4. Results

4.1 Expression and Purification of Recombinant Mouse Caspase-3 Protein

Cell death of AKR-2B cells was demonstrated in earlier publications [126-129]. After serum removal, death starts after a delay of 90 min. and 50% of AKR-2B cells die within 6h. Considerable importance of the caspases during cell death of AKR cells has been revealed by using specific caspase inhibitors and by determination of the specific activity of the caspases [129]. Beside DEVDase activity, constitutive VEIDase and IETDase activities have been also detected. Partially purification by ion-exchange chromatography showed that the caspase activity is mainly due to DEVDase suggesting that caspase-3 was activated [129]. But, unlike in the literature the molecular weight of active caspase-3 was reported to be 19 kDa whereas the human homologue was 17 kDa. Furthermore, compared to the human homolgue (11 μM and 250 μM for DEVD.pNA and VEID.pNA, respectively) untypical high K_m values of the endogenous caspase-3 for the substrates DEVD.pNA and VEID.pNA of (132 $\pm$ 4) μM and (530 $\pm$ 140) μM respectively, were reported [129].

Therefore, in order to clarify these discrepancies and to assign the DEVDase activity to caspase-3 two strategy were pursued: 1) Expression and purification of recombinant mouse caspase-3. Recombinant mouse caspase-3 protein should be used: for comparison of K_m values of both mouse recombinant and endogenous caspase-3, for the definition of the precise molecular weight of processed mouse caspase-3 protein and also in further *in vitro* experiments during the study. 2) Affinity labeling in combination with two dimensional gel electrophoresis.

4.1.1 Cloning of caspase-3 in to the expression vector pET21b

For cloning of the caspase-3 gene in to a bacterial expression vector, the pET21b (5.4 kb) expression vector was chosen which carries an N-terminal T7 tag and a C-terminal His$_6$ tag. A previously engineered pcDNA+Caspase-3 construct was used as template. Firstly the caspase-3 gene was amplified from the construct (pcDNA 3.1+Casp-3) by PCR by using the Dovetail primers Casp-3 2-1 , Casp-3 2-2 as forward and reverse primers respectively (Fig. 4.1.). Both forward and reverse primers were designed to introduce the restriction sites BamHI and XhoI in to the 5' and 3' ends of caspase-3 insert, respectively. The reverse primer was designed without a STOP codon in frame with the His$_6$ tag. The amplified full length caspase-3 gene was inserted into the BamHI and XhoI sites of the pET21b plasmid to have the His$_6$ tag at the C terminus (Fig. 4.1.) The constructed plasmid was then used for transformation of the competent E.coli (Nova Blue) cells for multiplication. Bacterial colonies were probed by PCR

by using an internal and an external primer of the insert. Plasmids were isolated from positive clones. Sequence analysis confirmed the correct integration of the insert in to the vector and no mutation was detected. The plasmid construct was then transformed into the expression host BL21(DE3) pLysS.
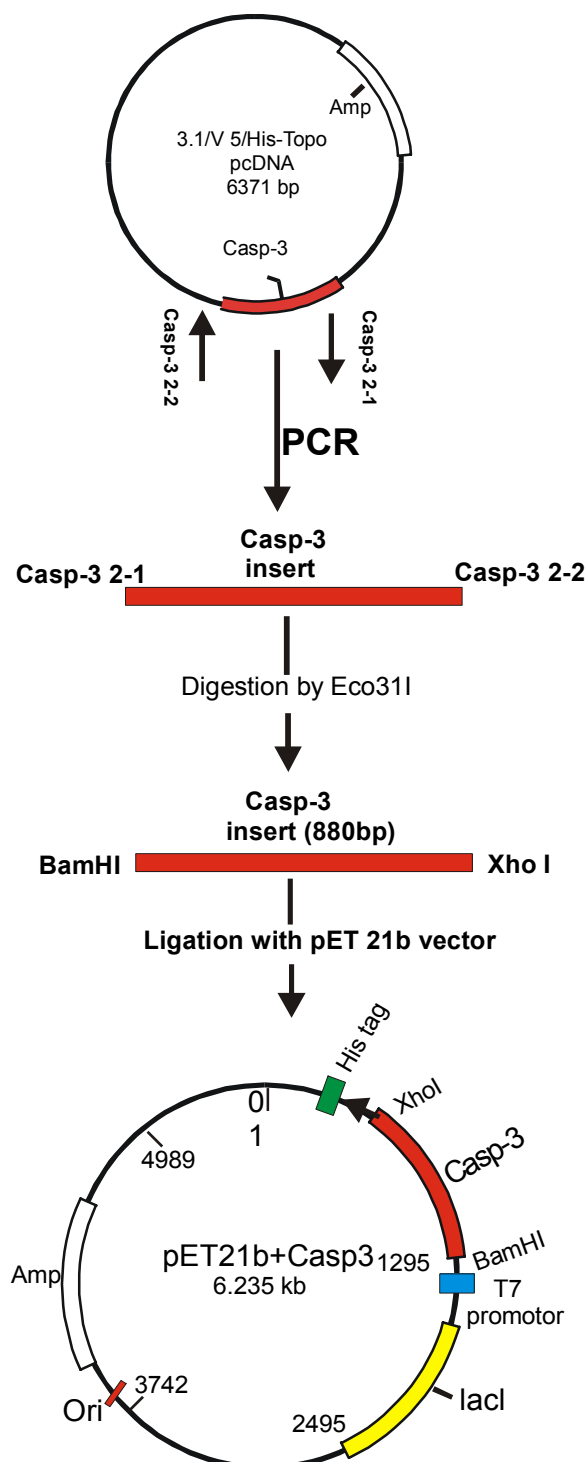

Fig. 4.1. Cloning strategy of caspase-3. Caspase-3 cloned in to the BamHI (N-term.) and XhoI (C-term.) sites of pET21b expression vector, without a stop codon in frame with the His tag at C-terminus.

4.1.2 Expression and purification of caspase-3

To find out the optimal protein production conditions, the induction and the expression of recombinant caspase-3 was analysed. One of the positive clones (Cl.4), was used for the induction and the expression analysis of caspase-3. 1l bacterial culture was prepared and allowed to grow at 37 ^{o}C around 3h until an OD was reached (OD_{600} : 0.4 –1). Induction was performed by adding 1mM IPTG for 3h at 30°C. During induction, at several time points samples were taken for the analysis of the expression profile. A sample was also taken before induction, as control. Bacterial cells were harvested by centrifugation and lysed by Bug Buster (protein purification reagent) and Benzonase® treatment. After centrifugation two fractions were obtained: a soluble part and inclusion bodies. Samples from both were analysed by SDS-PAGE and followed by Coomassie Blue staining to monitor the expression profile of Caspase-3 protein. As illustrated in Fig. 4.2., although the expression of Caspase-3 protein was mostly detected in soluble part, a small amount of the protein was detectable in inclusion bodies. Starting from the 1st hour an increase in the expression of a ~36 kDa (most likely Caspase-3 proenzyme) species was detected. The highest expression level was detected after 2h induction. Additionally increases in the expression of three other species corresponding to 21, 19 and 14 kDa (prodoamin+large, large and small subunits of active caspase-3, respectively) were detected which indicate most likely the autoactivation of caspase-3 during expression as reported in literature [16] (Fig. 4.2.).
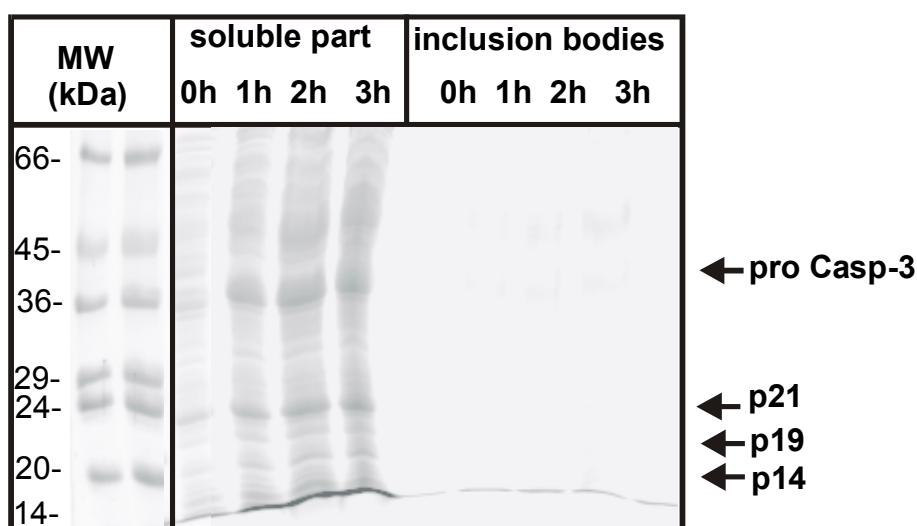

Fig. 4.2. Caspase-3 protein was expressed after IPTG induction and the major part was detected in soluble part. Samples from the both soluble part and inclusion bodies were separated by SDS-PAGE and followed by Coomassie Blue staining.

To further analysis of the expression profile, Western Blotting was applied by using the antibodies which recognize the p17 subunit of the active caspase-3 or the integrated His-tag at the C-terminus of caspase-3. As seen in (Fig. 4.3.) with an antibody directed against the p17

subunit of the active caspase-3, those species corresponding 21 and 19 kDa (prodoamin+large, and large, respectively) were detected. In contrast only procaspase-3 (36 kDa) and the small subunit (p12) were detected by the His-tag antibody, as expected (Fig. 4.3.). Thus these results showed that caspase-3 was expressed after IPTG induction and the major part was detected in soluble part. An induction time of 2h was found to be optimal for further analysis. Since caspase-3 was almost exclusively recovered in the soluble part, the soluble fraction from Bug Buster treatment was used to further purify the native protein by immobilized metal affinity chromatography.
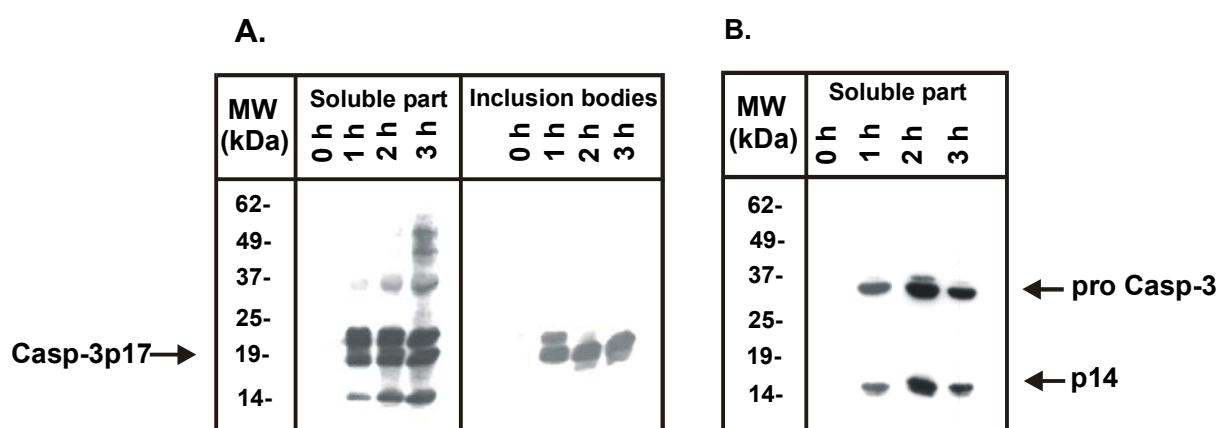
A.

MW (kDa)	Soluble part 0 h	1 h	2 h	3 h	Inclusion bodies 0 h	1 h	2 h	3 h
62-								
49-								
37-								
25-								
19-								
14-								

Casp-3p17➤

B.

MW (kDa)	Soluble part 0 h	1 h	2 h	3 h	
62-					
49-					
37-					← pro Casp-3
25-					
19-					
14-					←p14

Fig. 4.3. Expression of Caspase-3 protein was detected either in soluble part or inclusion bodies by using **A.** Casp-3p17 **B.** His-Tag antibodies (only for solublepart).

Affinitiy-tag purification was performed by using TALON® Cobalt Resin. For purification, 1l culture was grown and after 2h IPTG induction cells were harvested by centrifugation and treated with Bug Buster reagent and Benzonase®.

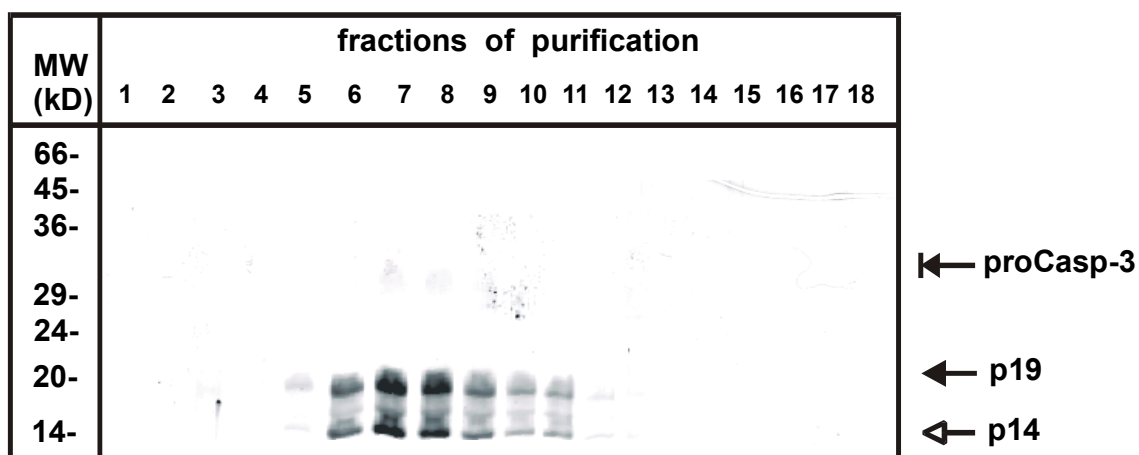| MW (kD)	fractions of purification 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
66-																			
45-																			
36-																			
29-																			◄— proCasp-3
24-																			
20-																			← p19
14-																			◁— p14

Fig. 4.4. Caspase-3 was purified as native active enzyme by affinity-tag purification. Purification profile was monitored by Coomassie Blue staining after SDS-PAGE separation.

The recovered soluble part was applied to the cobalt resin (see methods for the detailed description of the purification step). Firstly, unspecifically bound proteins were removed by 15 mM imidazole washing (intermediate-washstep). Pure native protein was recovered by 100

mM imidazole washing (main-wash step). Fractions of 1ml were collected and analysed by SDS-PAGE followed by Coomassie Blue staining to check the purity of the eluted protein. Fig. 4.4. shows the Coomassie staining profile of the fractions from the purification after SDS-PAGE separation. As seen in figure, by 100 mM imidazole elution, starting from fraction number 5 to 11, two species corresponding ~19 and ~14 kDa were detected. Very faint bands corresponding to 32 kDa in the fractions 7 and 8 (most likely pro caspase-3) were also detectable. No other band indicating unspecific binding or impurity was detected, thus no further purification step was needed.
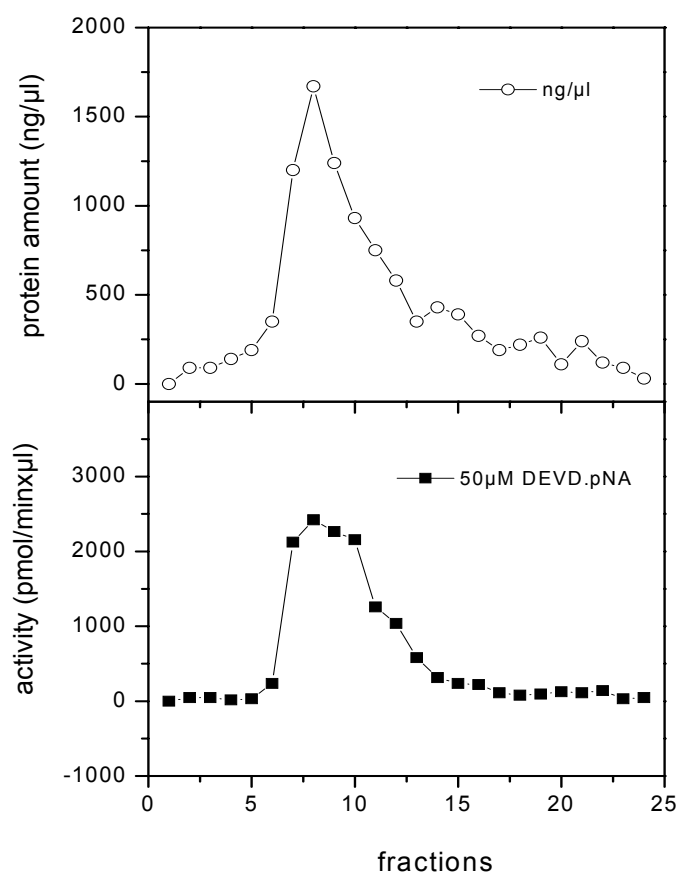

Fig. 4.5. Protein content and DEVDase (50μM) activity profile were found consistent during the purification step of Casp-3 protein .

All fractions from this purification step were analysed for protein content and caspase activity by using 50 μM DEVD.pNa (Fig.4.5.). Determination of the protein content showed that starting with fraction 5. the protein amount increased and reached the highest value as 1.6 μg/μl in fraction 8., and started to decrease with fraction 9. By using 50μM DEVD.pNa, DEVDase activity of the fractions was detected, as well. Activity assay profile was found consistent with the protein content profile. Maximum DEVDase was detected as 2700 pmol/min x μl for fraction 8. which was also containing the maximum protein amount

(Fig.4.5.). Determination of the protein content and the activity assays verified the Coomassie Blue staining data. In addition, the detected species by Coomassie staining corresponding to ~19 and ~14 kDa as well as the 32kDa species were used for N-terminal amino acid sequencing to confirm that the purified protein is indeed active caspase-3 and its subunits. Protein sequencing was performed by Dr. Viviane Hoppe by Edman Degradation using an automated system from Applied Biosystems (Model 476A).
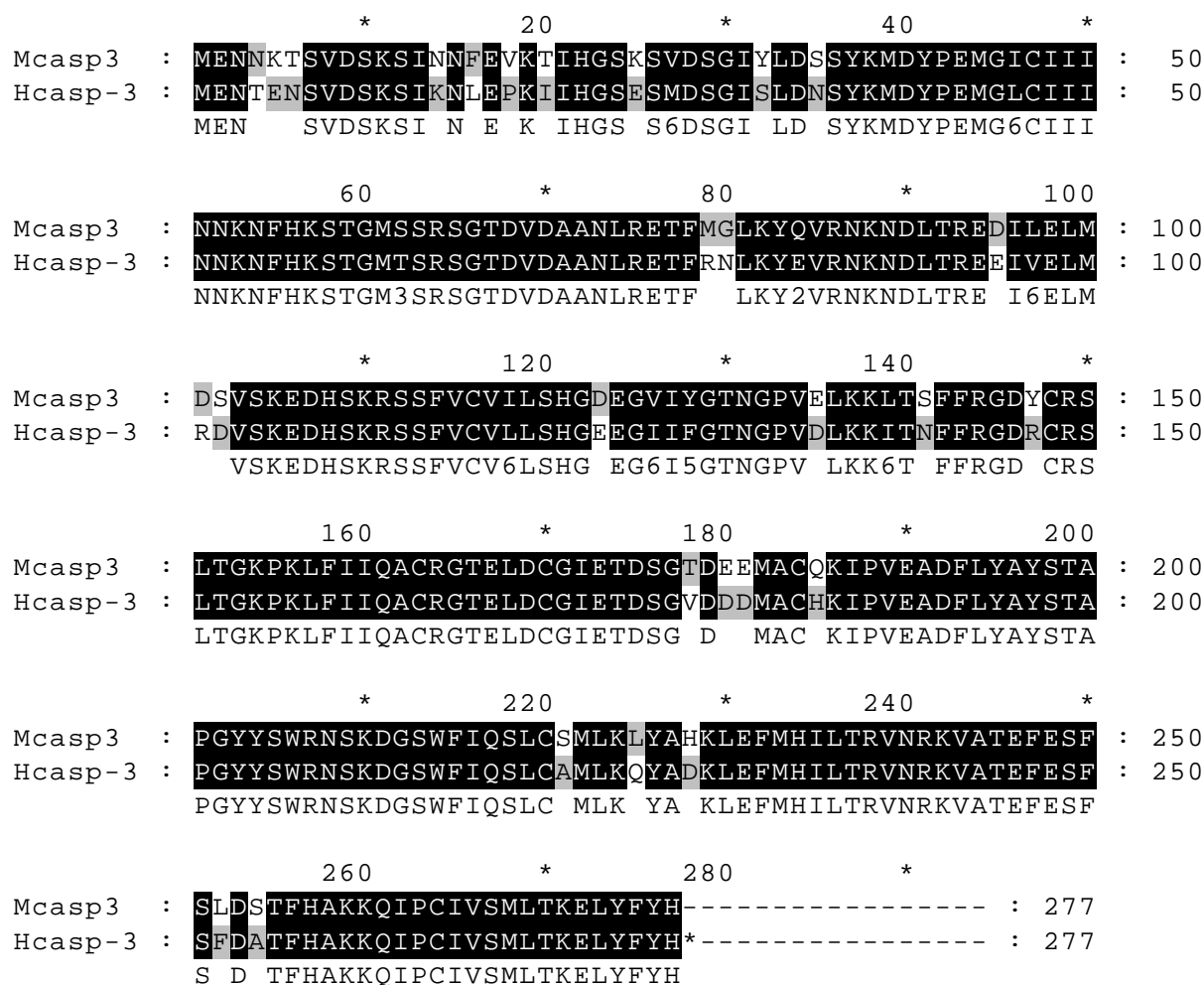
```
                      *         20         *         40         *
Mcasp3   : MENNKTSVDSKSINNFEVKTIHGSKSVDSGIYLDSSYKMDYPEMGICIII : 50
Hcasp-3  : MENTENSVDSKSIKNLEPKIIHGSESMDSGISLDNSYKMDYPEMGLCIII : 50
           MEN    SVDSKSI N E K IHGS S6DSGI LD SYKMDYPEMG6CIII

                 60         *         80         *        100
Mcasp3   : NNKNFHKSTGMSSRSGTDVDAANLRETFMGLKYQVRNKNDLTREDILELM : 100
Hcasp-3  : NNKNFHKSTGMTSRSGTDVDAANLRETFRNLKYEVRNKNDLTREEIVELM : 100
           NNKNFHKSTGM3SRSGTDVDAANLRETF  LKY2VRNKNDLTRE I6ELM

                      *        120         *        140         *
Mcasp3   : DSVSKEDHSKRSSFVCVILSHGDEGVIYGTNGPVELKKLTSFFRGDYCRS : 150
Hcasp-3  : RDVSKEDHSKRSSFVCVLLSHGEEGIIFGTNGPVDLKKITNFFRGDRCRS : 150
            VSKEDHSKRSSFVCV6LSHG EG6I5GTNGPV LKK6T FFRGD CRS

                160         *        180         *        200
Mcasp3   : LTGKPKLFIIQACRGTELDCGIETDSGTDEEMACQKIPVEADFLYAYSTA : 200
Hcasp-3  : LTGKPKLFIIQACRGTELDCGIETDSGVDDDMACHKIPVEADFLYAYSTA : 200
           LTGKPKLFIIQACRGTELDCGIETDSG D  MAC KIPVEADFLYAYSTA

                      *        220         *        240         *
Mcasp3   : PGYYSWRNSKDGSWFIQSLCSMLKLYAHKLEFMHILTRVNRKVATEFESF : 250
Hcasp-3  : PGYYSWRNSKDGSWFIQSLCAMLKQYADKLEFMHILTRVNRKVATEFESF : 250
           PGYYSWRNSKDGSWFIQSLC MLK YA KLEFMHILTRVNRKVATEFESF

                260         *        280         *
Mcasp3   : SLDSTFHAKKQIPCIVSMLTKELYFYH---------------- : 277
Hcasp-3  : SFDATFHAKKQIPCIVSMLTKELYFYH*--------------- : 277
           S D TFHAKKQIPCIVSMLTKELYFYH
```

Fig. 4.6. Protein sequence alignment of mouse and human Caspase-3 (Genedoc alignment).

The sequenced first 10 amino acid of 19 kDa (**SKSINNFEVK**) and 14kDa (**SGTDEEMAC**) species showed that those are the large and the small subunits and of mouse caspase-3 protein, respectively. The first 20 amino acid of the 32kDa species was sequenced (**ASMTGGQQ-MGRDPENNKTS)** and then identified as procaspase-3 plus the fused part of the pet21b vector. Importantly, sequencing of the 20 kDa species showed that prodomain cleavage site of mouse caspase-3 is different from that of the human homologue. Tetrapeptide cleavage motif in the prodomain in mouse caspase-3 protein was detected as **TSVD**, where **D** corresponds to the 9th a.a of mcaspase-3. In contrast it is known that human homologue is cleaved after the

ESMD motif where D corresponds to the 28th amino acid of hcaspase-3. Thus mcaspase-3 prodomain was found to be ~2 kDa smaller than the human homologue in agreement with the sequence comparisons (see Fig. 4.6. and 4.7. for sequence alignments and comparison of the molecular weight of subunits of Caspase-3 protein in human and mouse).
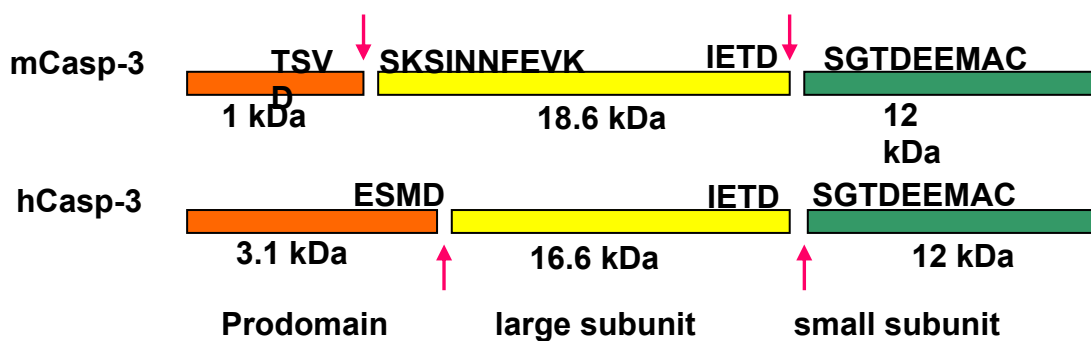

Fig. 4.7. Molecular weight comparison of mouse and human Caspase-3 subunits.

4.1.3 Generation of mt ($K_{25}E$) mcaspase-3 by site directed mutagenesis

Evidently, the clevage site of mCaspase-3 prodomain was different from that in the human protein. Importantly, the 4th amino acid **E** in the tetrapeptide motif is changed to a **K,** which is not accepted by caspase-3 (*i.e.* ESMD/KSVD, see in the sequence alignments Fig.4.7.). To find out if this different processing leads to altered functions, expression and the purification of the protein was decided after restoring the corresponding site in mouse Caspase-3 protein. Caspases are more stringent at P_4 rather that P_2 or P_3 in the tetrapeptide recognition site (P_4-P_1). Therefore it was decided to mutate the 4th amino acid **K** into **E** as in the human homologue. For this purpose a site directed mutagenesis was employed by using two mutagenesis primers forward and reverse, Casp-3 $K_{25}f$ and Casp-3$K_{25}r$ respectively. Primers were designed to be containing the mutated **K** in the mittle. Site directed mutagenesis was performed by using *Pfu Taq* polymerase and the pET21b +mCasp-3 construct as a template (Fig.4.8.), as described under methods in part 3.6.2. Thus, as seen in Fig.4.8. the whole construct (pET21b +mCasp-3) was amplified by using the above mutagenesis primers. After amplification, the parental plasmid DNA was digested by DpnI. To multiply the amplified mutant plasmid construct of caspase-3, the transformation was performed (as described under part 3.2.4.) by using the commercially available competent cells (Novablue). Bacterial clones were probed by PCR by using an internal and an external primer of the insert. Positive clones were used for plasmid isolation. Sequence analysis confirmed the introduction of $K_{25}E$

mutation in to the caspase-3 gene without any further mutation. Thus generation of the mt $K_{25}E$ caspase-3 expression construct was achieved by site-directed mutagenesis. The construct was then used for transforming in to the expression host BL21(DE3) pLysS.
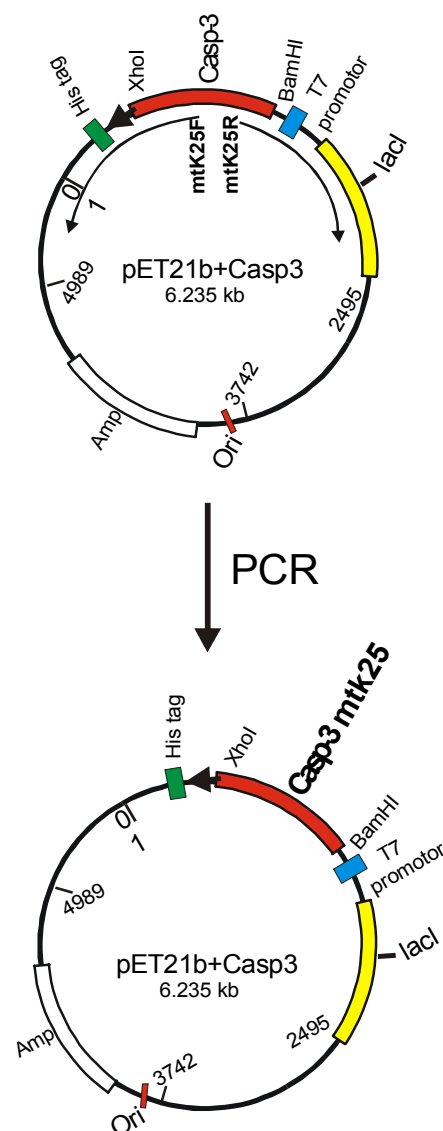

Fig.4.8. Cloning strategy of mtK$_{25}$E Casp-3 in to the pET21bvector by site directed mutagenesis .

4.1.4 Expression and purification of *mt* K$_{25}$E mcaspase-3 protein

As decribed in part 4.1.2. for *wild type* mcaspase-3 protein, again 1l bacterial culture was prepared and allowed to grow at 37 ^{o}C around 3h until the OD was reached to (OD$_{600}$: 0.4-1). Induction was performed by adding 1mM IPTG addition for 3h at 30°C. During induction, at several time points samples were taken for analysis of the expression profile to find the optimal protein production conditions. A sample was also taken before induction, as control.

Bacterial cells were harvested by centrifugation and used by Bug Buster (protein purification reagent) and Benzonase® treatment. After centrifugation two fractions were obtained; a soluble part and inclusion bodies. Samples from both were analysed by SDS-PAGE and followed by Coomassie Blue staining to monitor the expression profile of mt $K_{25}E$ *m*Caspase-3 protein. As monitored in Fig. 4.9., the major part of the mt $K_{25}E$ Caspase-3 protein expression was also detected in soluble part, less amount of the protein was detectable in inclusion bodies.

MW (kD)	inclusion bodies				soluble part				
	0	1h	2h	3h	0h	1h	2h	3h	
66									
45									
36									←p32
29									
24									←p21
20									
14									←p17 ←p14
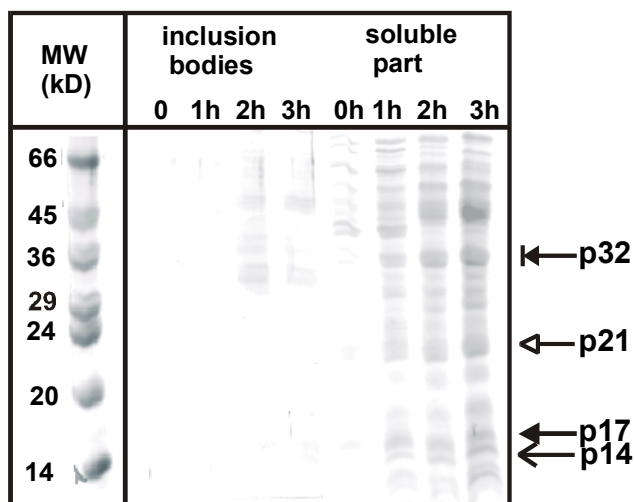
Fig. 4.9. Expression profile of mt Caspase-3 in the soluble part and in the inclusion bodies was monitored by SDS-PAGE separation and Coomassie Blue staining.

Starting from the 1st hour again an increases in the expression of 36, 21, 17 and 14 kDa species were detected (Fig. 4.9.). The highest expression level was detected after 2h-3h induction. An induction time of 3h was found to be optimal for further analysis. For purification, again 1l bacterial culture was grown, harvested and used for Bug Buster and Benzonase® treatment . The recovered soluble part was applied to TALON® Cobalt resin. Firstly unspecifically bound proteins were removed by 15 mM imidazole washing (intermediate wash-step). Pure native protein was then recovered by 100 mM imidazole washing. Fractions of 1ml were collected and analysed by SDS-PAGE separation and Coomassie Blue staining to check the purity of the eluted protein (Fig.4.10). Fig. 4.10. shows the analysis of the fractions by SDS-PAGE followed by Coomassie Blue staining. As clearly seen in the figure by 100 mM imidazole elution, starting from fraction number 4 to 17. prominent two species corresponding ~17 and ~14 kDa (most likely p17 and p12 subunits of active mt caspase-3) were detected. A very faint band corresponding to 32 kDa in fraction 8 (most likely pro caspase-3) was also detectable. No other band indicating to the unspecific binding or impurity was detected. Thus no further purification step was needed.
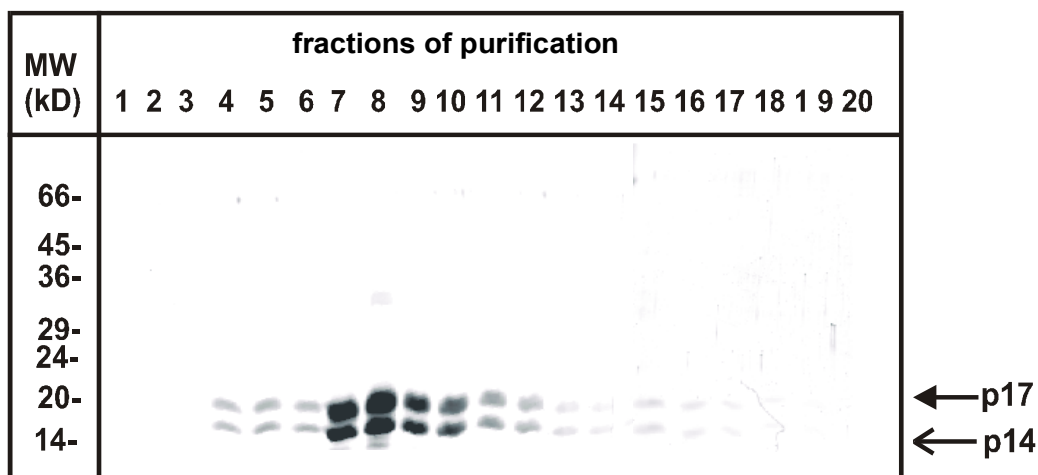
MW (kD)	fractions of purification
	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 1 9 20

66-
45-
36-
29-
24-
20- ← p17
14- ← p14

Fig. 4.10. mt Caspase-3 was purified as active enzyme by affinity tag purification. Purification profile was monitored by Coomassie Blue staining after SDS-PAGE separation

All fractions of the purification step, were also used for determination of protein content and caspase activity assay (50 µM DEVD.pNa.), (Fig.4.11).
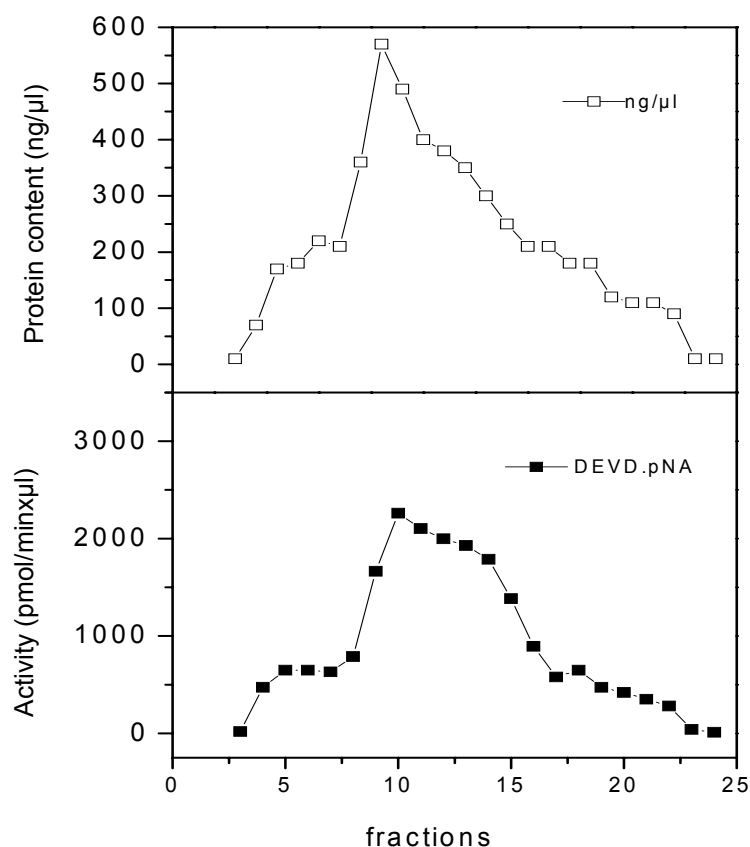
Fig. 4.11. DEVDase activity (50µM) and protein content profile of the purification of $mtK_{25}E$ Caspase-3 protein was found consistent.

Determination of the protein content showed that with fraction 3. protein amount started to increase and reached the highest value as 0.58 µg/µl with fraction 8, and started to decrease

with fraction number 9 (Fig. 4.11.). By using 50µM DEVD.pNa, DEVDase activity profile of the fractions were found consistent with the protein content profile(Fig. 4.11.).

Maximum DEVDase was detected as around 2300 pmol/minxµl for fraction 8 which was also containing the maximum protein amount (Fig. 4.11.). Additionally the detected species corresponding to ~17 and ~14 kDa as well as the 32 kDa band were used for N-terminal amino acid sequencing again to confirm that the purified protein is indeed active mutant caspase-3 protein. Protein sequencing was performed by Dr. Viviane Hoppe as described before. By sequencing of **mt K$_{25}$ECasp-3** the first 10 amino acid of the 17 kDa species were identified as **(SGIYLDS)** which indicates that prodomain is cleaved from the same site as in human caspase-3 after mutating the 25th K into the E. Sequencing informations of the 32 kDa and 14 kDa species were found identical as in wildtype Caspase-3 as **(ASMTGGQQMGRD-PENNKTS)** and **(SGTDEEMAC)** respectively.
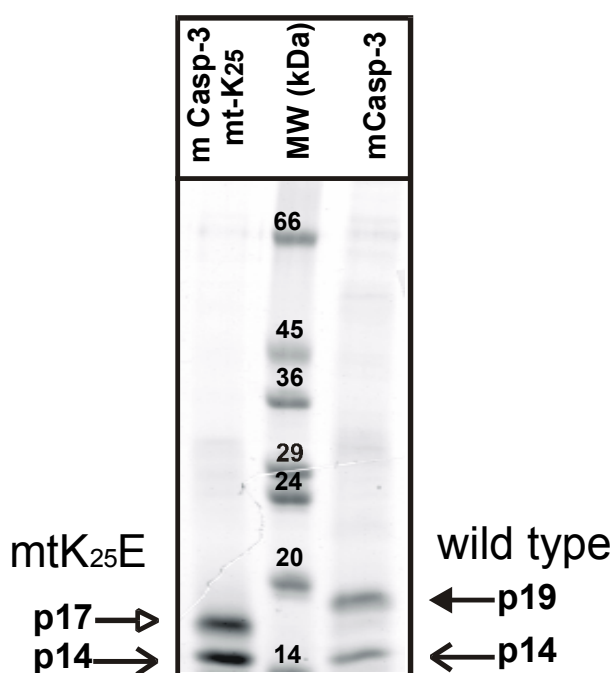
Fig. 4.12. Comparison of the subunits of purified, mutated (K$_{25}$E) and *wild type* active mCasp-3 were achieved by SDS-PAGE separation and Coomassie staining. Open and closed arrow indicates the large subunit of mutated and wildtype mCasp-3 corresponding 19 kDa and 17kDa respectively.

Fig. 4.12. shows the Coomassie Blue staining of the both purified active *mt* (K$_{25}$E) and *wild type* mCaspase-3 side by side after SDS-PAGE separation. As seen in the below, SDS-PAGE separation clearly showed that the large subunit of active mouse Caspase-3 protein is corresponding to 19kDa, whereas the large subunit of mt K$_{25}$E Caspase-3 has a molecular weight of 17 kDa. Small subunit was detected as 14 kDa for both proteins. Thus, these results showed that the purified protein is indeed active mtK$_{25}$E caspase-3 protein. Furthermore, sequencing of the 17 kDa band showed that by restoring the **K** (first amino acid of the

prodomain cleavage motif) to the **E**, the mouse prodomain was cleaved at the same site as the human homologue.

4.1.5 Determination of K_m and V_{max} values of the recombinant *wild type* and *mt* $K_{25}E$ mcaspase-3 proteins for the substrates DEVD.pNA and VEID.pNA

Since the large subunit of mouse active Caspase-3 has been found to be 2kDa larger than the human homologue, it was important to examine if it has any effect on the enzymic properties. Thus, the K_m and V_{max} were determined for the recombinant *wild type* and *mutant* mCaspase-3 for substrates DEVD.pNA and VEID.pNA at different concentrations between 25- 400 µM.
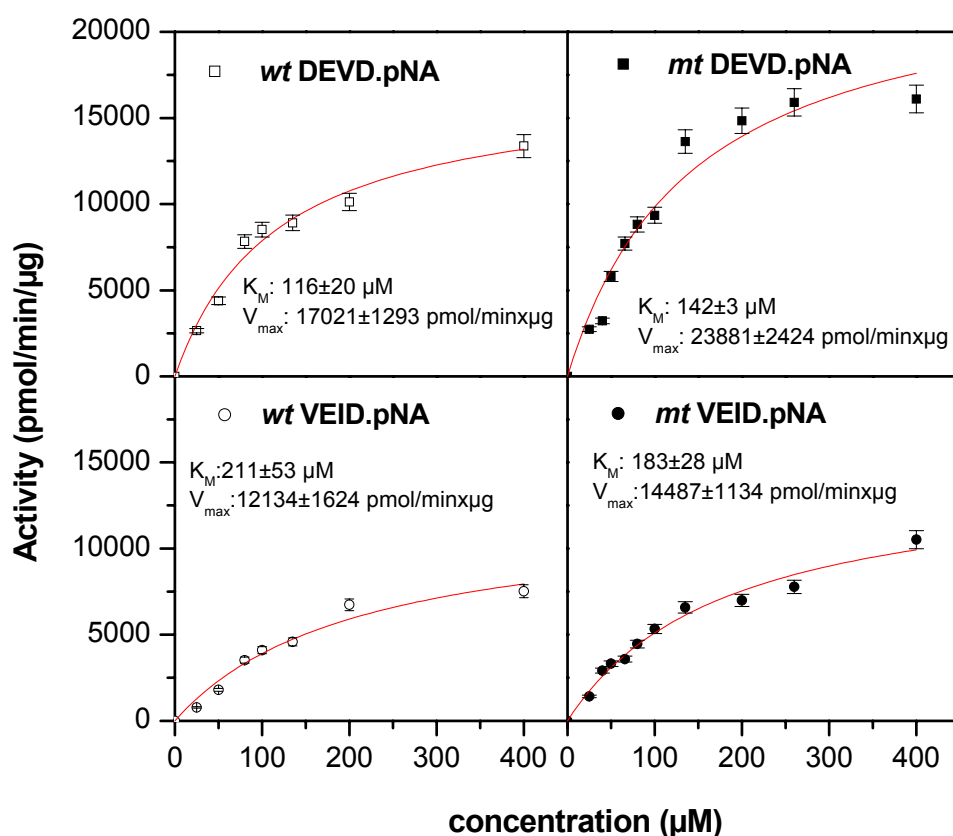
Fig. 4.13. Activity assay of *wild type* and *mtK₂₅E* recombinant Caspase-3 proteins, by using different concentration of the substrates DEVD.pNA and VEID.pNA. Closed and open symbols either square or circle indicate to recombinant *wild type* and *mt K₂₅E* Caspase-3, respectively.

Enzyme activities of the both recombinant *wild type* and *mt*K$_{25}$E mCaspase-3 proteins were detected as in Fig. 4.13. **K$_m$** and **V$_{max}$** values for caspase substrates DEVD.pNA and VEID.pNa were calculated from the *Michaelis-Menten* model (V= V$_{max}$ [S]/K$_m$+[S]) by using the program Origin (microcal). All calculated **K$_m$** and **V$_{max}$** values were summarized as

in (Fig.4.13.). K_m values of the substrates DEVD.pNA and VEID.pNA for recombinant mCaspase-3, were found as (99±19 µM) and (205±42 µM) respectively, whereas for mt $K_{25}E$ Casp-3 determined as (102±16 µM) and (244±32), respectively. Apparently almost identical K_m values of the recombinant *wild type* and *mt*$K_{25}E$ mCaspase-3 proteins were obtained for the substrates DEVD.pNA. and VEID.pNA. The K_m values for the substrate VEID.pNA were found higher than DEVD.pNA . Interestingly a slight increase in V_{max} value of the *mt*$K_{25}E$ caspase-3 were found for the substrate DEVD.pNA, compared to that of the *wild type*. Thus, these results show that, the extension of 2 kDa has no additional effect on substrate binding or enzyme activity. Additionally as reported in literature here it was also shown that DEVD.pNA is a better substrate for caspase-3 than VEID.pNA. Importantly, K_m values of the recombinant *wild type* mCaspase-3 protein were similiar to that of the endogenous Caspase-3 in AKR-2B lysates for the respective substrates (DEVD.pNA: (132 ± 4) µM and VEID.pNA: (530 ± 140) µM)

4.2 The Main Active Executioner Caspase of AKR-2B Mouse Fibroblasts

4.2.1 Caspase 3 is activated as the main active executioner caspase during cell death of AKR-2B fibroblasts

4.2.1.1 Cell death induced by serum deprivation.

It has been demonstrated previously that caspases are present and activated in cell death of AKR-2B cells in response to serum removal. Caspase activities are maximal 3h after serum removal. By using Ion-exchange chromatography partially purification of caspase activities from cytosolic extract showed that caspase activities are largely due to DEVDase activity [129]. In the current study to further define the executioner caspases that are activated and responsible for DEVDase activity in apoptotic AKR-2B cells, affinity labeling and 2D-Gel Electrophoresis (2D-GE) was applied. For affinity labeling, the biotin conjugated peptide inhibitor YVK(bio)D-aomk was used which is able to react with caspases -3, -6 and -7 . Since this peptide inhibitor of caspases mimics a tetrapeptide cleavage site, some selectivity between caspases whould be expected. Previously it has been demonstrated that the irreversible inhibitor YVK(bio)D-aomk does effectively label many caspases when it is used in an extended labeling times at optimized concentrations [142]. For labeling, 500 µg of cytosolic extracts from non treated and 3h starved AKR-2B cells were used. Labeling was performed by incubating extracts at 37 ºC for 1h with 10µM of the biotinylated inhibitor YVK-(bio) D.aomk. Thus, by this way the key requirement of labeling (decrease in the

selectivity of the inhibitor to be able to label all active caspases) was fulfilled. Since the large subunits of caspases have similar molecular weights, one dimensional gel electrophoresis was found to be insufficient to resolve all labeled caspases. Therefore it was decided to use 2D-GE.
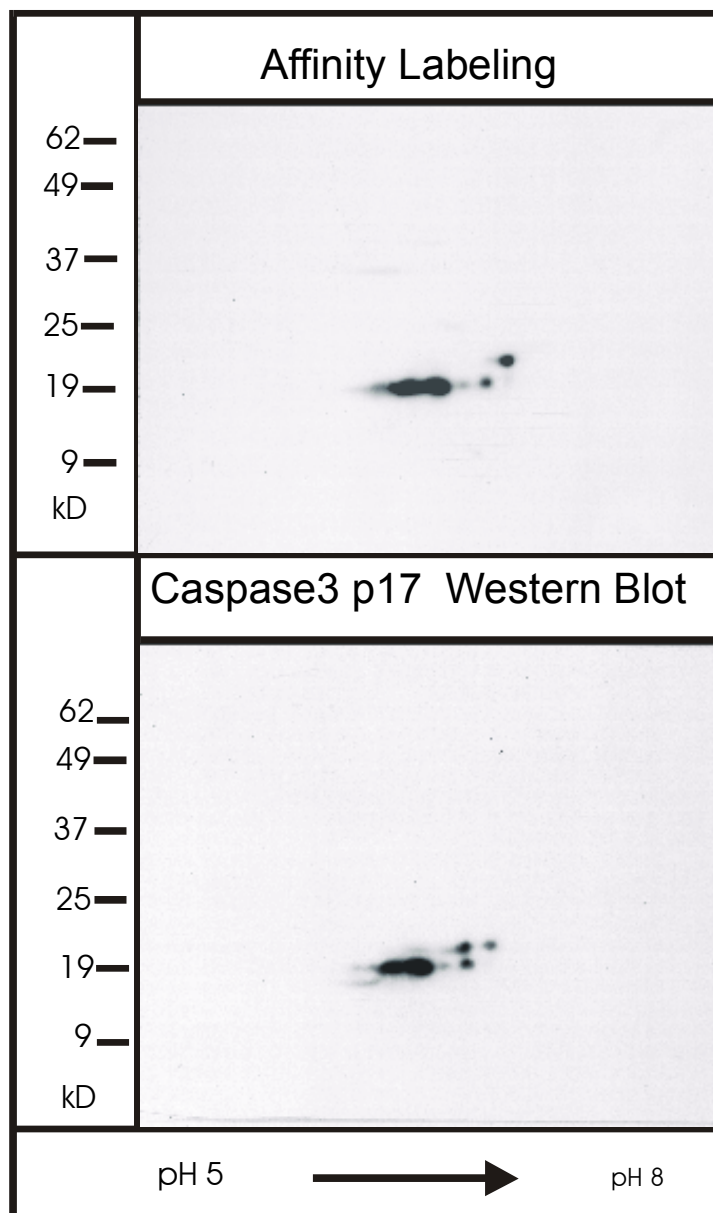

Fig.4.14. By 2D-GE separation identical spot patterns was detected (19 kDa and pI 6.5) by Casp-3p17 antibody and the affinity labeling.

A broad pH range (3-10) was used for isoelectrical focusing. Proteins were separated firstly by isoelectrical focusing, and then by SDS-PAGE. Proteins resolved by 2D-GE were transferred to membranes which were then incubated either with peroxidase conjugated streptavidin referred to as affinity blot or with antibodies which recognize the zymogen or the p17 subunit of active caspase-3. Labeling was then visualized by enhanced chemiluminescence (ECL), (Fig. 4.14.). As clearly seen in Figure 4.14. identical spot patterns

were detected corresponding to the molecular weight of 19 kDa and pI 6.5, (since no spot was detected between pI 3-5 and 8-10, these parts were excluded from the figure) by the affinity labeling and by the antibody which recognizes the p17 subunit of caspase-3. The presence of multiple spots both in affinity and western blot patterns indicated the presence of multiple species of caspase-3 in AKR-2B mouse fibroblasts as described before for human cell lines Jurkat or CEM [142].

Cytosolic extracts of non starved AKR-2B cells were used as a control for 2D-GE separation. From the control extract (non starved), only the zymogen of caspase-3 was detected by the antibody at a molecular weight of 32 kDa and at pI 6.5 (Fig 4.15.). Caspase-3 zymogen was also detected as two or three spots similar to the active enzyme indicating once again the presence of multiple species of caspase-3 protein in AKR-2B mouse fibroblasts.

MW (kDa)	WB-Casp-3 zymogen
62- 49- 37- 25- 19- 14-	
	ph 5 ⟶ 8
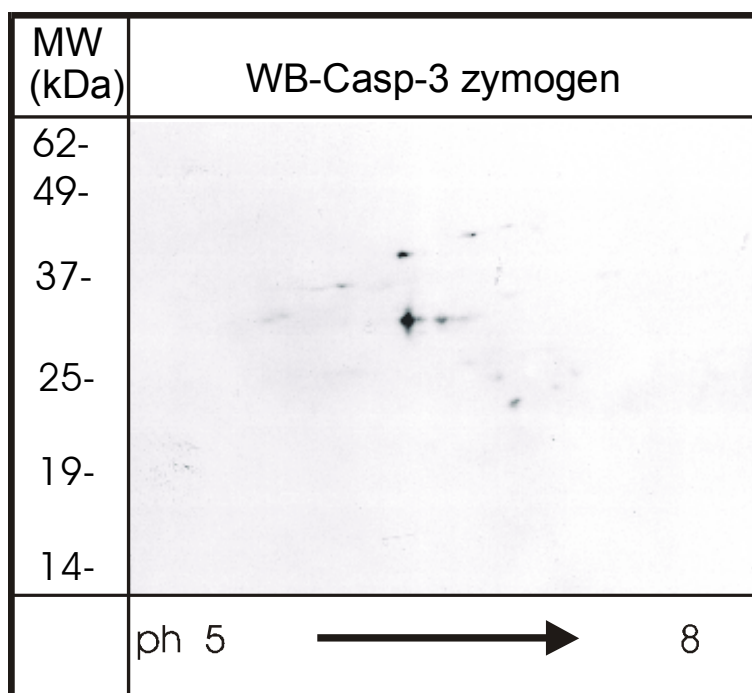
Fig.4.15. Only the zymogen of Caspase-3 was detected (32 kDa, pI 6.5) from the non starved control extracts by Western blot after 2D-GE separation.

4.2.1.2 Cell death induced by Anisomycin treatment

It has been recently demonstrated that Anisomycin (a potent protein synthesis inhibitor) induces cell death in AKR-2B cells, similar to serum deprivation. Cell death starts about 30-60 min after the addition of Anisomycin, about 40% of the cells die after 6h and cell death is completed after 24 h. Activation of caspases has been also studied by means of caspase activity assay. A maximum for DEVDase activity has been found after 6h of Anisomycin treatment corroborating with a substantial cell death at that time [143]. Thus, those previous

results showed that Anisomycin effectively induce cell death in AKR-2B cells which is paralleled by an activation of the DEVDase activity.
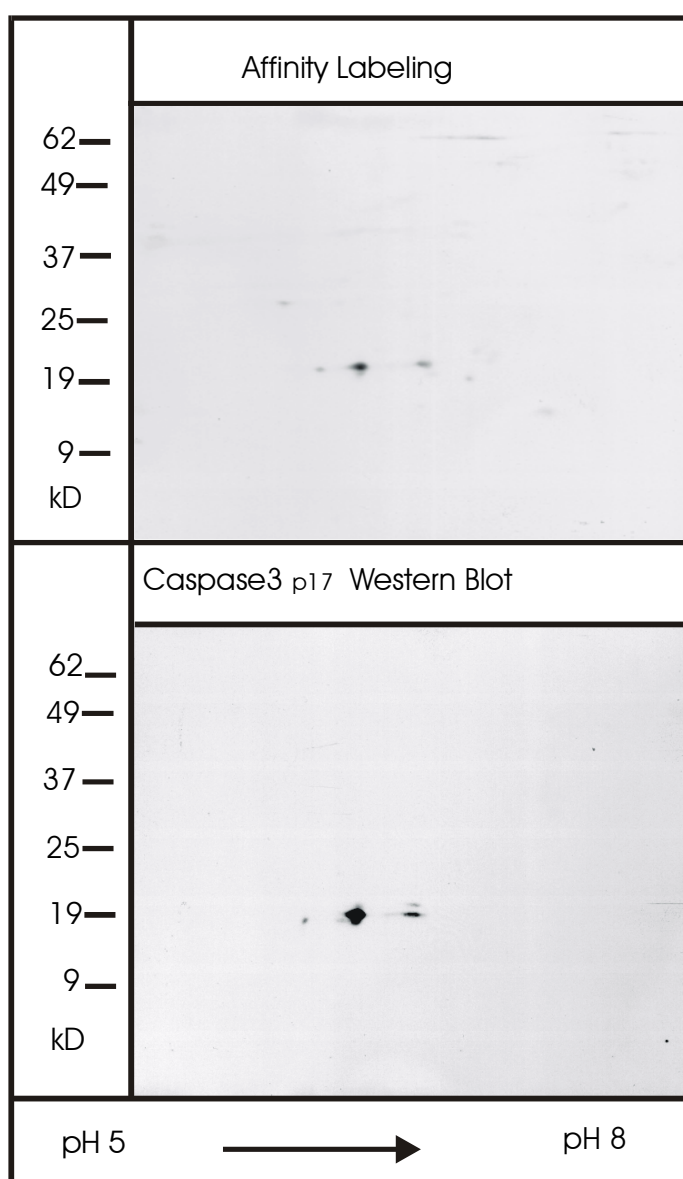

Fig. 4.16. Caspase-3 was detected as the active affector caspase by 2D –GE separation in the extracts from 10µM Anisomycin terated cells as displayed by either affinity labeling or staining with Casp-3p17 antibody.

To further characterization of DEVDase activity in response to Anisomycin treatment again affinity labeling and 2D-Gel electrophoresis was applied as described in 4.2.1.1 by using the biotinylated inhibitor YVK(-bio)D.aomk. AKR-2B cells were treated with 10 µM Anisomycin for 4h and used for preparation of cytosolic extract. 500 µg of this extract was labeled and separated by 2D-GE as described before. As seen in Figure 4.16. identical multiple spots were also detected corresponding to the molecular weight of 19 kDa and pI 6.5, by the affinity labeling and by staining with the antibody which recognizes the p17 subunit of

caspase-3. 2D-GE separation showed that during cell death induced by Anisomycin again caspase-3 is activated as the major executioner caspase.

4.2.2 Caspase-7 protein is not expressed in AKR-2B fibroblasts

Another effector caspase, Caspase-7 cleaves the substrate DEVD.pNA as effectively as caspase-3. This raises the possibillity that caspase-7 might be also responsible for the DEVDase activity in AKR-2B. In order to examine the involvement of caspase-7, expression of caspase-7 protein was analyzed by Western blot with an antibody which recognizes both the zymogen (p35) and the large subunit of (p21) active caspase-7. As a control, either non treated or cytochrom c treated Jurkat lysates were used beside the AKR-2B cell lysates. Both Jurkat and AKR-2B lysates were separated by SDS-PAGE and stained with the antibody as indicated above (Fig. 4.17.). As illustrated in Fig.4.17. Caspase-7 protein was detected as zymogen around 35 kDa from the non treated Jurkat cell lysates. The large subunit of caspase-7 was also detectable around 21 kDa from cytochrome c treated Jurkat lysates. Compared to Jurkat cell lysates, neither zymogene nor active caspase-7 protein was detectable in AKR-2B lysates, although Caspase-7 mRNA is expressed [129].
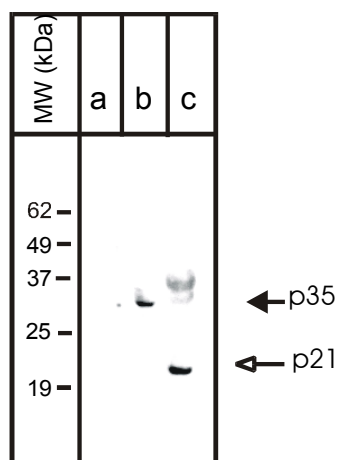
Fig. 4.17. Expression of caspase-7 analysed by Western Blotting . a) Lysates of 3h starved AKR-2B cells. b) Non treated Jurkat lysates. c) Cytochrom c treated Jurkat lysates.

Thus, these results confirmed that caspase-3 is activated as the main executioner caspase during cell death of AKR-2B cells induced by either serum starvation or Anisomycin treatment. Expression and purification of recombinant mouse caspase-3 protein revealed that the large subunit of active caspase-3 has a molecular weight of 19 kDa unlike the human homologue. In addition comparison of K_m values showed that the endogenous and the recombinant mouse caspase-3 have simliar K_m values for the substrates DEVD.pNA and VEID. pNA which allows the assignment of the DEVDase activity to caspase-3 in AKR-2B cells.

4.3 Analysis of The Activation of Caspase-3 During Cell Death of AKR-2B Fibroblasts

4.3.1 Caspase-3 is activated in complexes in response to serum starvation

Since caspase-3 was found to be activated as the main active executioner caspase in AKR-2B mouse fibroblasts, it was important to examine its process of activation. Activation of caspases involves intracellular caspase cascades and very often large protein complexes are formed such as Apoptosome, a 700 kDa caspase activating complex in which Apaf-1 interacts with caspase-9 in the presence of dATP and cytochrome c, leading to activation of caspase-9 which then cleaves and activates down stream effector caspases such as, caspase-3 and -7 [109]. Therefore, initially the possible involvement of high molecular weight complex formation during the process of caspase activation was examined during the cell death of AKR-2B cells induced by serum starvation. For this, gel filtration chromotograpy was used to fractionate the large molecules on the basis of their sizes.
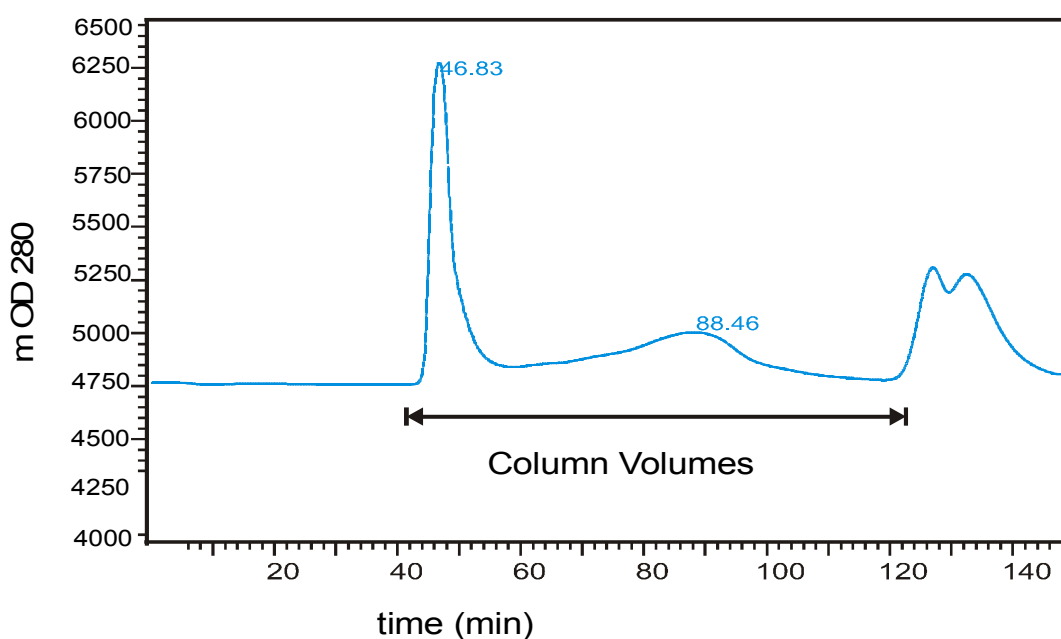
Fig.4.17. **Proteins were eluted mainly in two peaks by separation of cytosolic extracts from AKR-2B cells. (S-300 Sephacryl-Column).**

To fractionate the extracts of AKR-2B cells, initially a S-300 Sephacryl Gel Filtration column (16/60 Amersham-Pharmacia) was used, which gives the best resolution within a molecular weight range from 10 to 1500 kDa in the connection with an inert HPLC pump (Merck-Hitachi L6210). The column was calibrated by using a Calibration Kit (Pharmacia). Cytosolic extracts from either 3h starved (apoptotic) or non starved (control) AKR-2B cells, were

applied. Figure 4.17. shows the typical protein elution profile. During the separation, proteins were eluted mainly in two peaks within the separation range (Fig.4.17).
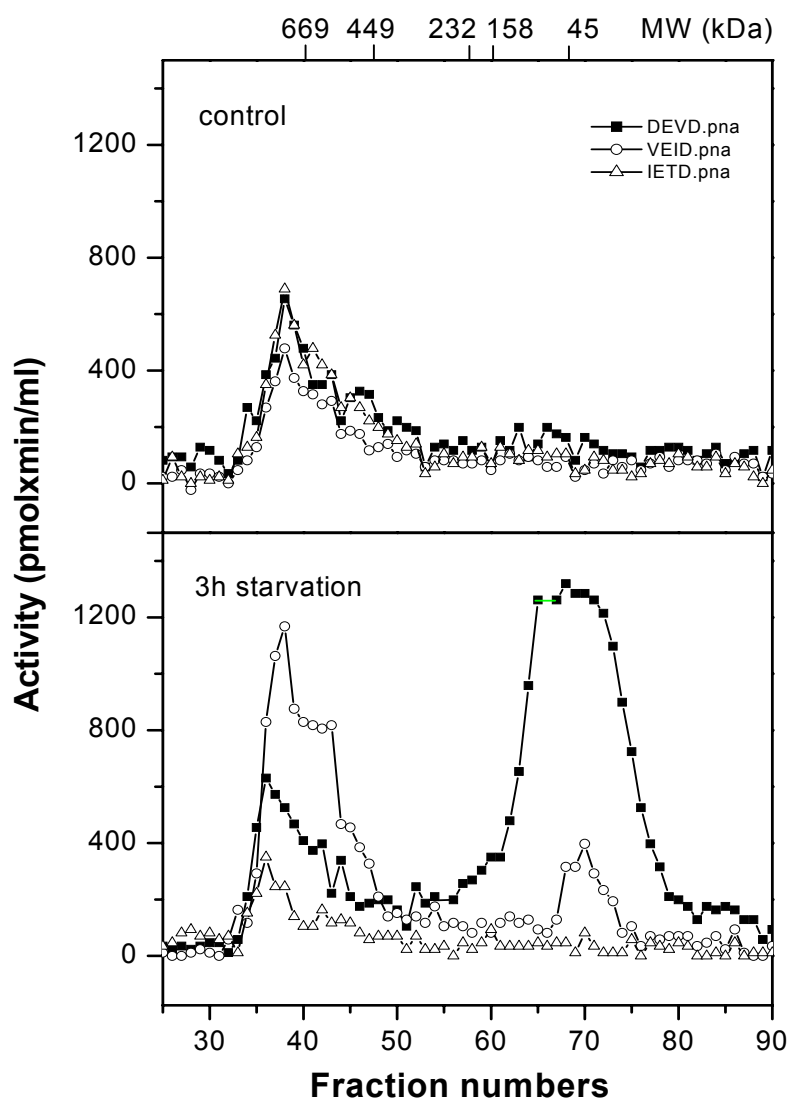

Fig. 4.18. Caspase activities were eluted mainly in two peaks in response to serum starvation. The elution profile shows the caspase activity of each fraction plotted against the respective fraction.

During the period of the separation, fractions were collected every 1.3 min. and therefore, elution time was referred as fraction number in the plots. All the fractions were examined for the caspases activity whereas every second one used for Western Blot analysis. Three different substrates were used to assay the activity of caspases. DEVD.pNa for the effector caspases such as caspase-3, VEID.pNa and IETD.pNa for the initiator caspases such as caspase-6, -8, -9. For the inhibition assay specific inhibitors of the corresponding substrate such as DEVD.cho, VEID.cho and IETD.cho, were used. Caspases activity assay was performed and analysed as described in the methods.

The fractions obtained after the separations of both extracts from apoptotic or control cells were initially analysed to identify the initiator and the activator caspase actvities. Fig. 4.18. shows the analysis of the extracts from control cells (upper panel) and 3h starved aoptotic cells (lower panel). In the control experiment, caspase activities were detected exclusively within the region of >700 kDa. When the extract of apoptotic (3h starved) cells was separated caspases activities were eluted mainly in two peaks corresponding to the molecular weight of ~700 kDa, and between 250kDa and 60 kDa, respectively (Fig. 4.18.) The initiator caspase activities VEIDase and IETDase were mostly found in the first peak correponding to the molecular weight of ~700 kDa in the fractions of the extract of apoptotic cells. In contrast, the effector caspase activity DEVDase was mostly found in the second peak which was generated between the 250 kDa and 60kDa. Thus, compared to the control after 3h starvation a substantial increase was detected for the initiator caspase activity VEIDase and the effector caspase activity DEVDase which indicate to the activation of the respective caspases (Fig. 4.18).

To further specify the caspase activities, especially to distingush the activities from each other, fractions from the extract of apoptotic cells were further examined by use of the various caspase peptide inhibitors whose structures are based on the substrate specificity motif. By using the specific inhibitor DEVD.cho, DEVDase activity was inhibited mainly in the region of the free enzyme around 60 kDa and in a lesser extent in the complexes of 600 kDa and 250 kDa, respectively (Fig. 4.19.). The high molecular weight activity of >700 kDa was not inhibitable by the inhibitor DEVD.cho, indicating to the unspecific activity. Low molecular weight VEIDase activity was completely inhibited by the inhibitor VEID.cho, whereas the inhibition of the activity in >700 kDa region was partially (Fig. 4.19.). In addition, inhibition assay showed that the IETDase which was detected at a molecular weight of 700 kDa, was not due to caspase activity (Fig. 4.19.). The activity peak which was detected from the control fractions corresponding to 450-700 kDa can be explained by the constitutive activities of the caspases in this high molecular weight region (Fig. 4.18.). Thus, the inhibition assay suggest that DEVDase and VEIDase activities are due to the activated caspases-3 and -6 respectively, in the respective regions. In contrast, no significant IETDase activity was detected concerning to the initiator caspases which are capable of cleaving the IETD motif necessary to activate caspase-3.
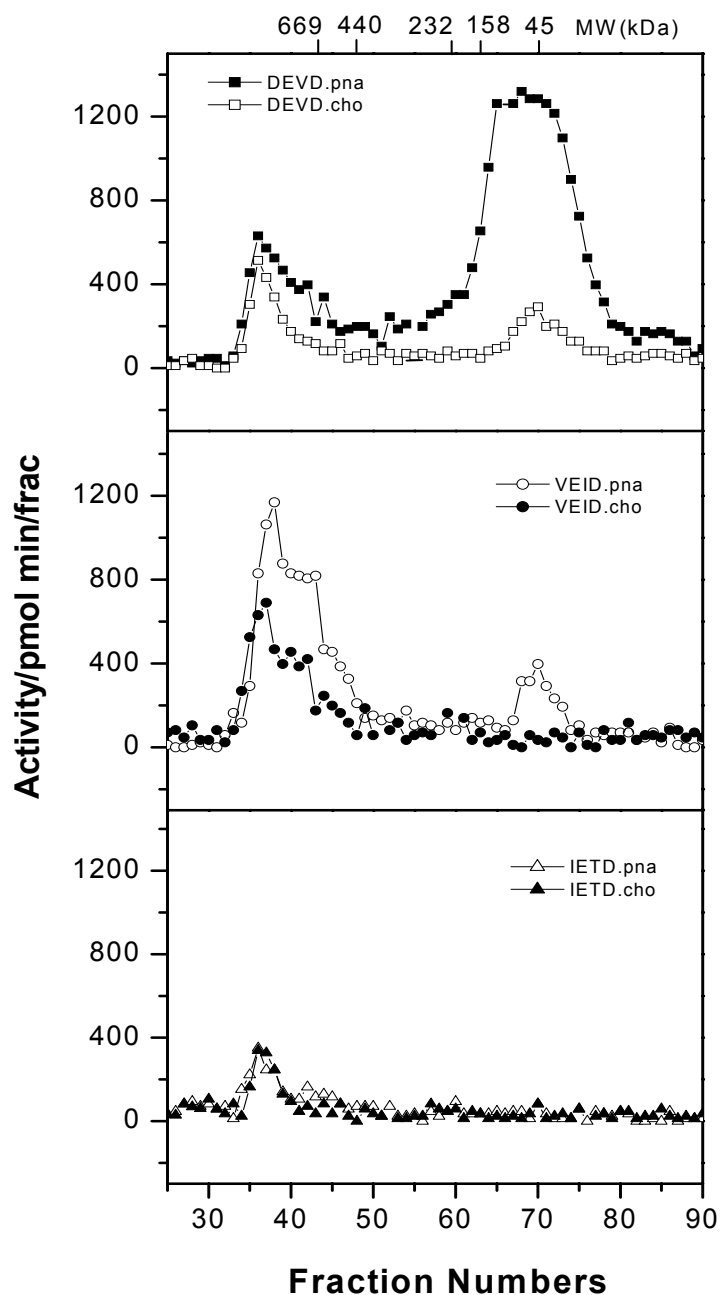
Fig. 4.19. **Specific caspase activities were determined in different complexes. Fractions from the sepration of the extract of apoptotic cells were assayed for the inhibition of caspase activities. The elution profile shows the specific caspase activity of each fraction plotted against the respective fraction.**

Furthermore, time dependency of the activitation of the caspases were also examined in the current study. AKR-2B cells were starved for 1 to 4 hours and then extracts were separated by using the Sephacryl S-300 column. The collected fractions were analyzed for the caspases activity assay. Activity assay showed that starting from the 1st hour caspases are activated (Fig. 4.20.). At the 1st hour mostly the initiator caspase activities were detected in the high molecular weight region (~700 kDa). In addition, a considerable increase was also detectable

for the effector caspase activity DEVDase at a molecular weight of 250 kDa (Fig. 4.20.). After 2h a further increase was found that the VEIDase increased at ~700 kDa. In contrast, the DEVDase activity was increased in the free enzyme region corresponding to the molecular weight of 60 kDa. No changement was detected for the IETDase activity after 2 hours (Fig. 4.20.). Considering the DEVDase and the VEIDase activities maximum activities were detected after 3h in the respective regions (Fig. 4.20.). After 4h caspase activities found to decreased, mostly the DEVDase at a molecular weight of 60 kDa (Fig.4.20.).
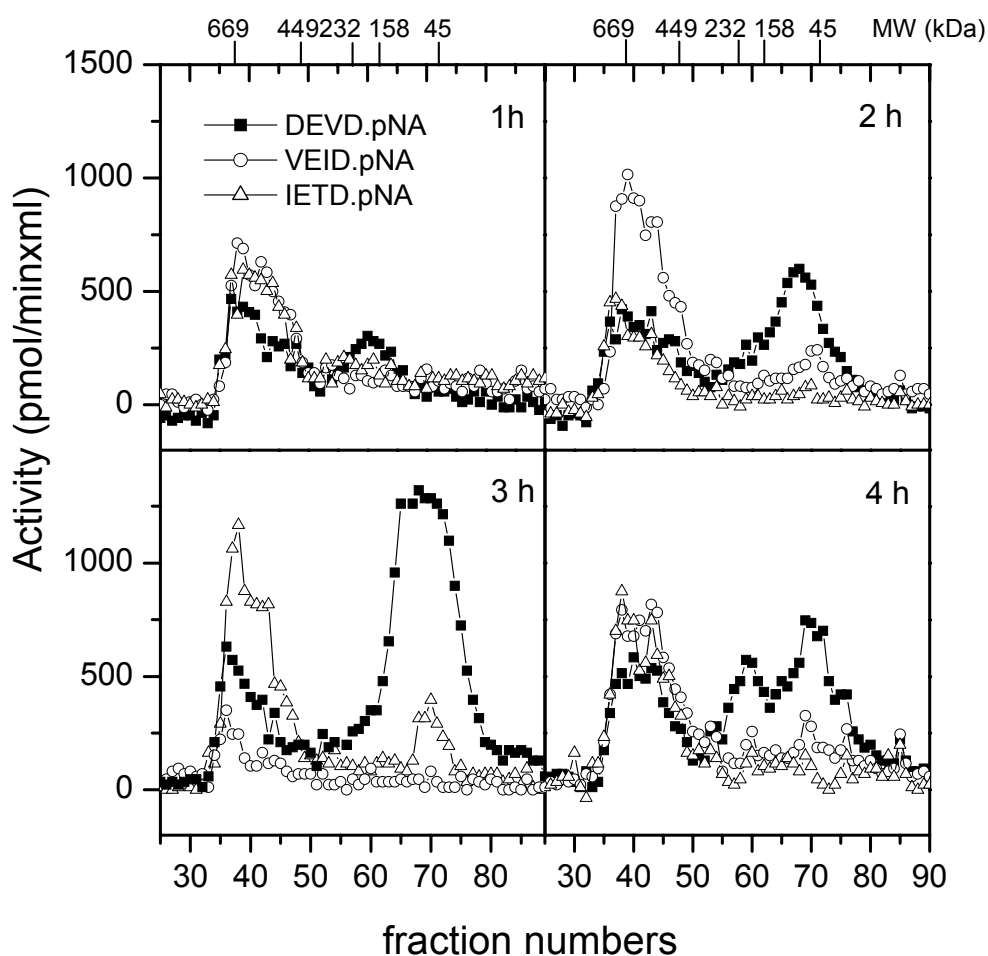
Fig.4.20. Time dependending activation of caspases were analysed by caspases activity assay.

Taken together, concerning the DEVDase activity, kinetic data suggest that after 1h, most likely caspase-3 started to be activated in the complexes corresponding to the molecular weight of 600 kDa and 250 kDa, and after 2 hours it is started to be released as a monomeric free enzyme at the 60 kDa. Furthermore, the slight increase of the VEIDase activity which was also observed in the high molecular weight regions is, probably due to the activation of

caspase-6, the lamin cleaving enzyme which is activated during apoptosis in AKR-2B cells [119].

To further characterize the complexes especially to find out the molecular composition of the complexes, fractions were analyzed by SDS-PAGE and Western Blotting. For all further experiments extracts from cells starved for 3h were used, since maximum caspase activity was detected at this time point. In the control extract, caspase-3 zymogen was detected mostly in the fractions corresponding to the molecular weight of 600 kDa and 60kDa (Fig.4.21. upper panel). For caspase-6, an antibody was used against caspase-6 which is recognizes the zymogen and the p12 subunit of active caspase-6. Caspase-6 zymogen was detected mostly in the fractions corresponding to the low molecular weights. Very small amounts were also detectable in the fractions corresponding to the molecular weight of 600 kDa (Fig.4.21. upper panel). To elucidate the role of caspase-9 in activation of caspases, an antibody against caspase-9 was also used which recognizes both the zymogen and the small subunit (10 kDa) of active caspase-9. Caspase-9 zymogen eluted in the fractions corresponding to the molecular weight of around 100 kDa. Additionally it was detected at the molecular weight of 600 kDa (Fig.4.21.). Cytochrome c was detected in the very low molecular weight region (Fig.4.21.). The active subunits of caspases were not detectable in any fraction, suggesting that activated caspases are not processed in the control cells (Fig.4.21. lower panel). Concerning the separation of extracts from apoptotic cells, procaspase-3 was still detectable in the fractions corresponding to 60 kDa (free enzyme) (Fig.4.21. lower panel). The p17 subunit of the active caspase-3 was detectable mainly as free enzyme in accordance to the DEVDase activity and in a lesser extent in the complexes of 250 and 600 kDa, respectively (Fig.4.21. lower panel). In contrast, p12 subunit of active caspase-6 was detected in a 450 kDa complex as well as a free enzyme (Fig.4.21. lower panel). Importantly from the fractions of the extract of apoptotic cells, caspase-9 remained again in those fractions corresponding to the molecular weight ~100 kDa, most likely as a dimer in its unactivated state. No cleavage subunit of active caspase-9 was detected indicative to its activation in apoptotic cells. Furthermore no cytocrome c coelution with caspase-9 was detected. Cytochrome c was found in the same fractions similar to the control (Fig. 4.21.). Thus, these results revealed that caspase-3 and caspase-6 were activated in different sized complexes during apotosis in AKR-2B cells. Caspase-3 is activated in complexes corresponding to 600 kDa, 250 kDa whereas caspase-6 in a complex corresponding to 450 kDa.
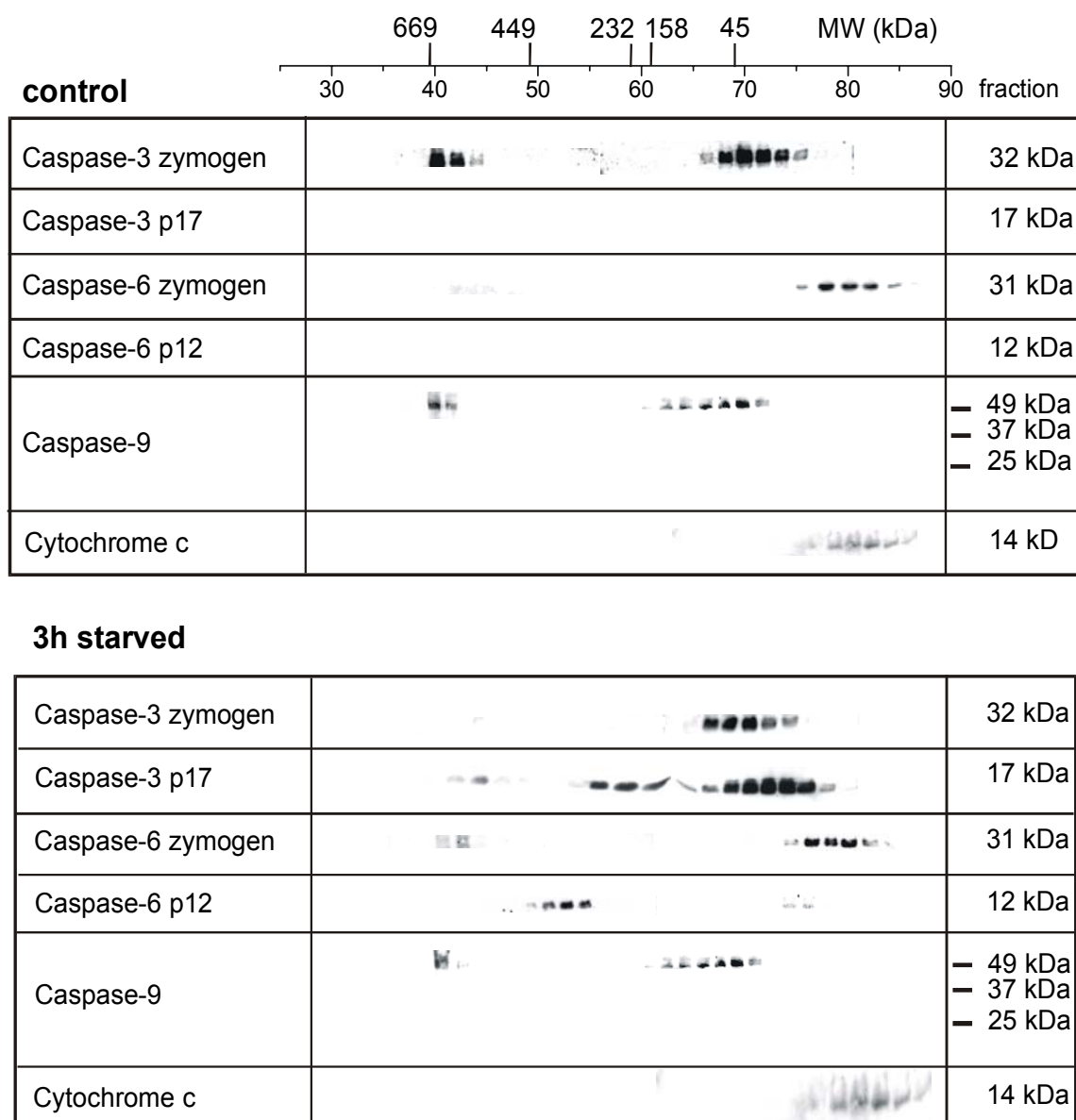
| | 669 | 449 | 232 | 158 | 45 | MW (kDa) | |

control

	30	40	50	60	70	80	90 fraction	
Caspase-3 zymogen								32 kDa
Caspase-3 p17								17 kDa
Caspase-6 zymogen								31 kDa
Caspase-6 p12								12 kDa
Caspase-9								49 kDa / 37 kDa / 25 kDa
Cytochrome c								14 kD

3h starved

Caspase-3 zymogen								32 kDa
Caspase-3 p17								17 kDa
Caspase-6 zymogen								31 kDa
Caspase-6 p12								12 kDa
Caspase-9								49 kDa / 37 kDa / 25 kDa
Cytochrome c								14 kDa

Fig. 4.21. Caspases are activated in complexes. Complex formation was examined by SDS-PAGE and Western blotting analysis after separation by Sephacryl S 300. Cytosolic extracts from non starved and 3h cells were used as control and apoptotic extracts, respectively.

4.3.2 Caspase-3 activating complexes devoid of Apaf-1, in response to serum stravation

Because the gel filtration column Sephacryl S-300 only poorly resolves complexes higher than 1000 kDa, another gel filtration column, Superose 6 HR (high-resolution) which can separate the complexes up to 4 MDa, was used to further characterize the high molecular weight complexes and to examine the oligomerization of Apaf-1 in to the high molecular weight complexes such as 700 kDa active and 1.4 MDa inactive apoptosomes [109-110].

Extracts from both control (non starved) and apoptotic (3h starved) cells were seperated. Fractions of the separations were analyzed initially for the caspases activity. Caspase activities were detected in the fractions corresponding to the very high molecular weight

regions from the separation of the extract from apoptotic cells. DEVDase activitiy appeared in a broad region starting from >2000 kDa to 700 kDa (Fig.4.23.) and it was not inhibitable by its specific peptide inhibitor in the very high molecular weight range > 1MDa (see Appendix 1. for the inhibition assay). VEIDase was also eluted in a broad peak from > 2000 kDa to 600 kDa. (Fig.4.23). Again the activity corresponding to the molecular weight of >2000 kDa was not inhibitable by the specific inhibitor VEID.cho (see Appendix 1. for the inhibition assay).
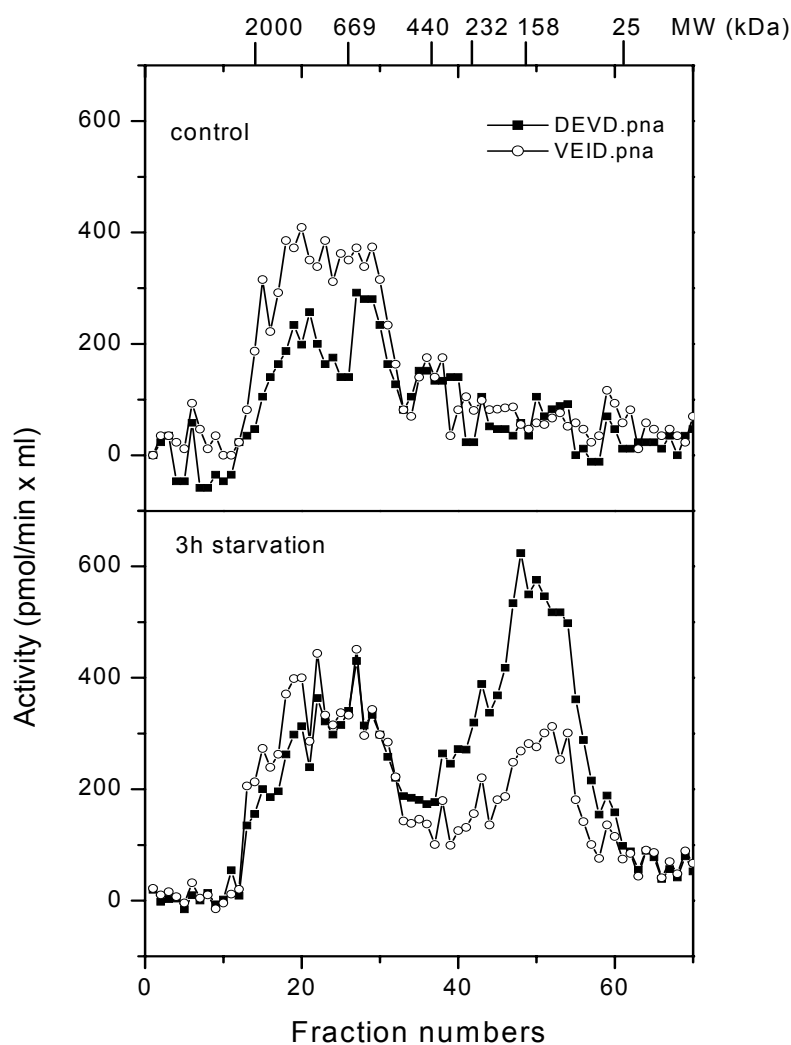
Fig.4.23. High molecular weight complexes were seperated by S 6 HR column. Cytosolic extracts from non starved and 3h starved cells were used as control and apoptotic, respectively.

Unexpectedly, the VEIDase, detected in the fractions corresponding to the region from 2000 kDa to 600 kDa was higher compared to the DEVDase activity. In addition, the VEIDase activity of the control was also found higher in the same region which is indicating possible unspecific protease activities in this region (Fig.4.23.). Because no specific IETDase activity was detected in the fractions of S-300 column, this substrate was excluded from the caspases

activity assays. Thus, activity assay data showed that both DEVDase and VEIDase activities which were previously detected at a molecular weight of ~700 kDa by Sepahacryl S-300 separation, are in a broad region from 2000 kDa to 700 kDa. Since these both activities were not inhibitable at a molecular weight of > 1MDa, most likely unspecific protease activities are present in this region.

Fractions of Superose 6 HR column were also analyzed by SDS-PAGE and Western blottings. In the fractions of the control, only caspase-3 zymogen was found in the very high molecular weights corresponding to ~ 2000 kDa. No active caspase-3 was detected in the fractions of the control and Apaf-1 was found entirely in those fractions corresponding to a molecular weight of ~200-300 kDa suggesting that it existed normally as a dimer. Following 3h starvation, active caspase-3 eluted in the complexes corresponding to the 600 kDa and 250 kDa and also as free enzyme as detected previously in the fractions of S-300 separation. Suprisingly Apaf-1 did not oligomerize into the high molecular weight complexes such as 700 kDa apoptosome and 1.4 MD inactive apoptosome, [109-110]. Apaf-1 eluted in its native state as detected in the fractions of control corresponding to ~200-300 kDa. The latter data suggested that, caspase-3 is activated in complexes devoid of Apaf-1.

Furthermore to analyze if the unspecific activities in the high molecular weight regions coelute with the proteosome as reported in literature [109], fractions were analyzed by Western blotting by an antibody which was directed against the α-subunit of 20 S-proteasome. In control cells this proteasome-subunit eluted mainly between 200-400kDa, most likely representing the precursor proteosome complexes, additionally to a lesser extent probably a single subunit was detected in the 25-100 kDa region. Some trace amounts were also detected in the very high molecular weight region ~2000 kDa. Following serum starvation the precursor proteasome complexes were still detectable, in addition a considerable increase was detected in the ~ 2000 kDa region most likely representing the catalytically active 26S-proteasome complexes. These results showed that unspecific activities in the region of ~2000 kDa are probably due to active proteasome complexes.
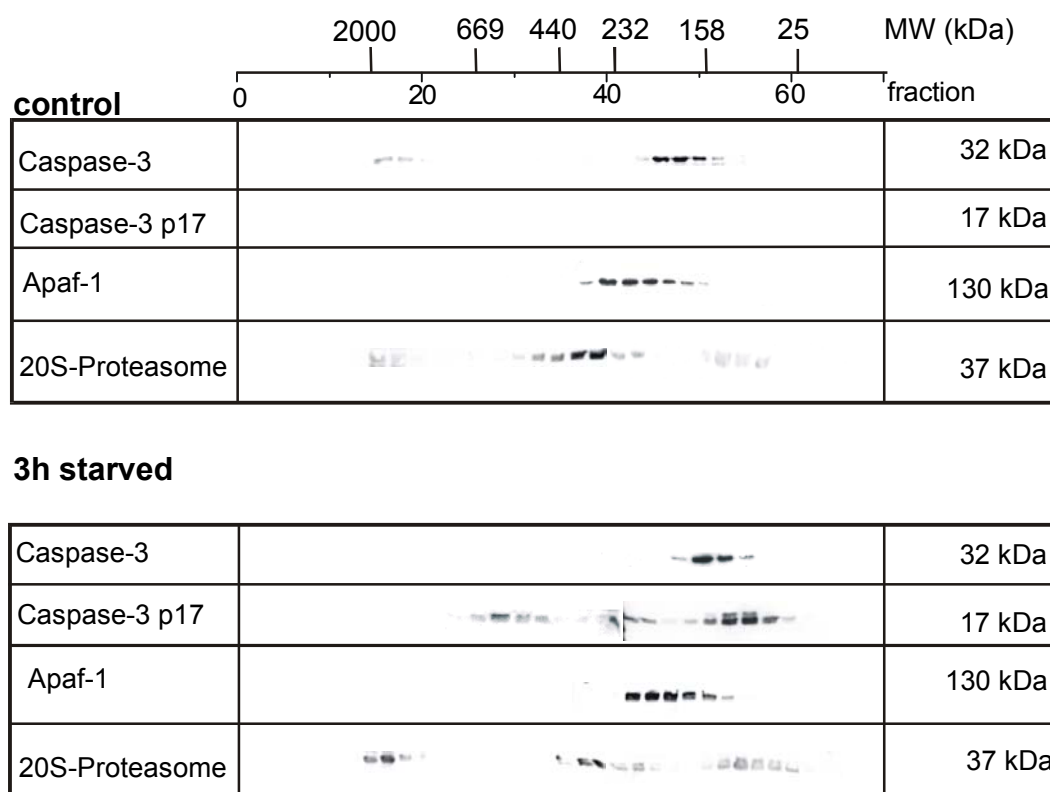
	2000 669 440 232 158 25	MW (kDa)
control	0 20 40 60	fraction
Caspase-3		32 kDa
Caspase-3 p17		17 kDa
Apaf-1		130 kDa
20S-Proteasome		37 kDa

3h starved

Caspase-3		32 kDa
Caspase-3 p17		17 kDa
Apaf-1		130 kDa
20S-Proteasome		37 kDa

Fig. 4.24. Caspase-3 is found to be activated in complexes devoid of Apaf-1. Extracts of control or apoptotic (3h starved) cells were separated by Superose 6 HR column. Fractions were analyzed by SDS-PAGE and Western blotting.

4.3.3 Analysis of involvement of complex formation in caspase activation during cell death of AKR-2B cells induced by Anisomycin.

Previous results have shown that Anisomycin effectively induce cell death in AKR-2B cells which is paralelled by activation of caspase-3. Cell death induced by Anisomycin exhibits similar characteristics like the death induced by serum starvation [141]. For example: as described in 4.2.1 Caspase-3 is activated as the main executioner caspase in both cases. Because, caspase-3 is found to be activated in complexes different than the classical apoptosomes in response to serum starvation, the process of activation of caspase-3 induced by Anisomycin was also examined. Gel filtration chromatography was again applied to examine the involvement of complex formation . Initially, a Sephacryl S 300 column was used for the separation of the both contol and apoptotic extracts. Apoptotic extracts were prepared from AKR-2B cells which were treated for 4h with 10μM Anisomycin. The results obtained by the analysis of the fractions of Sephacryl S-300 column were similar to serum starvation. Active caspase-3 is detected mainly as free enzyme and in a lesser extent in complexes at a molecular weight of 600 kDa and 250 kDa, in cell death induced by Anisomycin. (see Appendix 2-3 for the data of S-300 column). In contrast to serum starvation,

zymogen or active caspase-6 was found to be as free enzyme. Therefore it is excluded from the further analysis of the complexes.

To examine whether Apaf-1 and caspase-9 involved in the formation of the caspase-3 containing complexes, gel filtration chromatography was repeated by using a Superose 6 HR column. In the extracts of Anisomycin treated cells DEVDase activity was found in the fractions corresponding to the very high molecular weight ~2000 kDa. Inhibition assay showed that the DEVDase was not inhibitable in the region, corresponding to the >1MDa in contrast to the other fractions. Thus, activity assay data suggest that the activities which were found at the high molecular weights, are most likely due to unspecific protease activities.
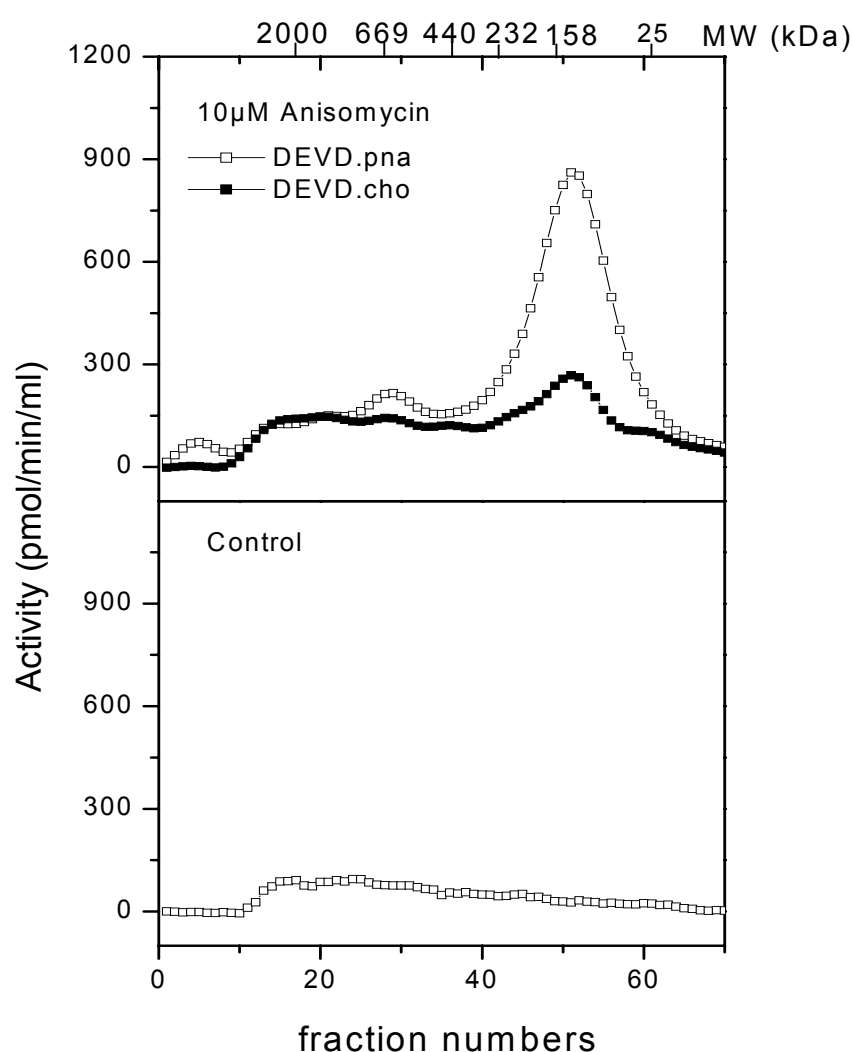
Fig.4.25. High molecular weight complexes were analysed by gel filtration chromatography. Cytosolic extracts from non treated or 4h Anisomycin treated cells were prepared and separated by S 6 H-R column.

Fractions were also analysed by SDS-PAGE and Western blotting. In the fractions of the control, the zymogens of caspase-3 eluted as free enzyme. Additionally it was detectable in

the high molecular weight region of >2000 kDa . (Fig. 4.26. upper panel). The active subunit of caspase-3 was not detectable in any fraction indicating that it was not processed in nontreated cells.

In the fractions from extracts of apoptotic cells, zymogen was still detectable as uncomplexed enzyme. The p17 subunit of activated caspase-3 was detectable mostly as free enzyme, in consistent with the DEVDase activity, and to a smaller extent in the 250 kDa and 600 kDa complexes, respectively (Fig. 4.26. lower panel). Caspase-9 was predominantly detected at ~100 kDa in the fractions from extracts of the both apoptotic and control cells. No subunit of active caspase-9 was detected (Fig. 4.26. lower panel). Importantly, Apaf-1 was found entirely in its native state most likely as mono-/dimers (Fig. 4.26. lower and uper panel). Thus these results revealed that Anisomycin treatment induce the formation of active caspase-3 containing complexes corresponding to 600 and 250 kDa similar to serum starvation. Importantly Anisomycin did not induce the formation of apoptosome complex as well as the serum starvation.
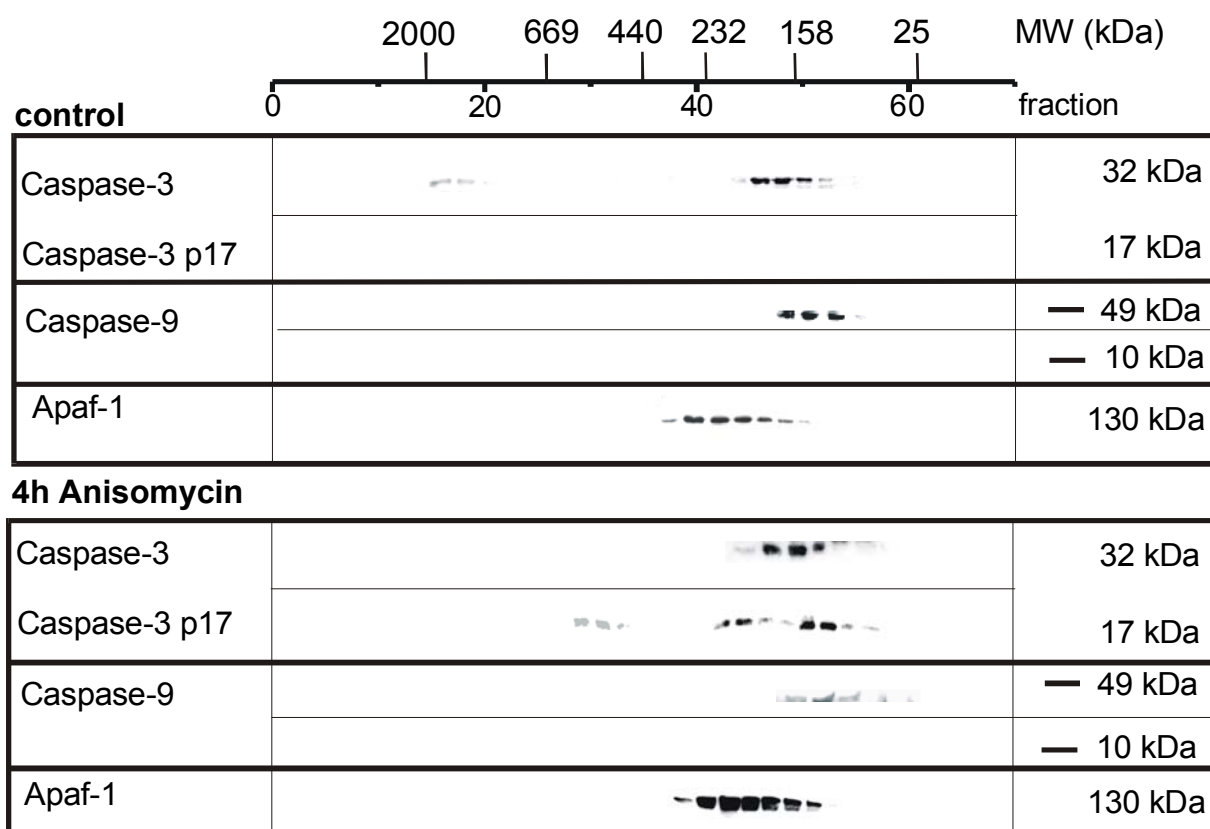
Fig. 4.26. Western blotting analysis showed that Caspase-3 is activated in complexes devoid of Apaf-1 in cell death induced by Anisomycin. (Superose 6 HR separation).

Taken together, remarkably all gel filtration experiments revealed that in AKR-2B cells caspase-3 is activated in noncanonical complexes devoid of Apaf-1 and caspase-9 during apoptosis induced by either serum starvation or Anisomycin treatment.

4.4 *In vitro* Reconstitution of Caspase Activating Complexes

4.4.1 Reconstitution of an Apaf-1 containing apoptosome complexes by the addition of cytochrome c, dATP and MgCl$_2$.

Apaf-1 did not coelute with caspase-9 and cytochrome c and did not oligomerize into the high molecular weight complexes in cell death of AKR-2B cells in vivo. Apoptosome complexes can be reconstitute *in vitro* by the addition of cytochrome c, dATP and MgCl$_2$. To compare the *in vivo* and *in vitro* situation the reconstitution of the complexes was studied. 200 μg of Cyt c, 2mM dATP and 5mM MgCl$_2$, added to the extract of nontreated AKR-2B and THP-1 cells. THP.1 cells (human monocytic) were used as a control in this assay, since these cells represent the Apaf-1 containing apoptosome complexes very well. Extracts of both cells were separated by a Superose 6 HR column as described before. Fractions were firstly analysed for the caspase-3 activity by the substrate DEVD.pNA (Fig. 4.27.).
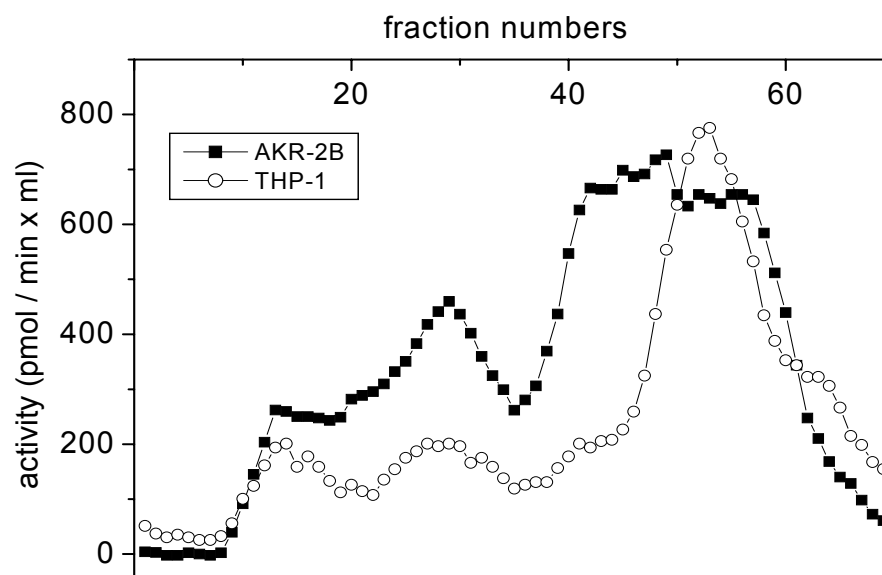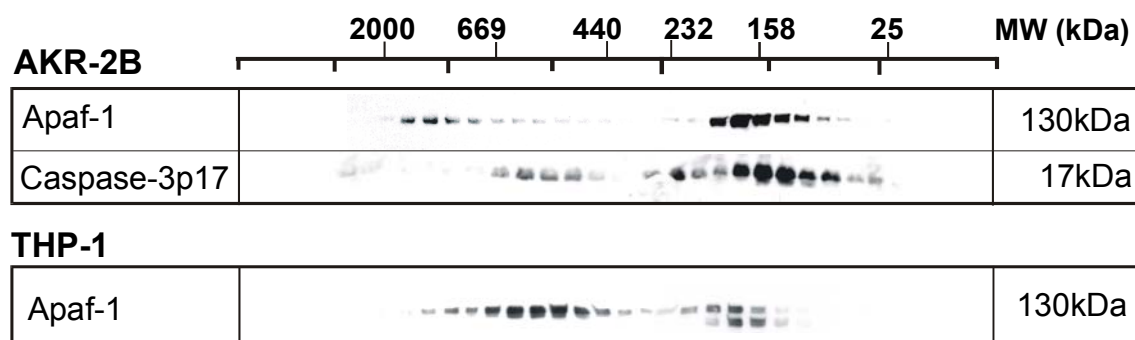
Fig.4.27. Reconstitution of Apaf-1 containing apoptosome complexes in vitro by addition of cyt c, dATP and MgCl₂. Upper panel shows DEVDase activity of both cells AKR-2B and THP.1. Lower panel shows western blotting of Apaf-1 protein for both cells.

Activity assay showed that in THP-1 cells DEVDase eluted mainly as a sharp single peak in the free enzyme region, whereas in AKR-2B fibroblasts the major part of the activity eluted in the fractions corresponding to the complexes at 600kDa and 250 kDa.

The distrubution of active caspase-3 and Apaf-1 into the complexes were analyzed by SDS-PAGE and Western blotting . In contrast to the *in vivo* situation (serum starvation), in AKR-2B cells *in vitro* experiment resulted in the distribution of a considerable fraction of Apaf-1 in to the 1.4 MD complex, though the major part still eluted in the mono-/dimer region. In THP.1 cells most of Apaf-1 was detected in the 700 kDa -apoptosome to a lesser content in the mono-/dimer region and in the 1.4 MD inactive apoptosome complex (Fig. 4.2.3.1 lower pannel). Thus, these results confirmed the previous gel filtration datas and showed that caspase-3 is activated in complexes which seem to be composed differently from the known described apoptosome complexes.

4.4.2 *In vitro* reconstitution of the complexes by the addition of recombinant caspase-3

To examine the role of active caspase-3 in the complex formation an *in vitro* experiment was decided. For this, 10 mg cytosolic extracts was prepared from non treated AKR-2B cells and incubated for 10 min with 1μg recombinant active and inhibited mcaspase-3, respectively. The amount 1μg was estimated as the approximate corresponding concentration of the endogenous caspase-3 under physiological conditions. In parallel experiment recombinant active caspase-3 was inhibited by specific inhibitor DEVD.cmk (100μM). The extract to which inhibited recombinant caspase-3 was added, was used as a control in the experiments. To examine the complexes a S 6 HR coumn was used for the seperations. Fractions were analysed for caspase-3 activity by the substrate DEVD.pNA. Compared to the control when the extracts to which recombinant active caspase-3 was added, was separated, DEVDase activity was detected in the fractions corresponding to the molecular weights of ~600 kDa, 250 kDa and 60 kDa (Fig.4.28). In contrast when extracts to which the inhibited recombinant

caspase-3 was added, was separated, a weak DEVDase activity was detected in the high molecular weight region corresponding to >1MDa (Fig.4.28). As described before in part 4.3.2. and 4.3.3. the activities which were detected in this region are considered as unspecific protease activity. No activity was detected in the other fractions.
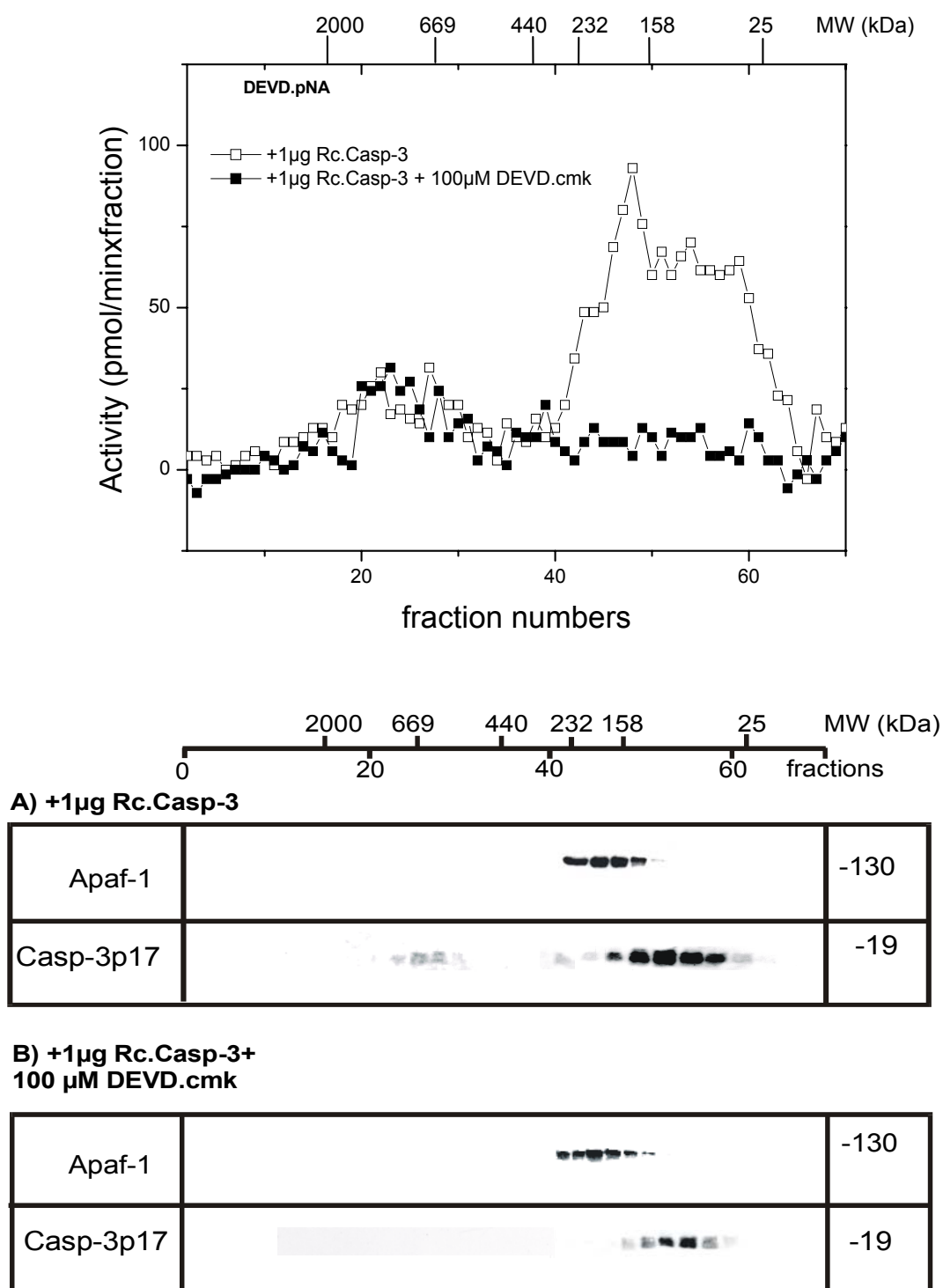

Fig.4.28. Recombinant active and inhibited caspase-3 was added to the extracts of non treated AKR-2B cells and separated by S 6 H-R column. Fractions were analysed by substrate DEVD.pNA for caspase-3 activity

and by Western blotting for Apaf-1 and active caspase-3. Inhibited recombinant caspase-3 added extract was analysed as a control.

The fractions from the separation of the both extracts were also analysed by SDS-PAGE and Western blotting to monitor the active caspase-3 in the complexes and to examine whether Apaf-1 distributed in to these complexes. As clearly seen from the Fig. 4.28. lower panel, compared to the control, from the extracts to which recombinant active caspase-3 was added, the p17 subunit of active caspase-3 was detected in consistently with the DEVDase activity, mainly as uncomplexed form and in the complexes of 600 kDa and 250 kDa (Fig. 4.28 lower panel). In contrast in the control, it was detectable at a molecular weight of 60 kDa, most likely it represents the inhibited recombinant caspase-3 which was added to the extract (Fig. 4.28 lower panel). In addition, Apaf-1 was again detected in its native state corresponding to ~200 kDa most likely as a dimer from the fractions of the both either active or inhibited caspase-3 added extracts. Thus these results suggest that most likely active caspase-3 is required for the complex formation.

4.5 Localization of Active and Procaspase-3 During Apoptosis in AKR-2B Fibroblasts

4.5.1 Active caspase-3 is localized in the membrane blebbs

Cell death of AKR-2B fibroblasts induced by either serum starvation or anisomycin, exhibits similar characteristics of apoptosis i.e. morphological changes such as membrane blebbing, nuclear condensation, cell shrinkage, but lack energy dissipation in mitochondria and intranucleosomal fragmentation [141]. So far, caspase-3 has been found to be responsible for most of the morphological changes and intranucleosomal fragmentation in apoptosis. Although caspase-3 was found as the main executioner active caspase in apoptosis, intranuclesomal fragmentation was not detected in AKR-2B cells. To find any information about the unusual behaviour of AKR-2B cells it was important to analyse the localization of active caspase-3 during apoptosis. Localization study were done by immunofluorescence and microscopy. AKR-2B cells were grown on coverslips to subconfluency and were then starved by serum deprivation and treated with 10μM Anisomycin for 4 hours. Immunofluorescence staining was performed by using the antibody which recognizes the p17 subunit of active Caspase-3 as the 1st antibody and labeled with a rabbit Cy3 as the secondary antibody. After staining, coverslips were mounted on cut edges and used for microscopy. Filter N. 1 was used to monitor fluorescence staining and photographs were recorded using a cooled digital camera. As illustrated in Fig. 4.29. A-B (superimposed fluorescence and phase-contrast pictures), active

caspase-3 was particularily detected in the membrane blebbs and also partially in the cytoplasm during apoptosis.
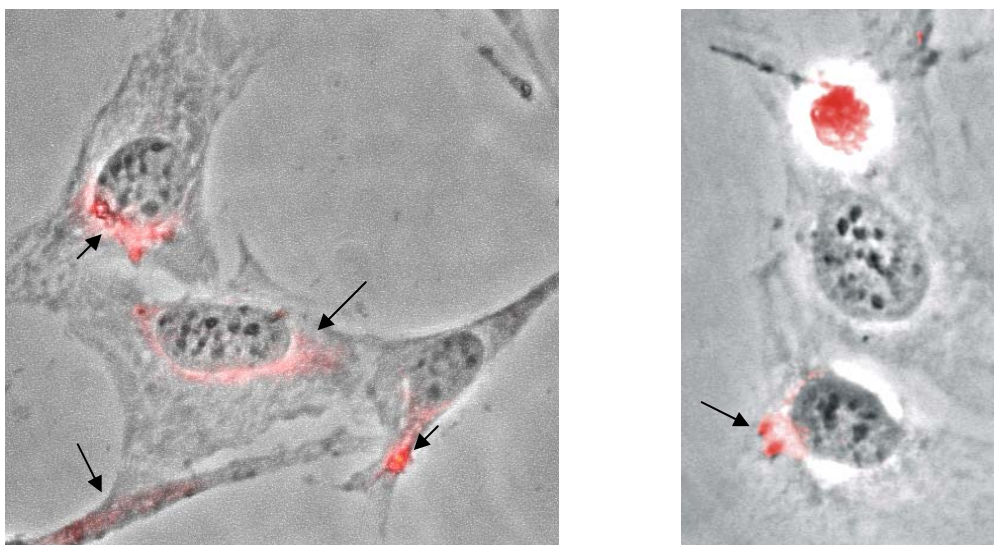

Fig. 4.29. Immunofluorescence staining of active caspase-3 during apoptosis. Fluorescence pictures were superimposed with phase contrast pictures. Arrows indicate the particular localizations of caspase-3.

4.5.2 Procaspase-3 is localized in the cytoplasm in AKR-2B cells

In order to examine the localization of procaspase-3, the caspase-3 gene was cloned into an GFP (Green Fluorescent Protein) expression vector pQBI25 under the control of CMV promotor. It is known that the overexpression of caspase-3 protein leads to its auto activation in cells, therefore to prevent the autoactivation the active site pentapeptide **QACRG** was changed to **QAARG** by introducing a **C/A** mutation by site directed mutagenesis. For this a two step PCR was employed by using two mutagenesis and two external primers (Fig.4.30.). In the first step, the previously engineered pET21b+caspase-3 construct was used as a template and the mutation was introduced by the internal mutagenesis primers. The resulting two products were used as a template in the 2nd PCR. During this reaction with the help of the external primers the full length caspase-3 gene was synthesized as having the desired mutation. The amplified full length caspase-3 gene was inserted into the BamHI and EcoRI sites of the plasmid pQBI25 in frame to generate an expression construct at the amino terminus of GFP gene. The Caspase-3–GFP fusion construct was then used for transformation of the competent E.coli (Nova Blue) cells for multiplication. Bacterial colonies were probed by PCR by using an internal and an external primer of the insert. Plasmids were isolated from positive clones. Sequencing data were analysed by using either GeneRunner or Bioedit Multiple alignment programs. Sequence analysis confirmed the correct integration of the insert and the presence of the desired mutation. No further mutation was detected. The plasmid construct was then used for the transfection of the cultured AKR-2B cells.
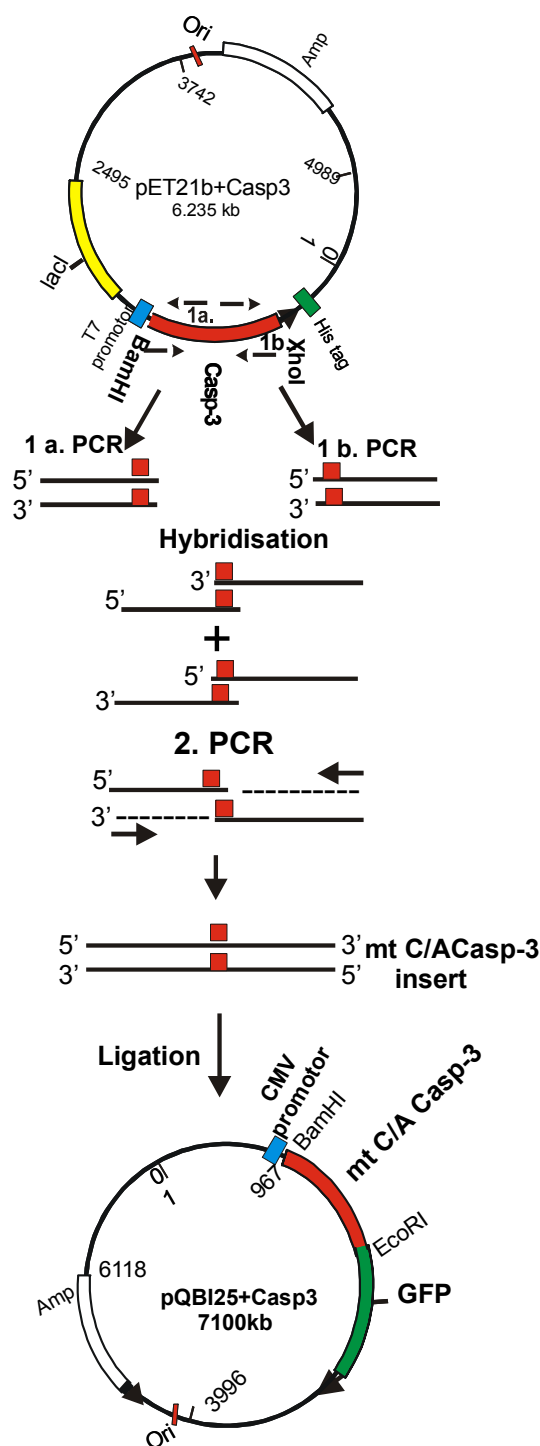
Fig.4. 30. Caspase-3 gene cloned into the GFP expression vector pQBI25 by using the two step PCR strategy.

Transfection was performed as described in methods part by electroporation. Transfection efficieny was examined by FACS analysis by Prof.Hoppe and determined as 20%. For the microscopy, transfected cells were seeded on coverslips. 24 h posttransfection the coverslips were mounted on cut edges and examined by the fluorescence microscopy. Photographs were recorded using a cooled digital camera
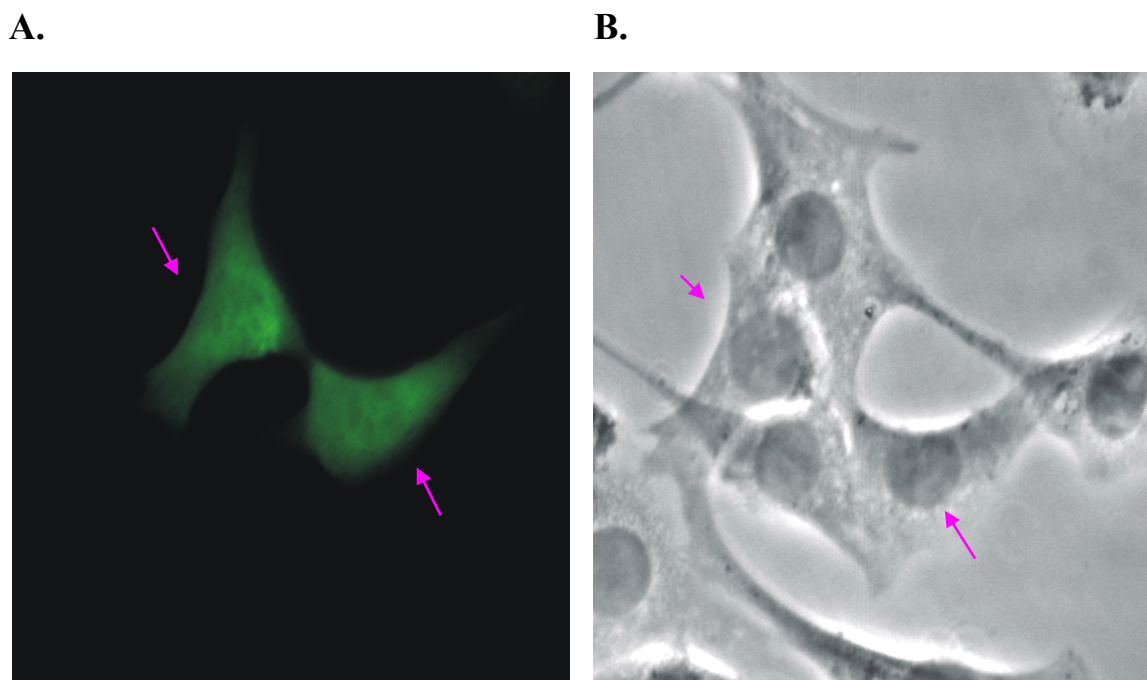
A.   **B.**

Fig.4.30. Localization of procaspase-3 was examined by the overexpression of a Caspase-3-GFP fusion construct in AKR-2B cells. Procaspase-3 was found mostly in the cytoplasm. Arrows indicate the identical cells visulaized either **A.)** by fluorescence or **B.)** by phase contrast microscopy.

As illustrated in figure 4.31 procaspase-3 was detected mainly in the cytoplasm of AKR-2B cells transfected with caspase-3-GFP fusion construct. Diffuse distrubution or in some cases nuclear localization of GFP was observed in cells transfected with the control plasmid (GFP expressing only). Therefore the partially nuclear localization was assigned to GFP. Thus, these results suggest that most likely procaspase-3 is started to be activated in the membrane blebs during apoptosis, probably to be involved in the initial apoptotic events which might happen exclusively in these parts.

4.6 Examination of The Activation of Caspase-12 During Apoptosis in AKR-2B Fibroblasts

Evidently, the mitochondria mediated caspase cascade which requires Apaf-1, caspase-9 and Cytochrome c complex formation, so called apoptosome is not involved in the cell death of AKR-2B cells. Recent studies have suggested the existence of a novel apoptotic pathway in which caspase-12 functions as the initiator caspase in response to a toxic insult to the ER [116,118,121]. Since the receptor mediated pathway has already been excluded in a previous study, activation of caspase-12 and the possible role of caspase-12 in the activation of the caspase cascade in AKR-2B cells was examined.

4.6.1 Caspase-12 is activated in response to serum deprivation

Initially the kinetic of the activation of caspase-12 was studied in response to serum starvation. Cytosolic extracts were prepared from serum starved AKR-2B fibroblasts at different times (0- 6 h) and analysed by Western blotting using an antibody which recognizes the zymogen (49 kDa) and the large (35 kDa) subunits of the active caspase-12. As shown in Fig. 4.30. starting from 1st hour (with a molecular weight of 47 kDa and 35 kDa) cleavage products were detected (reached a maximum between 2.5 to 4 h and decreased after 5 hours). It is believed that the specie corresponding tho the molecular weight of 37 kDa is a splice variant of caspase-12 which was also seen in the control. Furthermore the kinetic of the activation of caspase-3 was also examined by an antibody which was directed against the p17 subunit of the active caspase-3. Remarkably, the kinetics showed that caspase-3 and caspase-12 were activated at the same time as moniotored by the appearance of the p17 subunit of the active caspase-3 (Fig.4.32.)
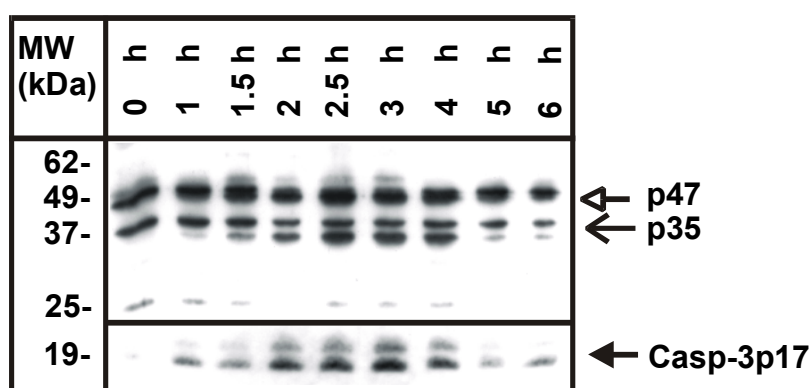

Fig. 4.32. Caspase -12 and Caspase -3 are activated at the same time points in response to serum deprivation of AKR-2B fibroblasts. Arrows indicate the appearance of 47 kDa, 35kDa cleavage products of caspase-12 and p17subunit of active caspase-3.

4.6.2 Caspase-12 is activated by Anisomycin treatment

Additionally, the kinetics of the activation of caspase-12 was studied in response to Anisomycin treatment. Cytosolic extracts from Anisomycin treated cells were prepared and analysed by SDS-PAGE and Western blotting. Caspase-12 was found to be activated in response to Anisomycin treatment, as well as serum starvation. A maximum of activation was detected around 4 –5 hours. Importantly, compared to the starvation, the 47 and 35kDa cleavage products appeared after 2h with a delay of 1h. In order to compare with the activation of caspase-12, kinetics of the activation of caspase-3 was also examined in response to anisomycin treatment. As shown in Fig. 4.33., interestingly the putative cleavage

products of caspase-12 (47 kDa and 35 kDa) appeared at the same time with the p17 subunit of the active caspase-3.
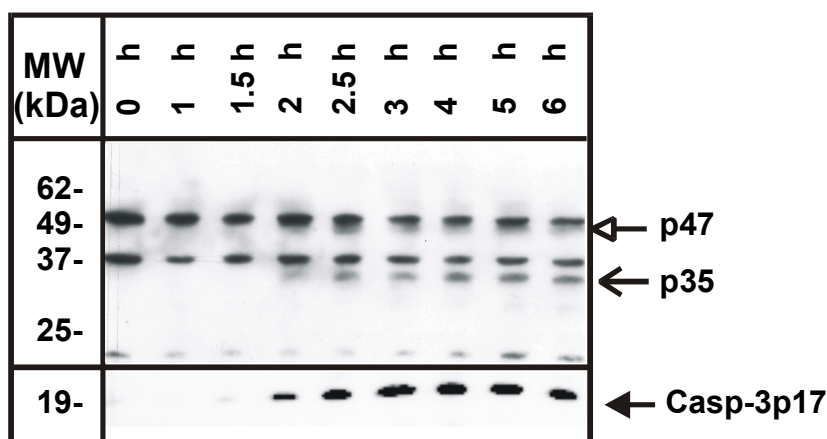

Fig. 4.33. In response to anisomycin treatment, Caspase -12 and Caspase -3 are activated at the same time points in AKR-2B fibroblasts. An arrow ($\rightarrow$) indicates the appeareance of a 35 kDa cleavage product of caspase-12 from the 2nd hour.

Thus, these results revealed that serum starvation or Anisomycin treatment, respectively induce the activation of caspase-12 resulting in the appearance of a cleavage products of around 47 kDa and 35 kDa molecular weight. Furthermore a strong time correlation between the activation of caspases-3 and −12 was also found in the both serum starvation or anisomycin induced cell death of AKR-2B fibroblasts.

4.6.3 Active Caspase-12 is released as uncomplexed free enzyme during apoptosis

In order to examine whether caspase-12 is activated in the same complexes with caspase-3 gel filtration chromatography was employed. S-6 HR column was used to separate the extracts from non treated/non starved (control) or 3h starved (apoptotic) or 4h Anisomycin (apoptotic) treated AKR-2B cells. Since the substrate specificity of caspase-12 has not been determined yet, the fractions from the separations were analysed only by SDS-PAGE and Western blotting. In the control extracts of non treated/nonstarved cells the zymogen of caspase-12 with a molecular weight of 49 kDa was detected at the very high molecular weight region corresponding to >2000 kDa (Fig. 4.34.). In contrast, in response to serum starvation or Anisomycin treatment caspase-12 is completely cleaved into distinct fragments as double bands of around 35 kDa and 25 kDa and released as free enzyme. After 3h starvation or 4h Anisomycin treatment unprocessed caspase-12 was not detectable anymore. The very faint bands which were detected from the control fractions indicate that caspase-12 is activated at very low levels in the control (nonstarved) cells, eluting from the column as uncomplexed

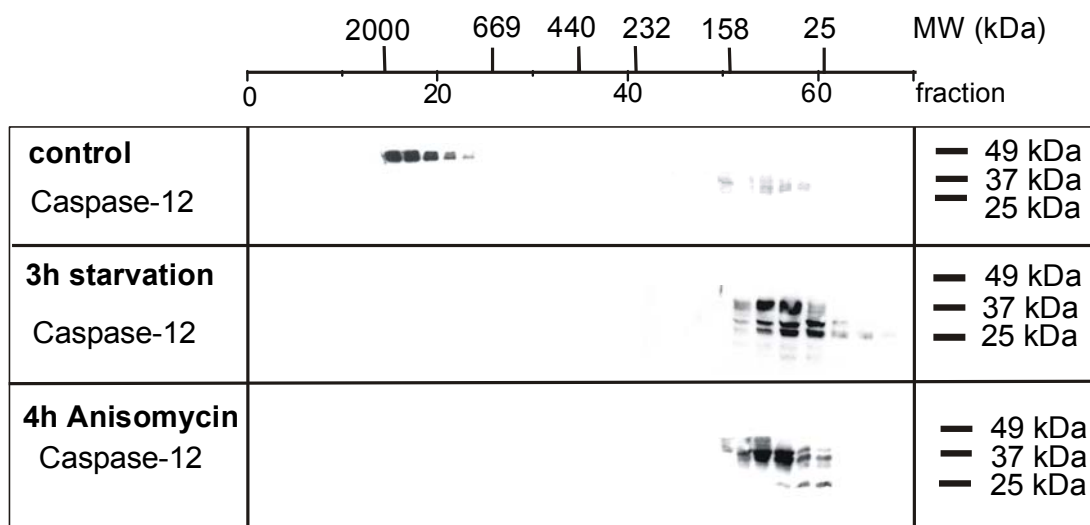enzyme (Fig 4.34.).Thus, these results showed that caspase-12 is activated and released as uncomplexed enzyme.

Fig. 4.34. Caspase-12 is activated and totally released as free enzyme in response to serum starvation.

4.6.4. The role of ER stress in the activation of caspase-12

Previous results have been showed that the ion-carrier A23187 (Ca^{+2} ionophore) that forms stable complexes with divalent cations and Thapsigargin, an inhibitor of the intracellular Ca^{+2}-ATP-transporters are both causing a rapid elevation of $[Ca^{2+}]$ in AKR-2B fibroblasts. By A23187 and Thapsigargin treatment of AKR-2B cells results in time-dependent cell loss, starting after 16 hours with 70% surviving cells for A23187 and 50% for Thapsigargin after 24h. The dose dependent induction of apoptosis by A23187 and Thapsigargin, showed that 2.5μM and 0.5μM are maximal effective concentrations for 24h treatment, for A2318724 and Thapsigargin, respectively (Hoppe et al.). ED_{50} values of A23287 and Thapsigargin have found as 0.5μM and 0.02 μM, respectively. In the current study the contribution of ER-stress to apoptosis in AKR-2B fibroblasts and caspase-12 cleavage was also examined. Thus, the influences of A23187 and Thapsigargin (well established ER-stressors) on ER, were analyzed. Since ER-stress increases the expreesion levels of chaperone Grp78 protein therefore firstly extracts from AKR-2B fibroblasts treated with 2.5 μM A23187 or 0.5 μM Thapsigargin were analysed by SDS-PAGE and Western blotting for the ER-specific chaperone Grp78. As shown in Fig. 4.33. Grp78 protein is elevated time-dependently and released to the cytosol with maximal amounts after 16 and 24 hours treatment in consistent with the time points of apoptotic events in response to A23187 and Thapsigargin treatment (Fig.4.35.).
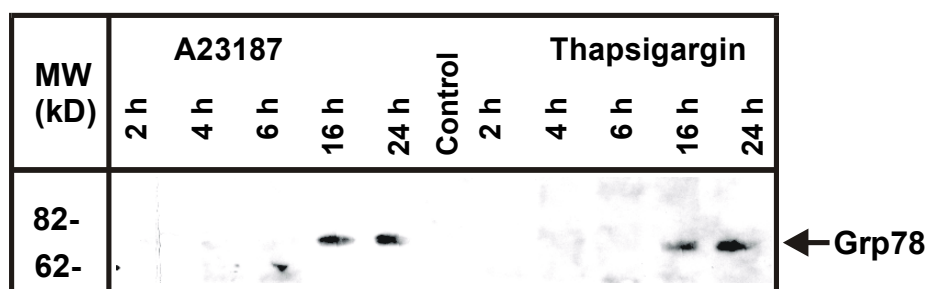
Fig.4.35. ER-specific chaperone Grp78 protein is elevated time-dependently and released to the cytosol in A23187 and Thapsigargin treated AKR-2B cells.

Secondly, in order to examine the effect of A23187 and Thapsigargin treatment in activation of caspases -12 and 3 the same extracts were analysed by SDS-PAGE and Western blotting using the antibodies of the respective caspases. Additionally the elevation of the expression level of Grp78 were also monitored by Western blotting in the total cell lysates from AKR-2B cells treated with A23187 and Thapsigargin. As shown in Fig. 4.36. caspase-12 started to degrade after 16h treatments and was almost not detectable at 24 hours.

At the same time points (16 and 24 hours) very faint bands were also detected for the p17 subunit of active caspase-3 which indicate the activation of caspase-3 at very low levels.

Thus, in response to A23187 or Thapsigargin treatment, the ER appears to be stressed and damaged since Grp78 is detected in the cytosol. Importantly, caspase-12 is degraded unspecifically and but not processed since no cleavage products of 47 kDa or 35 kDa were detectable.
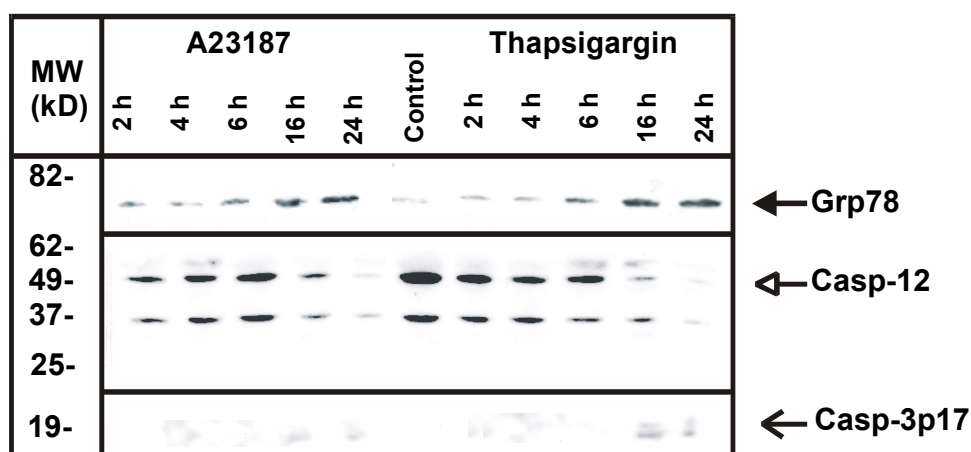

Fig. 4.36. In response to A23187 and Thapsigargin treatment, Grp 78 protein is elevated, Caspase-12 is totally degraded and caspase-3 is activated at very low levels.

Finally the expression levels of Grp78 protein in response to serum starvation or anisomycin treatment were also examined. Total cell lysates of serum starved or Anisomycin treated AKR-2B fibroblasts were analysed by SDS-PAGE and Western blotting (Fig.4.37).
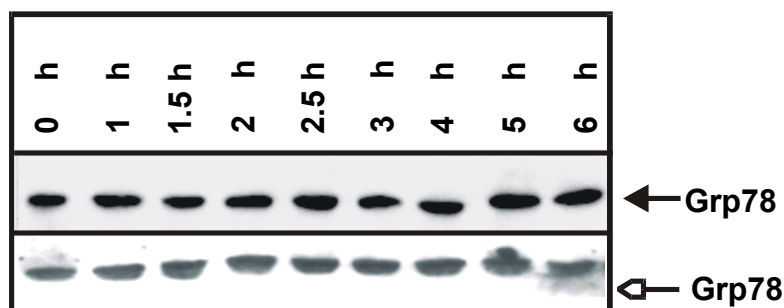

Fig. 4.37. Grp78 protein levels was examined by WB, in the total lysates of either anisomycin (open arrow) treated or serum starved (closed arrow) AKR-2B fibroblasts.

As shown in Fig. 4.37. during 6 hours no elevation in the expression pattern of Grp78 protein was detected either after serum starvation or anisomycin treatment suggesting that Anisomycin or serum starvation do not induce ER stress, and probably caspase-12 is activated independentlyr from ER stress in AKR-2B fibroblasts.

4.6.5 Activation of caspase-12 by recombinant mcaspase-3 or Cytc /dATP

The analysis of the protein expression level of Grp78 suggests that Anisomycin and serum starvation do not induce ER stress. Thus, ER stress is not involved in caspase-12 activation in AKR-2B cells. One possibility is that caspase-3 might cleave and activate caspase-12. To examine this an in vitro experiment was designed. Cytosolic extracts from non treated AKR-2B fibroblasts were incubated with 1μg recombinant mCaspase-3 and subjected to SDS-PAGE and Western blotting (caspase-12 antibody). It is known that *in vitro* cytochrome c and dATP addition can induce caspase-12 activation via caspase-9 [122], therefore the extract of non treated AKR-2B cells was separately incubated with cytochrome c and dATP as well. As a control, extracts of non treated and 3h starved cells were also analysed side by side with the recombinant caspase-3 or cytochrom c/dATP added extracts. As shown in Fig. 4.38, Western blotting analysis clearly showed that by addition of 1μg recombinant caspase-3, cleavage products of caspase-12 corresponding to 47kDa and 35 kDa appeared which were also detectable after Cytochromc +dATP addition or 3h starvation. These results showed that *in*

vitro, recombinant caspase-3 can cleave caspase-12 generating the identical cleavage products as *in vivo* (3h starvation) (Fig.4.38).
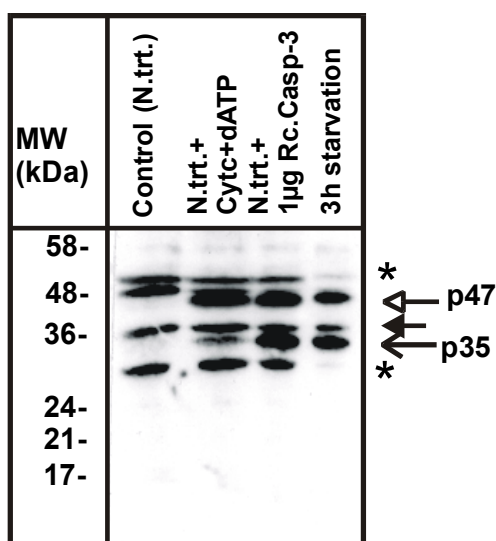

Fig.4.38. 47kDa and 35 kDa cleavage products of caspase-12 were detected by WB, either in Cytc+dATP treated or Rc. Casp-3 added extracts. Extracts from 3h starved and non treated cells were also analysed as control. Closed arrow indicate the possible splice variant of caspase-12. The star indicates the nonspecific bands.

Thus, ER stress induced pathway is not involved in cell death of AKR-2B cells. But, caspase-12 is activated as free enzyme. Unexpectedly, caspase-3 probably plays a direct or indirect role in activation of caspase-12 in AKR-2B cells, whereas the vice versa is unlikely.