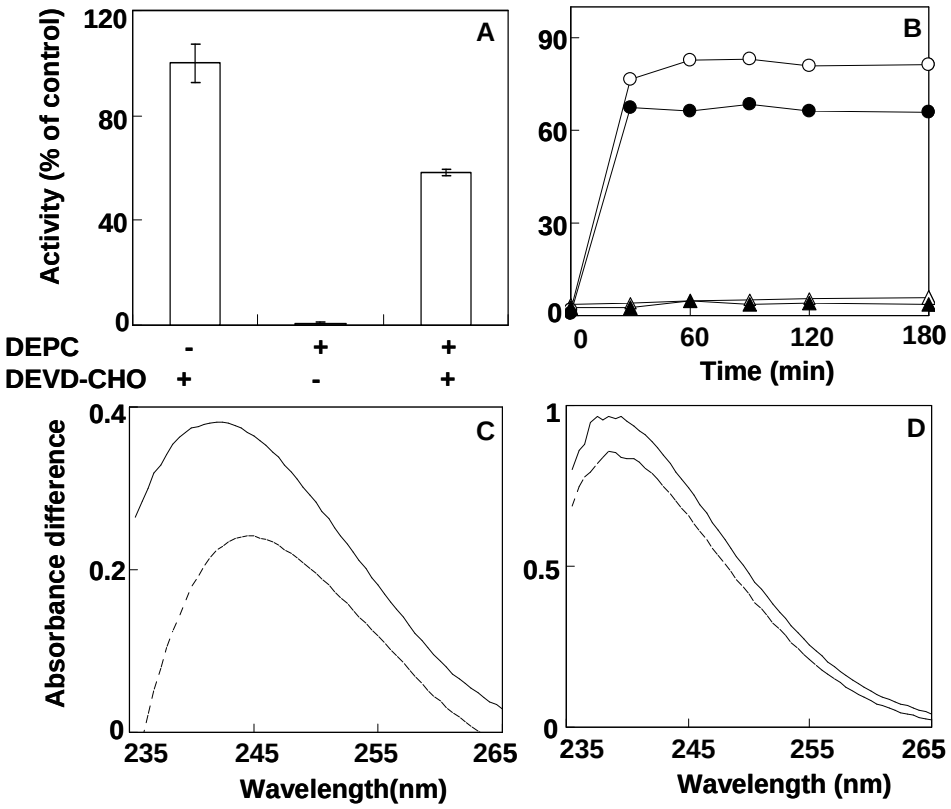

Fig. 5. Effect of caspase-3 inhibitor and hydroxylamine on DEPC inactivation of caspase 3 and procaspase-3. (A) Protection assay: DEPC was added in enzyme pretreated with DEVD-CHO and then enzyme assay was performed with DEVD-AMC. Error bars represent ± mean (n=3). (**B**) Reactivation assay: Following the inactivation of caspase-3 and procaspase-3 with DEPC, hydroxylamine was added and the enzyme assay was done with DEVD-AMC. (**C), (D**) Differential UV absorption spectra between native and DEPC treated enzyme (solid line) and between enzyme with inhibitor in presence or absence of DEPC (dotted line) for caspase-3 **(C)** and procaspase-3 **(D)**, respectively.

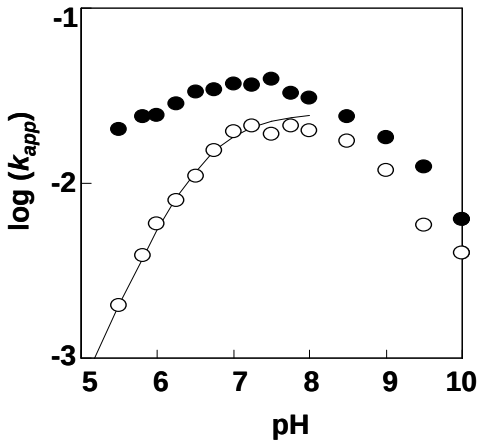

Fig. 6. The pH dependence of DEPC inactivation of caspase 3 and procaspase-3. Inactivation was carried out with DEPC for caspase-3 and procaspase-3 from pH 5.5-10 and enzyme assay was performed for inactivated enzyme with DEVD-AMC at pH 7.0. Effect of pH on inactivation rate constant for caspase-3 (○) and procaspase-3 (●). The solid line represents the fits to equation (8).

with equation (8) in > pH 8.0 that were ignored for the calculation of pK_a. For caspase-3, pK_a value was determined as 6.61±0.05. This pK_a value almost matched with that determined in pH dependency profile (Fig. 2D).

III-1-4. Inactivation of caspases with IAM

The active site cysteine residue of caspases is vulnerable to various types of modifications e.g.; oxidation, S-nitrosylation and thus causing potential inhibition of the enzyme activity [69]. IAM is considered as the specific modifier of the thiol group of cysteine residue and it caused the irreversible inactivation of the caspases. To measure pK_a values of the catalytic cysteine, inactivation study with IAM was performed. The pseudo-first-order loss of enzyme activity was observed with IAM in both forms of caspase (Fig. 7A, data not shown for procaspase-3). The k_i's of the two form of caspase-3 were estimated using equation (5). There was a little variation in their degree of sensitivity towards these inhibitors (Fig. 7B) with k_i values ~5.52×10^2 $M^{-1}min^{-1}$ and ~4.23×10^2 $M^{-1}min^{-1}$ at pH 7.0 for procaspase-3 and caspase-3, respectively. In the presence of DEVD-CHO, IAM can no more inactivate the enzyme (Fig. 7C), the indication of the residence of the modified residue on the active site. The inactivation rate in caspase-3 was slightly variable throughout the pH range (Fig. 7D), which, however, did not fit well to equation (8) and was not sufficient for the measurement of pK_a. It appeared that the affected residue may have very high or low pK_a values. The results

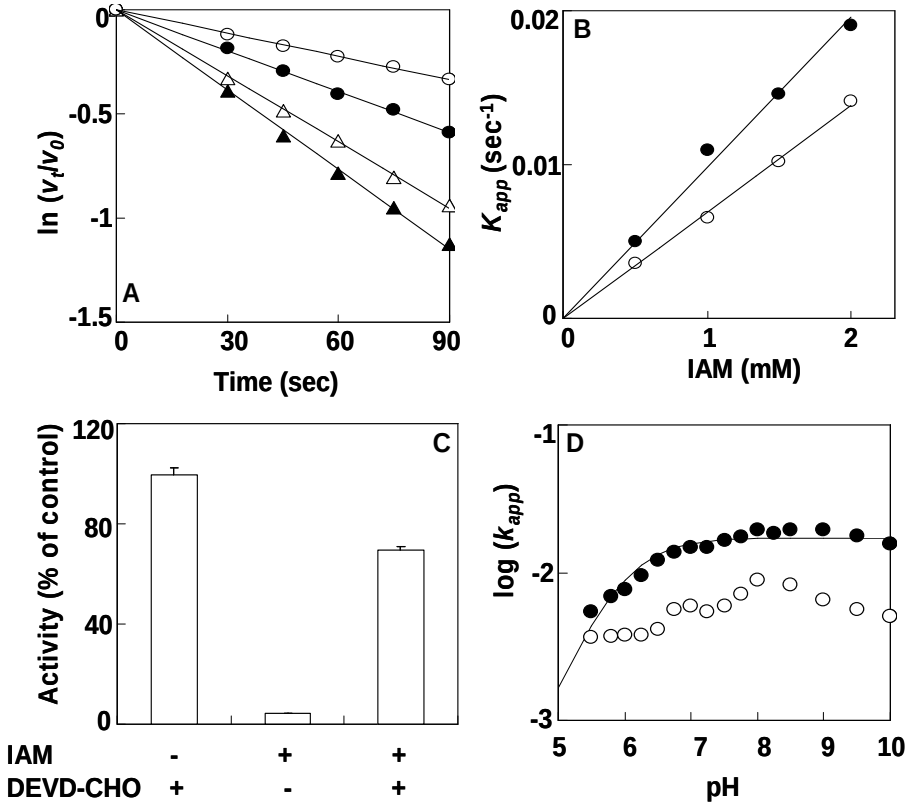

Fig. 7. IAM inactivation of caspase-3 and procaspase-3. Inactivation and enzyme assay were done with IAM in the place of DEPC as described above. (**A**) Pseudo-first-order loss of enzyme activity with 0.5 (○), 1 (●), 1.5 (△) and 2 (▲) mM IAM. (**B**) The apparent inactivation rate constant as a function of inhibitor concentration. (**C**) Protection assay was carried out as in Fig. 5. Error bars represent ± mean of three individual experiments. **(D**) Effect of pH on inactivation rate for caspase-3 (○) and procaspase-3 (●). The solid line represents to the fits to equation (8).

are consistent with the independence of V_{max} on pH in this enzyme (Fig. 2C). In procaspase-3, the inactivation was affected by pH especially below 7.0. The pK_a was determined to be 6.01±0.05 which almost matched to that determined in its pH profile (Fig. 2C).

Crystallographic structure of caspase-7 proenzyme and its mature enzyme was informative for understanding the activation process. The study indicates that the structural hindrance imposed by the covalent linkage between the large and small subunits was incompatible for substrate binding and also non-conducive to catalysis. The active site comprises four surface loops, L1 through L4, all from same monomer. L1 and L4 constitute two sides of the substrate binding groove; L3 forms the base. The catalytic cysteine resides in loop L2, poised for catalysis. Importantly, the L2' loop, which comes from the adjacent monomer, plays a significant role in stabilizing the activated conformation of the active site through intimate interactions with loop L2 and L4. In the inhibited active site conformation in the unprocessed procaspase-7 zymogen, the L2' loop is flipped by 180 degrees, which is necessitated by the uncleaved peptide linkage [27]. In the related enzyme caspase-3, the similar relationship should be applicable because the active site residues are well conserved. Other important result to understand the process was that even procaspase-3 has some activity. The result insisted that the proform of caspase-3 has high optimum pH and lower activity. Initially, we performed this study to explain the shift of optimum pH by measuring pK_a values of the involved residues and finally to understand the activation process. However, it was found that the shift was observed only under some specific conditions. It

appears that the shift observed in other report might be due to use of single fixed concentration of substrate. The results in this study indicated that the optimum pH of procaspase-3 and its mature enzyme were almost identical. However, in spite of this similarity the characteristic difference between the two forms of enzyme was noted. The pH dependency of caspase-3 was largely due to the change of K_M, while it was due to V_{max} for procaspase-3.

For the mature caspase-3, the plot of K_M as a function of pH indicated the existence of a residue with pK_a value of 6.89±0.13. In the subsequent inactivation assay with DEPC, a residue with the pK_a of 6.61±0.05 was allocated. The correspondence of these pK_a values and the specificity of DEPC as a histidine modifier implied the involvement of histidine residue on substrate binding. The crystallographic structure of the enzyme also illustrated that histidine residue on the active site could play roles in substrate binding [76]. In the comparison of the structure of procaspase-7 and mature caspase-7, it appears that the position of the histidine residue rarely changes during the activation process (Fig. 3). With the reports that caspase-3 and –7 share the structural similarity, it could be assumed that the histidine residue of caspase-3 also maintain its location during the process. In this context, it was unexpected that pH had little effect on substrate binding and DEPC modification for procaspase-3. One speculation is that the changes of the microenvironment around the residue rather than the histidine residue may account for this difference.

Further, pro- and mature caspase-3 also exhibited their different nature in the dependency of V_{max} on pH. In mature caspase-3, V_{max} was

hardly influenced by pH but it was clearly affected in procaspase-3, especially at lower pH regions. For procaspase-3, a residue with pK_a of 6.21±0.06 could be assigned. Inactivation assay with cysteine-specific modifier IAM also showed the existence of a residue with similar pK_a value (6.01±0.05). Furthermore, it was shown that the affected residue is located on the active site. IAM is a cysteine-specific modifier and only one cysteine (it is one of two ionizable residues) was found in the active site, suggesting that the pK_a of catalytic cysteine residue of procaspase-3 is 6.0~6.2. Although the measured pK_a was quite lower than that of free cysteine (~9.0), it can be influenced by neighboring residues as reported in serine or cysteine proteases [77]. In the case of caspase-3, the pK_a was not measurable, which might be due to a much lower value (Fig. 2).

In summary, although the optimal pH appeared similar in the both forms of caspase-3, its biochemical natures were different. Mature caspase-3 is shown to have different pK_a values of the catalytic histidine and cysteine residues, compared to procaspase-3. This difference implied that rearrangement of the active site residues should occur during the activation process. Finally, DEPC was shown to be effective inhibitor for caspase-3. The second-order rate constant (k_i, ~3.24×10^4 $M^{-1}min^{-1}$ for caspase-3) is quite high, comparable with that of urease (~6.0×10^4 $M^{-1}min^{-1}$) [78]. It has been suggested that caspase may be the potential target for the control of many diseases. The information on the inactivation of caspase-3 by DEPC might provide with a biochemical basis for development of efficient inhibitors for caspase.

III-2. Biochemical mechanism of caspase-4 activation

III-2-1. Improvement in specific activity of caspases by citrate

Antichaotropic cosolvents, also known as kosmotropes increase the specific activity of proteases by dimerization without changing the inherent substrate or inhibitor specificity [79, 80]. Although the citrate activation generally employs for apical caspases that are activated by dimerization [79], this study involves the effector and inflammatory caspases as well, to obtain the clear comparative picture of their activation (Table 3). In agreement with the earlier results, caspase-9 was activated substantially with ~ 75 times increase in its k_{cat}/K_M value [49, 79]. The uncleavable mutant had ~ 5 times lower catalytic efficiency to that of wild-type and similar difference in citrate activation implies that cleavage may help to stabilize the dimers [79]. The increase in k_{cat}/K_M was mainly due to reduction in K_M rather than an increase in k_{cat} that accounts for the improvement in binding affinity facilitated by dimerization. Similarly, the significant improvement was found in caspase-4 where citrate could produce more than 40 times increment largely affecting its K_M. The similar degree and nature of citrate activation between caspase-4 and caspase-9 indicates that caspase-4 belongs to the group of initiator caspases. If dimerization is the activating event for caspase-4 inside the cell, then oligomerization should be possible only in the presence of the adaptor proteins [28], but it is not known till now. The catalytic activity of uncleavable procaspase-4 mutant (E93D104D270D289/A) was not detectable (confirmed

Table 3. Catalytic parameters for various caspases with or without sodium citrate.

Enzyme	Parameters*	Normal buffer	Citrate Buffer	Enzyme	Parameters*	Normal buffer	Citrate Buffer
NC Casp-9	K_M	>1mM	332	ΔCARD Casp-9	K_M	>1mM	89
	k_{cat}	0.11	0.21		k_{cat}	0.39	1.2
	k_{cat}/K_M	0.04	0.62		k_{cat}/K_M	0.18	13.65
NC Casp-4	K_M	ND*	193	Δp Casp-4	K_M	133.±18	8.6
	k_{cat}	ND*	0.03		k_{cat}	0.14	0.37
	k_{cat}/K_M	<0.01	0.16		k_{cat}/K_M	2.1	88.1
NC Casp-2 [55]	K_M	27±5	ND	ΔP Casp-2	K_M	30.3±3.5	10.8
	k_{cat}	0.04	ND		k_{cat}	2.55	4.14
	k_{cat}/K_M	1.6	ND		k_{cat}/K_M	84	382
NC Casp-8 [26]	k_{cat}/K_M	1% of casp-8	ND	ΔDED Casp-8	K_M	13.1±0.7	6.2
					k_{cat}	0.39	0.74
					k_{cat}/K_M	30	119
NC Casp-3	K_M	9.7±1.6	9.3	ΔP Casp-3	K_M	10.7±0.6	10.1
	k_{cat}	0.04	0.05		k_{cat}	10.08	20.31
	k_{cat}/K_M	3.9	5.0		k_{cat}/K_M	940	2020
NC Casp-6	K_M	235±118	ND*	ΔP Casp-6	K_M	283±114	244
	k_{cat}	0.01	ND*		k_{cat}	3.31	4.25
	k_{cat}/K_M	0.044	0.032		k_{cat}/K_M	11.6	17.4

K_M, k_{cat} and k_{cat}/K_M are in μM, sec^{-1} and $X10^3$ $M^{-1}sec^{-1}$, respectively. Casp-, caspase; NC, non-cleavable; ND, not determined; ND, could not be determined; Δp, prodomainless; ΔCARD, without caspase recruitment domain and ΔDED, without death effector domain. The enzyme assays were performed as described in Materials and Methods. and values ± standard errors on the fit to equation (1) are presented.

by utilizing several fold higher enzyme and substrate concentrations to that of caspase-4) and the minimal level of activity could be gained on citrate activation. So, dimerization could activate caspase-9 and -4 to high level but they differ in the requirement of proteolytic processing. In case of caspase-4, the interdomain cleavage appears to be indispensable for its activation as illustrated by the catalytic difference between the uncleavable mutant and processed forms of enzymes (Table 3). Proteolysis could result in the activation of caspase-4 but it is far from complete as processed enzyme was further activated by citrate. These observations were supportive on the requirement of both dimerization and proteolytic processing for the complete activation of capase-4. The other initiator capsases, caspase-2 and -8 were able to get activated only up to 5 fold because of their occurrence in dimeric state unlike to caspase-9 which is predominantly a monomer [55, 79]. For these enzymes, citrate activation was followed by improvement in both kinetic constants k_{cat} and K_M, still the reduction in K_M was albeit better. As expected, none of the effector caspases (-3, -6 and -7) showed significant improvement on citrate activation and just about two fold increment was the result of increased k_{cat}, leaving the K_M almost unaffected.

III-2-2. Effect of dimerization and proteolysis on caspases activation

Table 4 illustrates the influence of dimerization and proteolysis on the activation of various caspases. For caspase-9, dimerization was sufficient for its activation that is in agreement with the unanimous view of negligible role

of proteolysis for its activation [81]. Caspase-4 shows a unique phenomenon of activation as both dimerization and processing seem to be prerequisite for its complete maturation. Another important feature of this activation was that none of the above two events were able to generate the fully mature enzyme in acting alone. However, other two initiator caspases (-2 and -8) also appear to require processing for their full activation. For these proteases, dimerization accounts for the initial acquisition of activity and cleavage is essential for the stabilization of dimers [55]. The huge increment in their activity after processing clearly indicates that at least the activation here does not truly coincide with that of caspase-9 where processing is largely meaningless. So, although the dimerization seems to be the common event of activation for initiator caspases, the significant difference exists between them in terms of role of proteolytic processing. As expected, the proteolytic processing was found to be an absolute requirement for the activation of naturally dimeric effector caspases-3, -6, and -7 (Table 4).

III-2-3. Activation mechanism of caspase-4

At lower concentrations of enzyme, the second-order component of activation would describe dimerization. To determine whether the activation of caspase-4 was a second-order process, the activity of caspase-4 was measured in 1.0 M citrate buffer as a function of enzyme concentration. The activity versus concentration plot demonstrated a second-order component of activation indicating a bimolecular process (Fig. 8A). The fit to the equation (9)

Table 4. Effect of cleavage vs. dimerization on the activation of caspases.

Caspases	Fold increase in specific activity after	
	Interdomain cleavage	Dimerization
Caspase-9	5	75
Caspase-4	>100	40
Caspase-2	>50 [55]	4.5
Caspase-8	100 [26]	4
Caspase-3	240	2
Caspase-6	265	2
Caspase-7	240 [82]	2

revealed a mixed order with $n =$ 1.67±0.04 (Fig. 8A) that was similar to the citrate activation of caspase-9 [49]. For comparisons, the concentration dependency of enzyme activity in citrate buffer was also done for caspases -2, -6 and -7 where the reaction was not a bimolecular one with $n =$ <1 (Fig. 8B, C and D). The reason behind this variation of the activation reaction was thought to be due to their occurrence in different state of dimer (caspase-2, -6 and -7) or monomer (caspase-4). The size-exclusion chromatography was performed to confirm the natural state of caspase-4 and the chromatogram profile clarified its sole occurrence as a monomer (~35 kDa) (Fig. 9A). The gel filtration fractions were checked for enzyme activity and the activity peak truly coincided with the protein peak (Fig. 9A). The very high level of citrate activation of caspase-4 led us to test the importance of proteolytic processing that could give the better picture of activation. The uncleavable procaspase-4 mutant (E93D104D270D289/A) protein did not show measurable catalytic activity even with several fold higher concentration of both enzyme and substrate. However, it was able to gain the minimal level of enzyme activity after pre-activating in 1.0 M citrate buffer proving its capability to be activated by dimerization (Fig. 9B). During the gel filtration of caspase-9, a small proportion of the protein could be found as dimer with maximal enzyme activity [81] but here we did not observe such phenomenon as the protein was eluted completely as a monomer with reasonable enzyme activity. This difference between these two enzymes may be explained by the uncleavable procaspase-4 mutant that could not correlate with the caspase-9 counterpart that is as much active as its mature form or it may be the outcome of higher

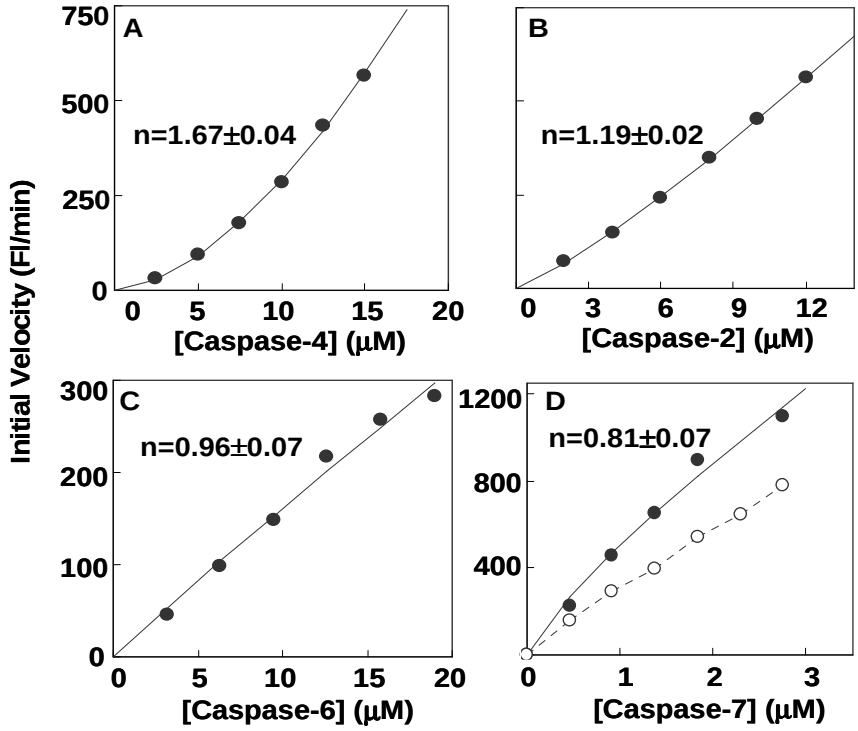

Fig. 8. Kinetics of caspases activation. The indicated concentrations of caspases were incubated at 30 ºC for 30 min in standard caspase assay buffer (pH 7.0) supplemented with 1.0 M sodium citrate and the enzyme assay was done with 50 μM of LEVD-AMC, 30 μM of VDVAD-AMC, 50 μM of VEID-AMC and 20 μM of DEVD-AMC for caspase-4, -2, -6 and -7 respectively. The plot of initial velocity versus enzyme concentration is shown for caspase-4 **(A),** caspase-2 **(B),** caspase-6 **(C)** and caspase-7 **(D)**. The activity of caspase-7 (○) in normal caspase assay buffer is shown as a control. In all cases, the line represents the fits to equation (9).

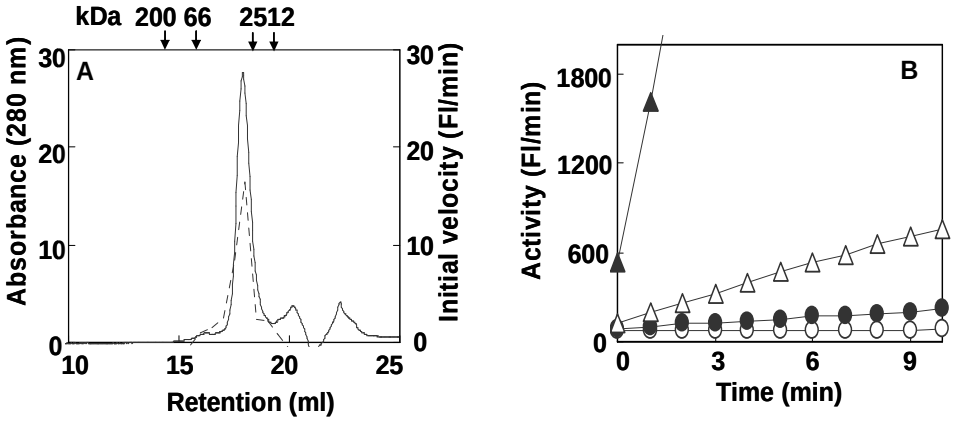

Fig. 9. Size-exclusion chromatography and caspase-4 activation. (A) The gel filtration was done with 80 μg of caspase-4 and the profile (solid line) showing the absorbance at 280 nm with elution volume is presented. The LEVDase activities (dotted line) of the fractions are also shown. The molecular markers β-amylase (200 kDa), BSA (66 kDa), Carbonic anhydrase (25 kDa) and cytochrome *c* (12 kDa) are indicated in the upper panel. **(B)** Citrate activation of caspase-4 and procaspase-4. The enzyme assay was done at spectrofluoremetre with 50 μM of LEVD-AMC for 500 ng of caspase-4 and 1 μg of procaspase-4 before and after citrate activation. The plot of enzyme activity versus time point is presented for caspase-4 (Δ), activated caspase-4 (▲), procaspase-4 (○) and activated procaspase-4 (●).

K_d values for caspase-4.

III-2-4. Chemical cross-linking of caspase-4

The kosmotrope induced activation of proteases correlates with the changes in the monomer-dimer equilibrium [80]. Although the chemical cross-linking can not be used to estimate the proportion of existing dimer, it can provide qualitative information regarding the existence of dimers if assisted by efficient interaction between proteins and cross-linker. In kinetic analysis, the high level of increase in the specific activity of enzyme in citrate buffer was largely contributed by reduction in K_M value that was considered to be the effect of dimerization. To confirm the citrate induced dimerization of the protease, chemical cross linking was carried out with a homobifunctional cross-linker DSS that has spacer length of 1.14 nm and is reactive toward primary amines on side chains of amino acids with lysine as the most reactive one [83]. In the presence of this cross-linker, the dimer was favored with increasing concentration of citrate (Fig. 10). This observation confirms that the high level of activation of the protease in citrate buffer is the outcome of citrate induced dimerization.

The present study revealed a unique feature of caspase-4 activation mechanism requiring both dimerization and proteolytic processing for its complete activation. The extensive studies carried out in the past decade have established that the initiators and executioners caspases exhibit differing activation mechanism- dimerization for initiator caspases and interdomain

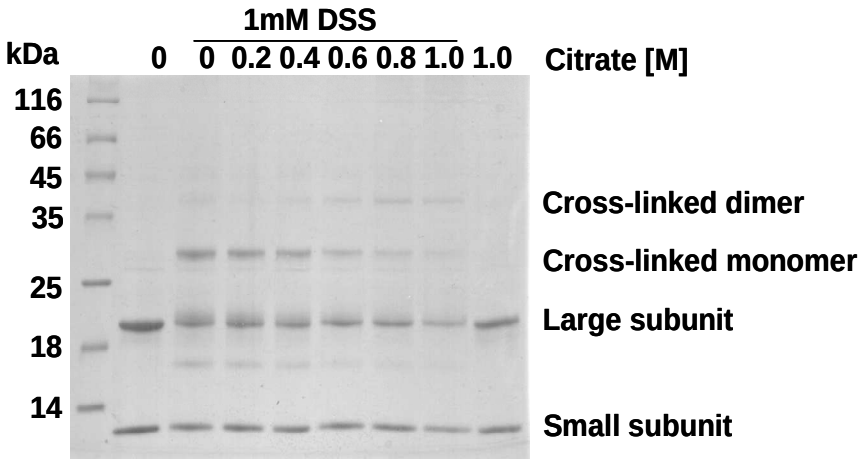

Fig. 10. Chemical cross-linking of caspase-4. The cross-linking reaction was carried out in HEPES buffer (pH 7.5) with 1 mM DSS in absence or presence of varying concentrations of sodium citrate and reducing SDS-PAGE analysis of cross-linked monomer and dimer of caspase-4 is shown. The controls without citrate, cross linker are included and the protein molecular weight markers in kDa are also presented.

cleavage for the later, still the fundamental mechanism of zymogen latency, requirement of translocation of activation loop and lack of self-amplification is conserved [16]. Our observations slightly contradicted with this classical paradigm of caspase activation and suggested a novel scenario of activation mechanism where both dimerization and interdomain processing appear to be indispensable.

In terms of citrate activation, caspase-4 was activated to such a high magnitude that it was only lower than that of caspase-9, exceeding all other caspases. This activation was largely contributed by the reduction in its K_M value, a clear indicative of dimerization. Further, the corresponding enhanced appearance of dimeric species with increasing concentration of citrate in presence of cross-linker illustrates the citrate induced dimerization of the protease (Fig. 10). The capability of uncleavable procaspase-4 mutant to become activated to some extent in the presence of citrate suggests dimerization assists to gain the initial acquisition of its activity. An emerging theme from current investigations is that an initiator caspase is always recruited into and activated within a specific adaptor protein complex but till now no any such activation platform could be established for caspase-4 [28]. Further, proteolysis can also generate the active enzyme and still its full maturity can be gained only after dimerization as illustrated by citrate activation (Fig. 9B). So, both dimerization and proteolytic processing seem to be crucial for complete maturation of caspase-4. The actual order and importance of these activating events inside the cell can not be predicted at this point. With our data, it can be speculated that caspase-4 could gain the

initial acquisition of activity after getting recruited in some protein complexes and following the proteolytic cleavage the stabilized dimers will be able to retain the full maturity. Actually, this type of activation mechanism is described for caspase-2 [55] but unlike uncleavable procaspase-2, procaspase-4 is devoid of measurable enzyme activity and processing also can not generate the fully mature enzyme as in caspase-2.

The catalytic parameters determined here for various forms of caspase-9 and their activation were in accord with the universal theme of its activation, described as Apaf-1 mediated oligomerization following the release of cytochrome c from mitochondria.. The only less than 5 fold activation of recombinant caspase-2 and -8 by dimerization can be justified by the fact that overexpression of these proteases could result in the production of a robust enzyme and these two caspases exhibit an increased preference for the formation of homodimer following the autocatalytic cleavage [79]. The citrate activation was specific for apical caspases, so constitutive dimeric executioner caspases failed to show any significant improvement in their specific activity. For these caspases, proteolytic processing is utmost essential to get rid of the structural hindrance imposed by the covalent linkage between the large and small subunits that is incompatible for substrate binding and also non-conducive to catalysis [32].

III-3. Biochemical characterization of caspase-9 dimer

III-3-1. Design of constitutive dimers

The striking difference between the initiator and executioner caspases is their native physiological state because effectors caspases always exist as dimers but initiator caspases like caspase-9 is monomer [16]. The crystallographic and gel filtration analysis have shown that caspase-9 is inactive monomer at concentrations in the micromolar range, and that activity requires dimer formation [81]. The structural and biochemical evidences have demonstrated that active caspases are obligate dimers of identical catalytic units, with each catalytic unit containing one active site and caspase-9 is no exception to this fact [81]. If the activity of caspase-9 is associated with its dimerization, then the constitutive dimeric form should replace the fully activated caspase-9. A direct comparison of the catalytic activity of the dimeric caspase-9 with that of apoptosome activated caspase-9 can assess the correctness of the activation mechanism proposed for the caspase-9. Considering these important features of caspase-9 structure and activation, the dimeric forms of wild type and uncleavable mutant caspase-9 were prepared by ligating two respective monomers (Fig. 11). The rationale behind this simple design was to reflect the structure of active caspase-9 where termini from each monomer were located very close to each other. Thus constructed dimeric as well as monomeric wild type and mutant proteins were overexpressed in *E. coli* and purified to homogeneity (Fig. 12).

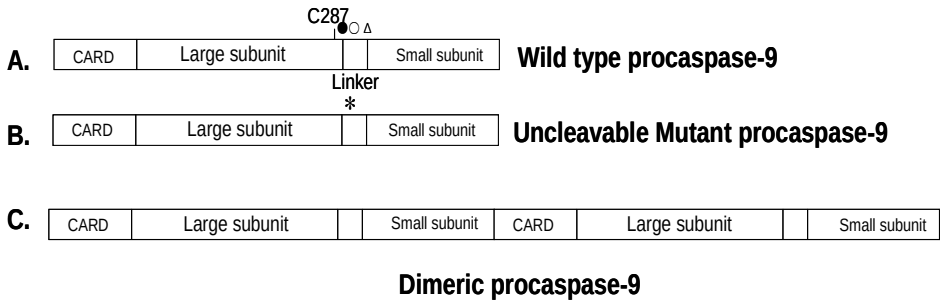

Fig. 11. Schematic representation of various caspase-9 constructs. A, Human wild type procaspase-9 showing autocatalytic cleavage site (○, Asp 315); caspase-3 cleavage site (Δ, Asp 330); minor autocatalytic cleavage site (●, Glu 306) and catalytic cysteine. **B**, Uncleavable procaspase-9 in which all the above three processing sites are mutated to alanine (*, E306, D315, D330/A). **C**, Dimeric caspase-9 formed after fusion of two procaspase-9 molecules. LS, large subunit; SS, small subunit.

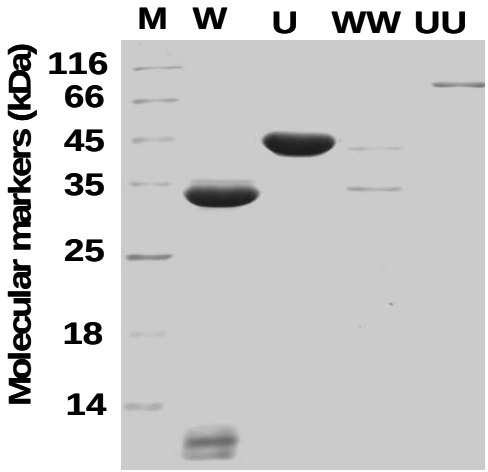

Fig. 12. Purification of caspase-9. The purified proteins were resolved in 15% SDS-PAGE and visualized with Coomassie staining. M, protein molecular weight marker in kDa; W, wild type procaspase-9; U, uncleavable mutant procapsase-9; WW, wild type dimeric procaspase-9 and UU, uncleavable mutant dimeric procaspase-9.

III-3-2. Recombinant dimers showed improved catalytic activity but lower than that of apoptosome

The constructed dimers were expected to represent constitutive dimeric caspase-9 and indeed they showed increased specific activity towards the optimal tetra peptide substrate LEHD-AMC (Table 5). The similar degree of increment in activity of non-cleavable dimer to that of wild type dimer suggested that proteolytic processing is not responsible for initial activation of caspase-9. The wild type dimer was ~5 times more active than non-cleavable dimer consistent with their monomeric counterparts. The dramatic increase in specific activity of initiator caspases can be achieved by citrate, but these recombinant dimers were no more activated to similar level (Table 5). However, wild type dimers showed a little bit higher level of citrate activation that may be due to possible presence of various forms of proteins after processing but still the value was much lower than its native monomer. So the dimers were able to show at least 2-fold enhanced activity but could not reach the value of citrate activation that is shown to be almost equivalent to Apaf-1 mediated activation [49]. The similar results were reported for the dimer interface mutant engineered dimeric caspase-9 [84] and so taking together with this, our observations indicate the distinctly differing nature of oligomerization of caspase-9 monomers by Apaf-1. Next, on examining the cleavage of natural substrate procaspase-3, the concentration and time dependent reactions illustrated the advancements of engineered dimer over its monomer in terms of catalytic activity (Figs. 13A and 13B).

Table 5. Catalytic parameters for monomeric and dimeric caspase-9.

Enzymes	*k_{cat}/K_M ($M^{-1}s^{-1}$)x10^3	
	Normal buffer	Citrate buffer
Wild type procaspase-9	0. 17	12.71
Wild type dimeric procaspase-9	0. 37	4.31
Uncleavable mutant procaspase-9	0.04	0.62
Uncleavable dimeric procaspase-9	0.08	0.26

*The kinetic constants were determined as described in Materials and Methods and only k_{cat}/K_M values are used for comparison due to very high K_M values.

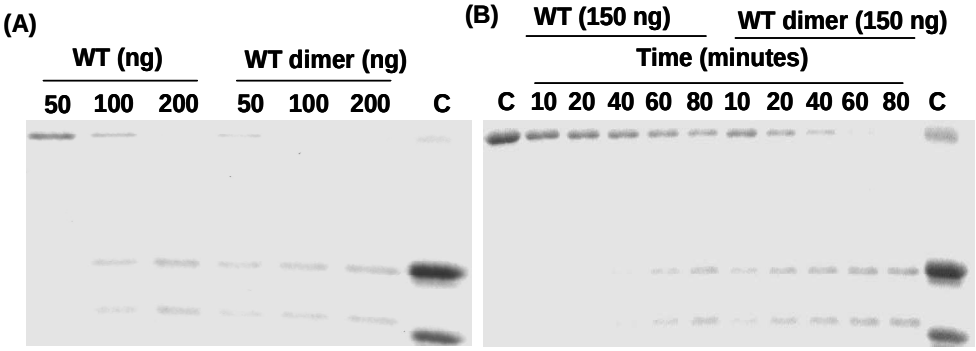

Fig. 13. Cleavage of procaspase-3 by various forms of caspase-9. (**A**) Concentration- dependent cleavage: Procaspase-3 (0.5 μg) was incubated at 25 ºC for 2 h with 50, 100 and 200 ng of monomeric and dimeric wild type caspase-9 and reacted samples were analyzed in 15% SDS-PAGE. (**B**) Time dependent cleavage: 150 ng of each form of caspase-9 was added to 0.5 μg of procaspase-3 separately and the reaction was carried out at 25 ºC. The samples from 10, 20, 40, 60 and 80 min of reaction were taken, mixed with 6X SDS sample buffer and stored at -20 ºC before loading on 15% SDS-PAGE. The gels were visualized with Coomassie staining. The procaspase-3 and cleaved caspase-3 are shown as controls on left and right respectively. WT, wild type.

The artificial dimerization and kosmotropes could activate recombinant caspase-9 [79, 85] but it is not clear whether dimerization is sufficient for generating fully active enzyme. The previous study of engineered dimeric caspase-9 had argued the dimerization of caspase-9 as insufficient for activation and qualitatively different from Apaf-1 activation [84]. However, some recent interesting findings demonstrated that leucine-zipper induced dimerization of caspase-9 or citrate activated caspase-9 could mimic the Apaf-1 activated caspase-9 [49, 86]. But these observations should be accepted cautiously as in interface mutants the gain of high level of catalytic activity may have been restricted due to the alterations of important interface residues which can not only influence in catalytic activity but also in interaction between two monomers during dimerization. On the other hand, leucine-zipper dimerized caspase-9, the possibility of other modifications in conjunction with dimerization can not be completely ruled out as exemplified by differing affinity of thus activated caspase-9 towards synthetic and natural substrates.

In this study, to reflect the inherent property of caspase-9 during dimerization and to maintain all possible interactions between two units, a simple approach of ligating two monomeric caspase-9 molecules was employed to generate a constitutive dimer. Both the cleavable wild type and non-cleavable mutant forms of dimers were designed to investigate the role of proteolysis during or after dimerization. Regrettably, the recombinant dimers could not replace the Apaf-1 activated caspase-9 as expected, but still it could provide some information regarding the activation mechanism of caspase-9. The enhanced catalytic activity of wild type and mutant dimers indicated the

dimerization as an activating event. The failure of these dimers to get activated to higher magnitude might have been contributed by faulty design or different scenario of dimerization from apoptosome. Further, the better capability of kosmotropes and leucine-zipper to enhance the catalytic activity of caspase-9 may imply that directing the individual caspase-9 molecules in free environment for dimerization is favorable than designed engineered dimers. At this stage, it can not be confirmed whether it is the effect of better dimerization or other mechanisms are also involved. The higher activation of cleavable monomers and dimers with citrate as compared to their uncleavable mutant counterparts explains the role of proteolytic processing in the stabilization of dimers and it is in agreement with the previous observation of albeit better activated fully processed forms by the apoptosome than partially processed ones [48].

In conclusion, the better understanding of caspase-9 activation has now provided many clear insights into previous elusive issues but still it is far from complete. The similar catalytic activity of leucine-zipper linked and citrate activated caspase-9 with Apaf-1 activated caspase-9 suggest the role of apoptosome as a dimerization machine rather than an allosteric modulator [49, 86]. But, until the underlying mechanisms by which the apoptosome complexes activate initiator caspases is elucidated by biochemistry, biophysics and structural biology, it is too early to accept these conclusions [28]. Our effort to design a constitutive dimeric caspase-9 using a simple approach may be helpful to some degree for future studies to elucidate the molecular mechanism of caspase-9 activation.

III-4. Efficient cleavage of Bid and procaspase-7 by caspase-2 at lower pH

III-4-1. Caspase-2 was efficient in cleaving Bid and it also cleaved procaspase-7 but not procaspase-3

Catalysis in caspases is mediated by a typical mechanism of cysteine proteases, in which a catalytic dyad composed of cysteine residue acts as the nucleophile activated by histidine residue [87]. The optimum pH for caspase-2 was found to be 6.5-7.2 from its bell-shaped pH dependency profile (Fig. 14A), which was in agreement with the previous report that pH optimum for all caspases lie between 6.5 and 7.5 [73]. However, the profile did not fit well to equation (1), which accounts for the complex ionic interactions involving more than one ionizing group, rather than a simple general acid-base catalysis. Unlike other initiator caspases, caspase-2 was found to be constitutively dimeric with the presence of a central disulfide bridge [55]. So, this special property of the enzyme also hints the complex nature of interactions to some extent. The enzyme showed sharp drop in its activity from pH 6.25 in the lower and 7.5 in the upper regions (Fig. 14A). Ionic strength has been described as a critical regulator in cellular commitment to apoptosis and these ionic effects on caspase activity are mostly contributed by monovalent cations [88]. On examining the effect of salt on caspase-2, the enzyme showed requirement of ~ 50 mM NaCl for slightly

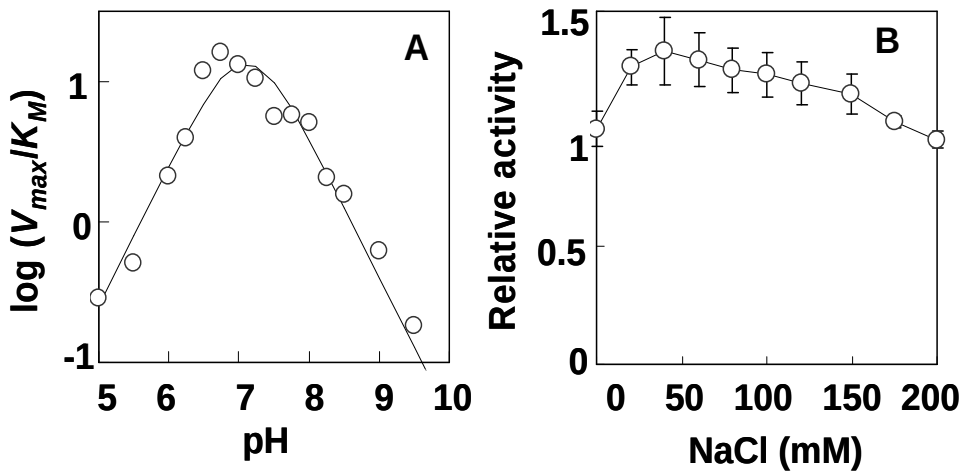

Fig. 14. pH and salt dependency of caspase-2 with synthetic substrate, Ac-VDVAD-AMC. The enzyme assay was carried out for 50 ng of caspase-2 at different pH, as described in Materials and Methods, and plot of log (V_{max}/K_M) vs. pH is presented **(A)**. Data are representative of three independent experiments and the line indicates the fit to equation (1). In **(B),** the caspase activity was done for 50 ng of caspase-2 with 10 μM Ac-VDVAD-AMC at pH 6.75 caspase assay buffer in presence of 0-200 mM NaCl. The relative activity, obtained after normalization of the initial velocities of substrate hydrolysis in each concentration of salt to the velocities in absence of salt, is shown. Error bars represent mean ± S.D. of three separate experiments.

better activity and retained almost stable activity up to physiological range of 150 mM (Fig. 14B), consistent with those reported for other caspases [87]. Considering their identical optimal pH values and similar behavior towards ionic strength, the pH 6.75 reaction buffer supplemented with 50 mM NaCl was used in the following cleavage reactions for caspase-2 and caspase-8, unless specified.

Bid is a pro-apoptotic Bcl-2 family member protein and following the cleavage, t-Bid fragment translocates from the cytosol to the outer mitochondrial membrane triggering the release of cytochrome c from the mitochondrial intermembrane space [89]. The cleavage of Bid has a potential significance in apoptosis to provide a cross-talk between mitochondria-independent and dependent pathway as the latter pathway is more efficient than the former [90]. Besides caspase-8, caspase-2 is also known to cleave Bid [57], but has already been challenged by some lines of studies [61, 65]. All of the previous experiments carried out to demonstrate Bid cleavage include *in vitro* transcription and translation methods which have certain limitations for quantitative interpretations of the cleavage reactions. Keeping this in mind, we opted to revisit the efficiency of caspase-2 cleavage of Bid with homogenously purified recombinant proteins. As shown in Fig. 15A, dose-dependent cleavage reactions confirmed that caspase-2 is only one-fourth less efficient than caspase-8 to cleave Bid.

It has been difficult to assign caspase-2 into the category of initiator or effector caspases because of its unique nature of sharing the properties of both of the groups [54]. Most of the studies prefer to place it into the group of

initiator caspases, considering the similarity with the fellow initiator caspases in structure, activation mechanism and ability to engage the mitochondrial pathway. But, this attempt has been thwarted by the failure of caspase-2 to cleave or activate any effector caspases. However, a recent study has proposed that caspase-2 could provoke the activation of caspase-7 [58]. We checked the cleavage of effector procaspases-3 and -7 by caspase-2. For this purpose, both of these procaspases were subjected to catalytic cysteine residue mutation to avoid auto activation of the proteases during over expression in *E. coli*. In agreement with previous studies, caspase-8 resulted in the processing of both procaspases, while caspase-2 failed to process procaspase-3 but was able to cleave procaspase-7 albeit weak (Figs. 15B and 15C) [57]. The resulting cleavage products were identical in caspase-2 and -8 mediated cleavages, consistent with two cleavage sites (Asp198 and Asp206) in the linker region of procaspase-7. Caspase-7 is also an important executioner caspase, capable of conducting most of the apoptotic program although the overall catalysis rates are lower than caspase-3 for the cleavage of multiple apoptotic substrates [91]. The promising possibility of procaspase-7 being a physiological target of caspase-2 needs to be explored with further comprehensive cellular studies. These observations of robust cleavage of Bid and somewhat processing of procaspse-7 by caspase-2 suggest that some variations in the biochemical conditions might be responsible behind the failure to observe such results in earlier studies. So, to test this hypothesis, we next conducted cleavage reactions for both Bid and procaspase-7 with caspase-2 and -8 in varying conditions of pH and ionic strength.

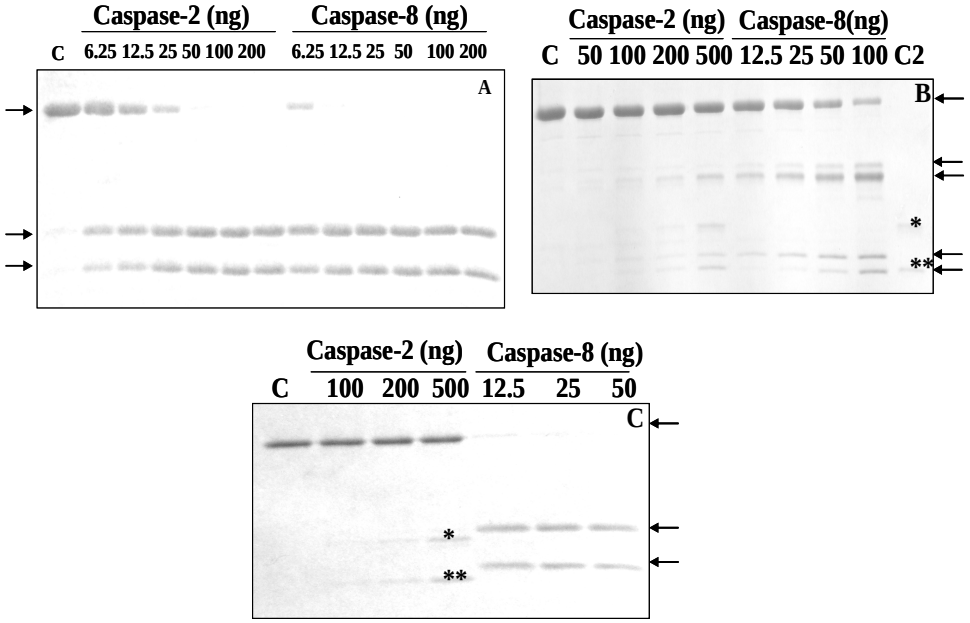

Fig. 15. Cleavage of Bid, procaspase-3 and -7 by caspase-2 and -8. The cleavage reaction was performed with 1.5 μg of Bid **(A)**, 1.2 μg of procaspase-7 **(B)** and 0.5 μg of procaspase-3 **(C)** by adding indicated amounts of caspase-2 and -8 in pH 6.75 caspase assay buffer. Following the reactions, 15% reducing SDS-PAGE analysis was performed. In each case, C represents the control (substrate only), arrowheads show the uncleaved proteins and their corresponding cleavage products, while * and ** denote large and small subunits of maximum amount of caspase-2 (C2) used in the reaction, respectively.

III-4-2. Caspase-2 cleaves Bid more efficiently at lower pH

Ionic charge and pH are two major biochemical parameters that regulate the activity of proteases *in vitro* as well as inside the cell. Various studies have concluded that the ionic strength must be maintained at a permissive level for the entire apoptosis to occur and the process of acidification precedes apoptosis, dropping the intracellular pH from 7.4 to ~ 6.8 during apoptosis [88, 92]. These notions highlight the important relationship between pH, salt and caspase activity. Interestingly, we noticed a strong favor towards lower pH in case of caspase-2 for Bid cleavage (Fig. 16A). In sharp contrast to the optimum pH determined with its synthetic pentapeptide (Fig. 16A), caspase-2 cleaved Bid much more efficiently at lower pH and the cleavage was significantly inhibited from pH >7.25 (Fig. 16A). Caspase-8 was fairly active throughout the range, showing best cleavage at pH 6.75 to 7.25 and the activity was reduced at upper and lower extremes, the latter being more inhibitory (Fig. 16A). The similar picture of inhibition of caspase-2 cleavage of Bid at higher pH was observed in SK-N-BE(2) cell extract (Fig. 16B). This inhibition of cleavage with increasing pH also provided an explanation for the failure of detection of Bid cleavage in the cell extract experiments in a previous study [64]. Caspase-8 was found to be equally active to cleave Bid in the cell extract throughout the used pH range (data not shown). From these results, caspase-2 appears to be quite sensitive to pH, showing an unusual inclination towards lower pH for maximal activity, but to cleave only protein substrates. In fact, the enzyme retained almost similar

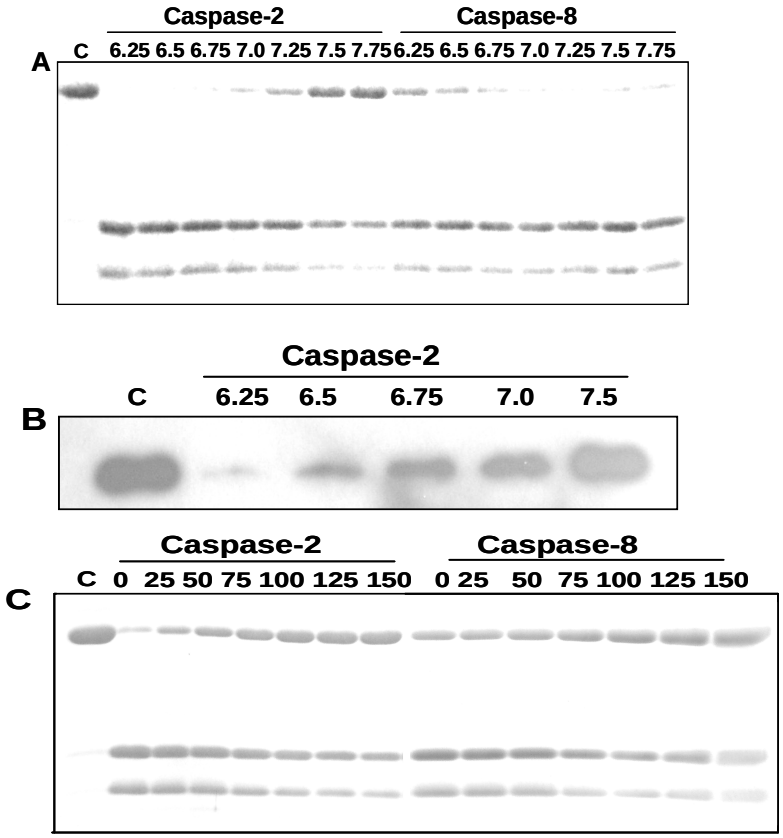

Fig. 16. Effect of pH and salt on caspase-2 and -8 mediated cleavage of Bid. Fifty ng of caspase-2 or 10 ng of caspase-8 were added to 1.5 μg of Bid in caspase assay buffer of varying pH **(A)** or increasing salt concentration at pH 6.75 buffer **(C)** and the reaction was continued for 1 h at 30 ℃. The samples were resolved in 15% SDS-PAGE and visualized with Coomassie staining. In **(B)**, equal amounts (50 μg) of SK-N-BE(2) cell extracts were reacted with 50 ng of caspase-2 at the indicated pH buffer and immunoblotting against Bid was done. C refers to the untreated substrate control.

high activity even up to pH 5.5 (data not shown). One of the outcomes of these observations is that the inconsistency to show the cleavage of caspase-2 substrates, especially Bid, in some earlier studies was largely contributed by unfavorable pH of the reaction buffers. These types of discrepancies often arise because of the variation in experimental procedures. For example, the failure to observe cytochrome *c* release in many reports was later found to be due to the use of different buffers lacking sufficient ionic strength [93]. The results from our study also raised a serious concern over the role of caspase-2 as the enzyme was found to be more active at pH values much lower than physiological range. The further studies are required to clarify whether the exceptional favor of caspase-2 to lower pH has contributed to its restricted function in apoptosis.

Similarly, unlike their fair tolerance to 0-150 mM NaCl in hydrolyzing synthetic substrates, both of the enzymes were found to be sensitive towards higher concentration of the salt during Bid cleavage (Fig. 16C). The inhibition of proteases activity by salt has been considered to be associated with attaining a different structure by protein substrates at higher salt concentrations and thus becoming resistant to cleavage. In previous studies, higher potassium ion concentration inhibited procaspase-3 activity and also inhibited cytochrome c-mediated procaspase-3 activation but not the activity of once activated caspase-3 [37, 88]. Together with this, our observation of selective effectiveness of salt to inhibit the cleavage of only protein substrates suggest that physiological concentration of salt provides a regulatory check point in the apoptotic process.

III-4-3. Effector caspases-3 and -7 also cleave Bid

Some reports have suggested that the induction of cytochrome *c* release from mitochondria is not only restricted to caspase-8 as the effector caspases-3, -6, and -7 can also facilitate the process in presence of other cytosolic factors [94]. Here, we tested the ability of various caspases to cleave Bid and only effector caspases-3, and -7 were found to process Bid (Fig. 17). The cleavage was much weaker than that of caspase-2 which is inconsistent with a previous report that showed similar low degree of cleavage of Bid by caspase-2 and -3 [95]. The results in this study from dose-dependent reactions with homogenously purified recombinant proteins have clearly shown that caspase-2 is much better than the effector caspases in cleaving Bid. The cleavage of apical caspases-9 and -8 by caspase-3 was described as feedback amplification of the apoptotic process [96]. In this perspective, the ability of effector caspases to cleave Bid and provoke cytochrome *c* release may have some roles in enhancing initiator caspases activity or facilitating a link between mitochondria-independent and dependent pathways.

III-4-4. The cleavage of procaspase-7 by caspase-2 is also pH- and salt- dependent

The surprisingly better cleavage of Bid by caspase-2 at lower pH led us to consider two possibilities. It could be either due to certain structural changes in the substrates, making it more favorable for the cleavage or the

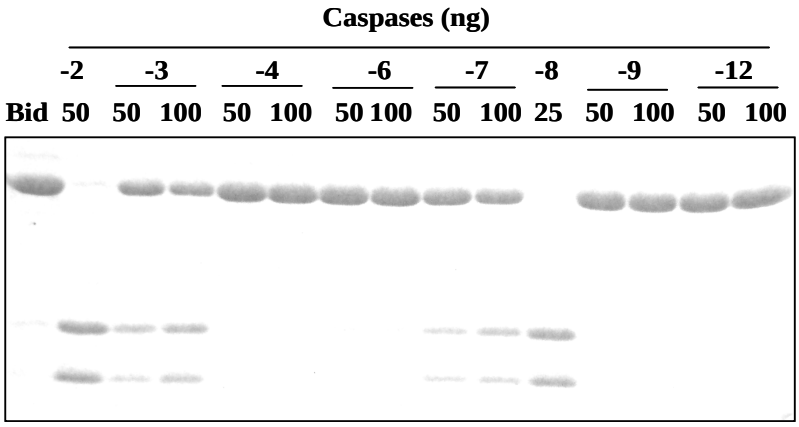

Fig. 17. Bid cleavage by caspases. Indicated amounts of caspases were added to 1.5 μg of Bid at pH 7.0 caspase assay buffer. Following the reaction, samples were analyzed in 15% SDS-PAGE.

unique characteristics of the enzyme to remain exceptionally active at lower pH. The better cleavage at lower pH only with caspase-2 and not that with caspase-8 precludes the former possibility. Actually, this notion appears to be true in the case of inhibition of cleavage at higher salt concentration where both of the enzymes were equally affected. So, to analyze the second possibility, we performed the cleavage reactions at varying pH with another substrate, procaspase-7. Caspase-2 could cleave procaspase-7 only poorly (Fig. 15C); still it showed comparatively better cleavage at lower pH (Fig. 18A). This consistent observation confirmed that caspase-2 prefers lower pH for the cleavage of its protein substrates. Similar to the case of Bid, caspase-8 showed better activity at the middle of the used pH range and both lower and upper extremes were slightly inhibitory (Fig. 18A). The higher concentration of the salt was inhibitory for both of the enzymes in this case, too (Fig. 18B).

In summary, the results from this study demonstrate that the effect of salt on proteases activity is not uniform with their synthetic and protein substrates. Moreover, caspase-2 showed a unique property of favoring lower pH to cleave its protein substrates that makes this controversial enzyme more interesting.

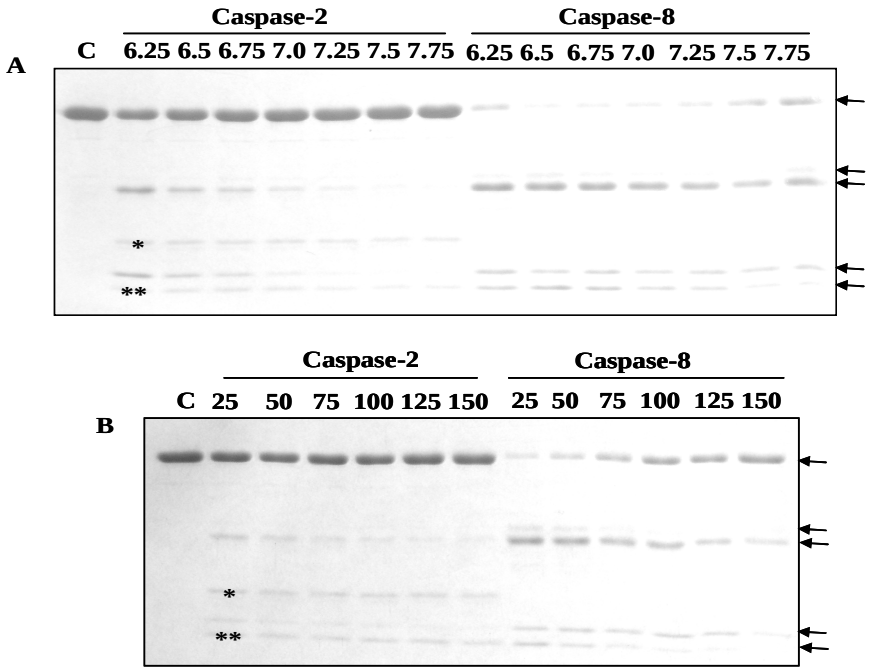

Fig. 18. Effect of pH and salt on procaspase-7 processing by caspase-2 and -8. Five hundred ng of caspase-2 or 100 ng of caspase-8 were added to 1.2 µg of procaspase-7 in caspase assay buffer of varying pH **(A)** or to 1.0 µg of procaspase-7 in pH 6.75 caspase assay buffer with different salt concentration **(B)**. C represents the substrate control, arrowheads show full length proteins and their corresponding cleavage products, * and ** denote large and small subunits of caspase-2, respectively.

IV. RELATED STUDIES AND PROSPECTS FOR FURTHER RESEARCH

IV-1. Role of caspase-7 prodomain in its activity and activation

All caspases possess N-terminal extensions, and in the case of initiators these are required for recruitment of the respective activation complexes. In contrast, a consensus role for the short N-terminal extensions of the executioners has yet to be established. Initial findings suggest that the N-terminal peptides of caspase-3 and -7 have no effect on activity or the ability to be activated *in vitro*. However, *in vivo* both caspases seem to require the removal of N-peptide for efficient activation. Indeed, in some cells, caspase-3 removes the N-peptide of caspase-7 before the later is activated by granzyme B. A recent study has postulated that the prodomain of caspase-3 acts as an intramolecular chaperone during assembly of the (pro)caspase subunits and increases the efficiency of formation of native conformation [37]. In this context, the role of N-peptide of caspase-7 in its catalytic activity and as a substrate of upstream caspases was investigated.

For this purpose, N-peptide cleavage site mutant (D23A) and N-peptide deleted (ΔN) caspase-7 constructs were prepared to compare the enzyme activity. The kinetic constants determined for both of these enzymes were almost identical, ΔN form being slightly more efficient (Table 5). The pH dependency profile of ΔN caspase-7 was consistent with that of other

caspases determined previously and optimum pH value was found to be ~6.8 (Fig. 19A). Finally, to check any difference between full length and N-peptide less caspase-7 as substrates of upstream caspases, catalytic cysteine mutant proteins were purified. The cleavage reaction with caspase-9 illustrated that both of the proteins were equally good substrate for the initiator caspases (Fig. 19B). These observations were consistent with earlier reports, ruling out any possible role of N-peptide for caspase-7 activity and activation [91]. However, a comprehensive kinetic comparison between uncleavable procaspase-7 and its mature N-peptide less form will provide important information regarding the potential role of its N-peptide for the activation. During the study, a surprising observation of failure of expression of wild type caspase-7 in *E. coli* was noted. The requirement of removal of its N-peptide by caspase-3 for the activation and lack of sufficient expression in bacteria indicates the possibility of N-peptide to be an intramolecular inhibitor for caspase-7. The further studies to enlighten this issue will be appreciated.

Table 6. Kinetic constants for full length and ΔN caspase-7

Parameters* / Enzymes	K_M (μM)	k_{cat} (Sec^{-1})	k_{cat}/K_M ($M^{-1}Sec^{-1}$)
Full length caspase-7	17.55	2.06	117378.81
ΔN caspase-7	17.16	3.03	176573.43

*The caspase activity assay was carried out as described in Materials and Methods for 39.4 ng of full length or ΔN caspase-7 with 10-90 μM of DEVD-AMC at pH 7.0 buffer. The kinetic parameters were determined using equations (1) and (2).

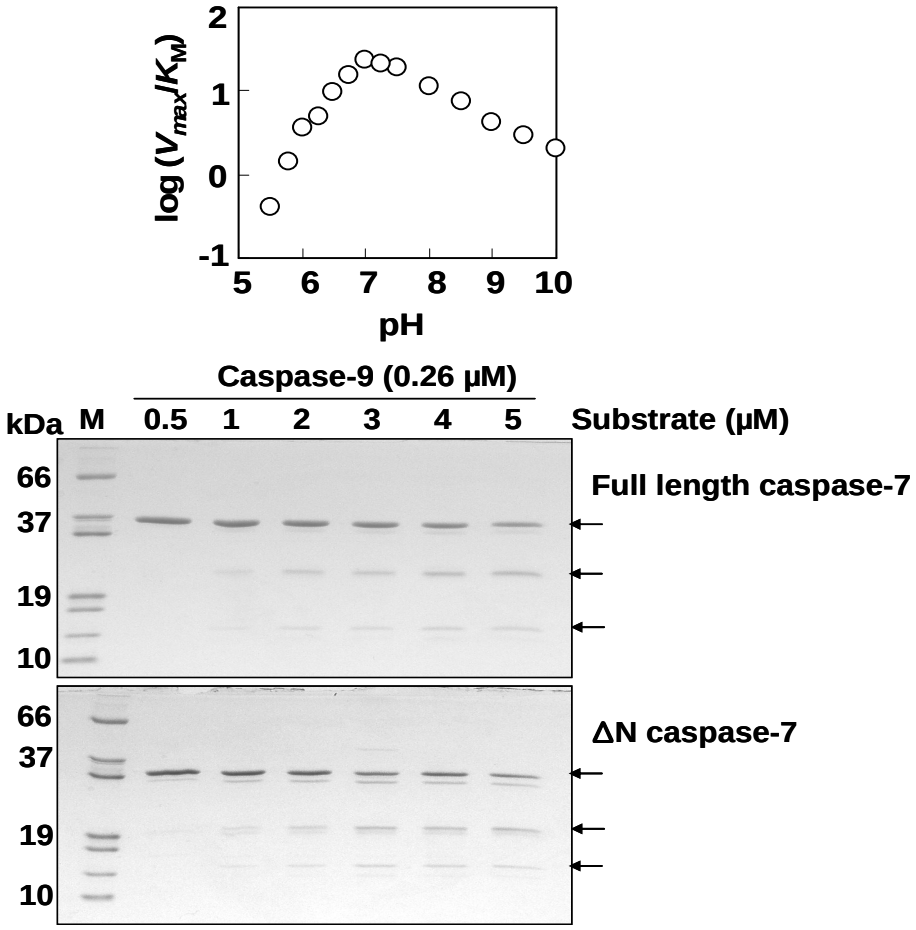

Fig. 19. pH dependency profile and cleavage of caspase-7. (A) The enzyme assay was carried out for 39.4 ng of ΔN caspase-7 at different pH, as described in Materials and Methods, and plot of log (V_{max}/K_M) vs. pH is presented. **(B)** The indicated concentrations of full length and ΔN caspase-7 catalytic cysteine mutant proteins were reacted with caspase-9 at 30 ºC for 1 h. The reacted samples were resolved in 15% SDS-PAGE and visualized with Coomassie staining. The arrows indicate full length protein and its corresponding cleavage products.

IV-2. Expression, purification and enzyme activities of dimeric hybrid caspase-9 mutants

The extensive studies have established that the activation of caspase-9 is associated with its dimerization and proteolysis has little to do in the activation process. With an aim to analyze the role of proteolysis and dimerization in activation, various dimeric hybrid caspase-9 proteins were purified. To study the possible interactions between two monomers, catalytic cysteine of one unit was mutated to serine. All these constructs were expressed, purified in *E. coli* and their enzyme activities and ability to be activated with citrate was studied.

The mutation in one catalytic unit of the dimer completely abolished the activity of the enzyme (Fig. 20A). The reason behind this is not clear and one catalytic mutant seems to act as a dominant negative for the other. Interestingly, during citrate activation, the dimers harboring the cleavable unit were much more efficiently activated (~300 times) than with non-cleavable ones (<5 times) (Fig. 20B). This difference was not due to the variation in their expression level as similar level of expression was observed in Western blot analysis (Fig. 20C). These data suggested that although dimerization is primarily responsible for caspase-9 activation, the accessory role of proteolytic processing to accomplish the full activation can not be completely ruled out. The further studies will unravel this issue.

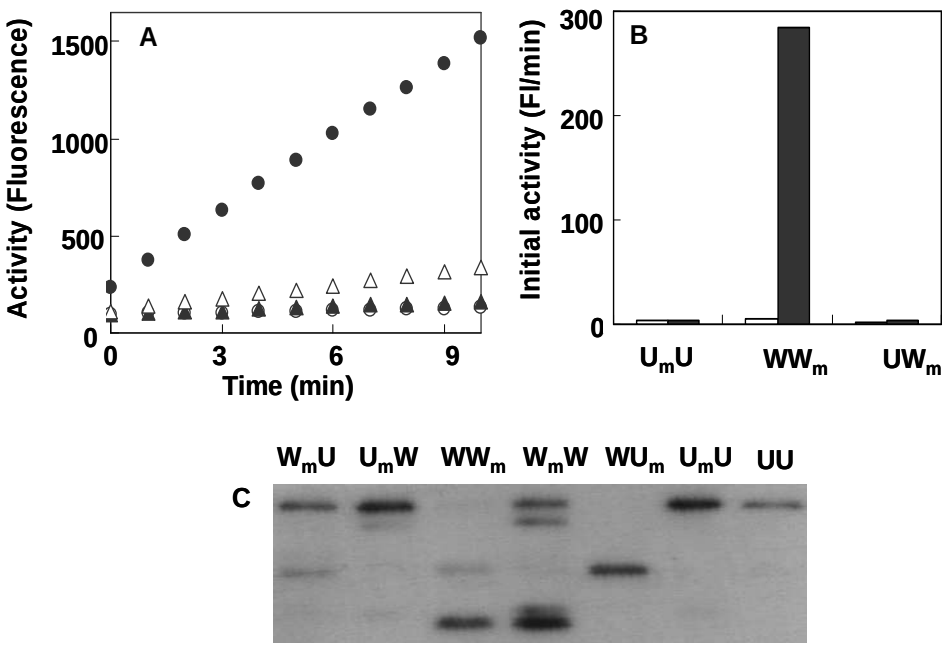

Fig. 20. Enzyme activity and citrate activation of dimeric hybrid caspase-9. (A) The caspase activity assay was carried out for 1 μg of Ni-column purified proteins with 50 μM LEHD-AMC. The activity during 10 min reading is presented for UU (●), U_mU (○), WW_m (▲) and UW_m (Δ). **(B)** Citrate activation was done as described in Materials and Methods and initial velocity of the substrate hydrolysis is shown. **(C)** Equal amounts of indicated proteins were resolved in 15% SDS-PAGE and immunoblotting was performed against caspase-9 antibody. W, wild type; U, Uncleavable mutant; $_m$, catalytic cysteine mutant.

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VII. ABBREVIATIONS

CARD	Caspase-recruitment domain
Apaf-1	Apoptotic protease activating factor-1
DED	Death effector domain
XIAP	X-chromosome-linked inhibitor-of-apoptosis protein
ER	Endoplasmic reticulum
Ac-DEVD-AMC	Acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin
Ac-WEHD-AMC	Ac-Trp-Glu-His-Asp-AMC
Ac-LEHD-AMC	Ac-Leu-Glu-His-Asp-AMC
Ac-LEVD-AMC	Ac-Leu-Glu-Val-Asp-AMC
Ac-IETD-AMC	Ac-Ile-Glu-Thr-Asp-AMC
Ac-VEID-AMC	Ac-Val-Glu-Ile-Asp-AMC
Ac-VDVAD-AMC	Ac-Val-Asp-Val-Ala-Asp-AMC
DEPC	Diethylpyrocarbonate
IAM	Iodoacetamide
CHES	2-(Cyclohexylamino) ethanesulfonic acid
MES	2'-(N-morpholino)ethanesulfonic acid
Tris	Tris[hydroxymethyl]aminomethane
DTT	Dithiothoureitol
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal bovine serum
HEPES	N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis

VII. 적요

Caspases-3, -4, -9 활성화와

caspase-2 에 의한 Bid 절단의 생화학적 연구

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보존된 시스테인 단백질 분해효소군인 caspase는 세포예정사에서 중요한 역할을 한다. 이 단백질 분해효소의 활성기작은 전체 세포사멸조절 과정을 이해하는데 아주 중요하다. 이 연구에서는 caspase-3, -4 그리고 -9의 생화학적 활성화 작용기작은 caspase군의 작용기, 염증유발 그리고 개시자라는 것을 규명하였다. 전구체와 완전한 caspase간의 역학연구에서 두 효소는 비슷한 최적의 pH값을 나타내었다. 하지만, $1/K_M$에 대한 pH연구에서 caspase-3에서는 잔기의 pKa는 6.89±0.13으로 확인 되었으며 V_{max}에 대한 pH역학 결과는 pK_a는 6.21±0.06으로 procaspase-3 돌연변이체(Asp9, Asp28 and Asp175/Ala)와 일치하였다. Diethylcarbonate 불활성화 분석에서 caspase-3 잔기의 pK_a는 6.61±0.05로 나타난 반면 idoacetamide가 있는 procaspase-3의 pKa는 6.01±0.05로 나타났다. 이 결과는 촉매 시스테인과 히스티딘 잔기의 pK_a 값은 caspase-3의 활성과정 중에 변한다는 것을 의미한다. Citrate와 같이 잘 알려진 kosmotrope의 단량체-이합체 전이를 높이기 위해, caspase-4는 caspase-9(75배 증가)

과 비교했을 때 40배 정도 활성이 있었다. 활성반응에서 단량체인 caspase-4는 거의 이량체로 존재한다. 내부에 존재하는 절단부위는 작용 caspase와 비교하였을 때, caspase-4를 100배 이상 활성화 시켰다. 이 결과는 caspase-4가 이량체화와 단백질분해를 동시에 활성화 할 수 있다는 것을 나타내는 새로운 활성화 작용기작 이라는 것을 나타낸다. 이 연구에서는 procaspase-9의 구조적인 이량체형성은 caspase-9분자의 말단부분들이 연결이 되어 형성되었다. 이량체는 대장균에서 과발현하여 분리하였다. 이 재조합 이량체는 각각의 단량체에에 비해 2배 높은 효소활성을 나타내었다. 이량체에 의해 본질적이 촉매 촉진을 하는데 실패한 것은 이 분자를 형성한 것과 Apaf-1에 의해 활성화된 caspase-9이 이량체를 형성하는데 차이가 있다는 것을 나타낸다. Caspase-2 활성의 생화학적 파라미터를 분석한 결과 caspase-8의 기질인 Bid는 caspase-2에 의해 1/4정도 낮은 효율로 절단되었고 procaspase-3보다는 훨씬 낮은 효율이지만, caspase-7 또한 절단하였다. 합성된 기질의 결정된 적정 pH와는 대조적으로, caspase-8은 7.0 이상의 pH에서 더 활성이 있는 반면, caspase-2는 Bid와 procaspase-7을 훨씬 낮은 pH에서 효율적으로 절단하였다. 합성된 기질이 150 mM의 NaCl이 존재하는 상태에서도 가수분해가 일어나는 것과는 달리 두 caspase 모두 높은 염 농도에서는 단백질을 절단하는데 더 민감했다. 이 실험은 caspase-2의 활성은 그것의 생리학적 기질이 pH에 의해 크게 영향을 받는다는 것을 의미한다.