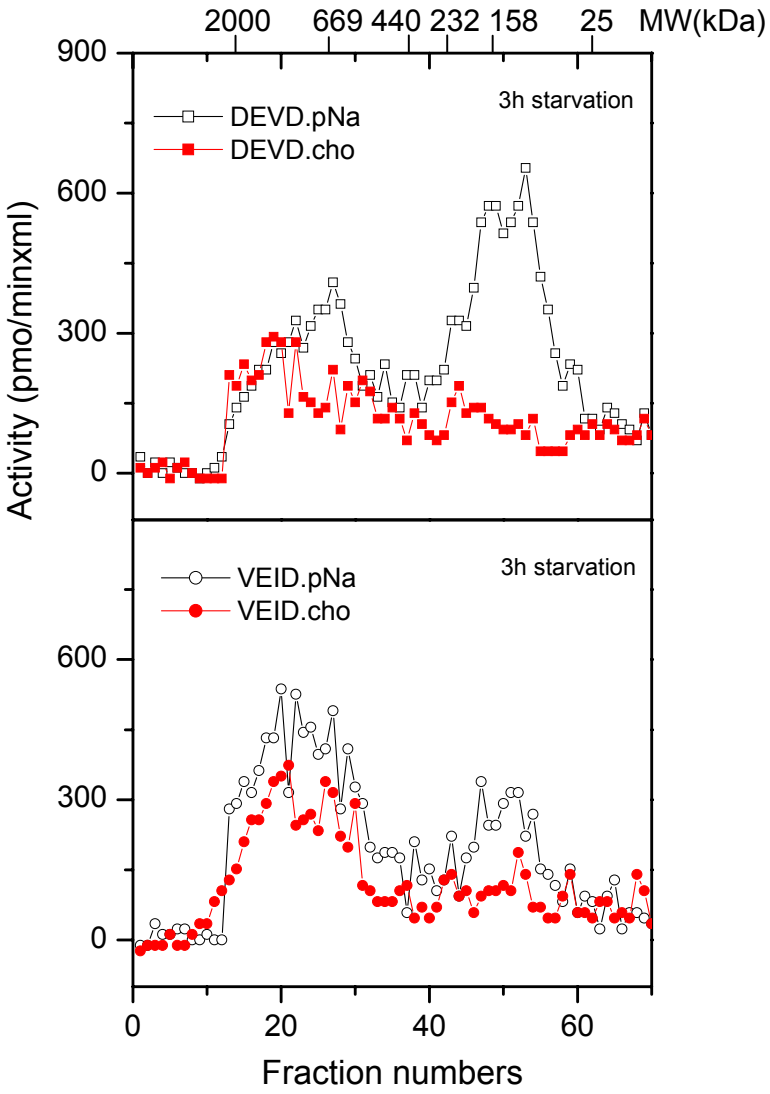
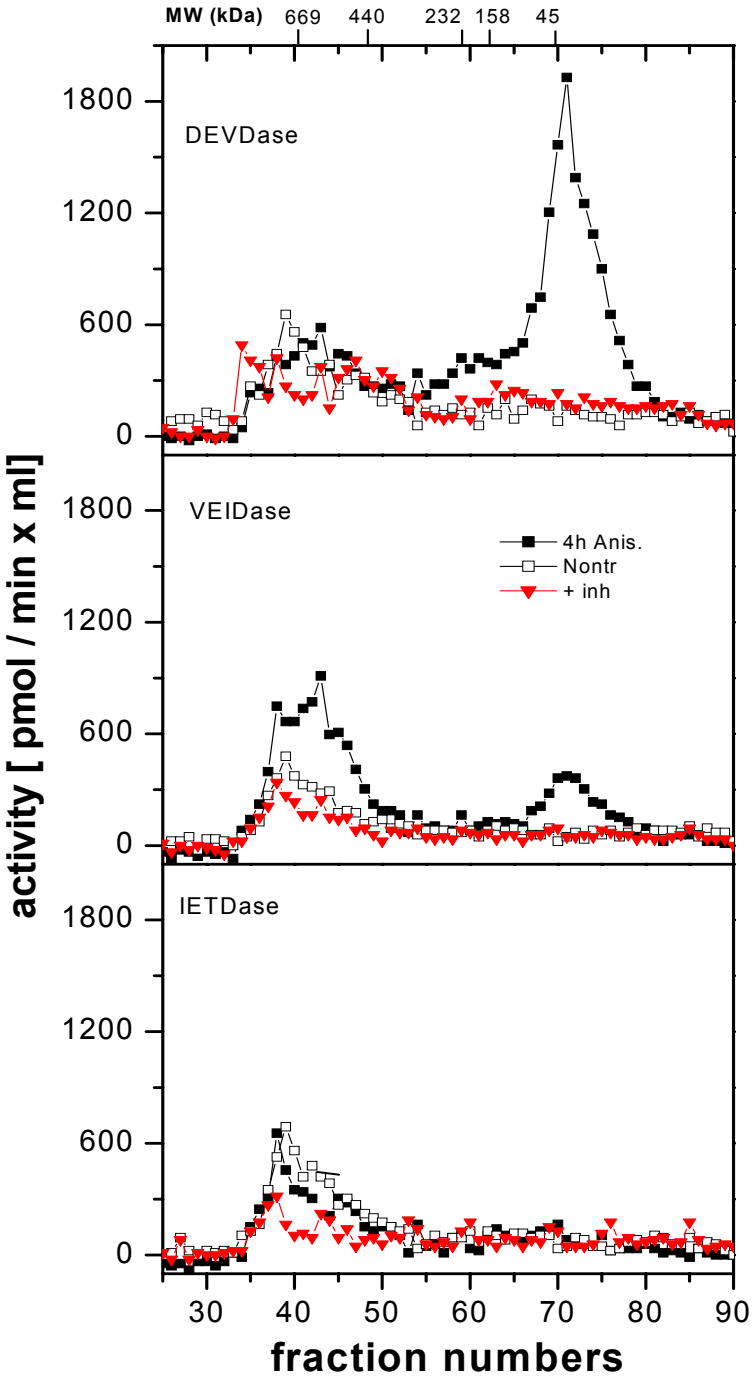
8. Appendices

Appendix 1 Caspases inhibition assay after S-6 separation of the extracts of 3h starved AKR-2B cells



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 (VEIDase was also eluted in a broad peak from > 2000 kDa to 600 kDa. Again the activity corresponding to the molecular weight of >2000 kDa was not inhibitable by the specific inhibitor VEID.cho.

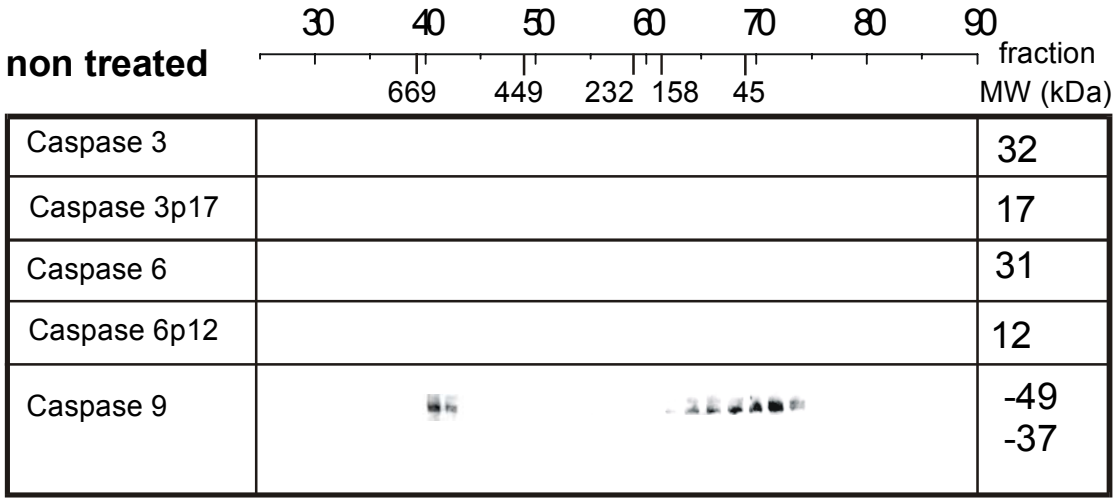
Appendix 2 Caspases activity and inhibition assay after S-300 separation of the extracts of Anisomycin treated AKR-2B cells.



Compared to control when the extract of Anisomycin treated AKR-2B fibroblasts was separated by Sephacryl S-300 column, caspase activities mainly eluted in two peaks corresponding to ~700 kD, and between 250 kD and 60 kD similar to serum starvation. Inhibition assay showed that specific DEVDase eluted mainly as uncomplexed enzyme around 60kD and in a lesser extent complexed at >700 kD, and 250 kD, respectively. The high molecular activity of >700 kD was only partially inhibitable in this region, by the inhibitor DEVD.cho, indicating mostly unspecific

activity was present. In contrast DEVD.cho inhibited the activities in the other regions. Specific VEIDase as confirmed by inhibition assay, associated mainly with the 700 kD apoptosome and with lower activities as uncomplexed enzyme in contrast to DEVDase. By Superose 6 HR seperation, VEIDase activity corresponding to 700 kD regionInhibition assay showed that, VEIDase was not inhibitable in this region, as well. Although IETDase which was detected at 700 kD apoptosome, was partially inhibitable in this region, no IETDase was detectable, in the very high molecular weight region .

Appendix 3 Western blotting analysis of the fractions from S-300 separation of the extracts from non treated and Anisomycin treated AKR-2B cells.



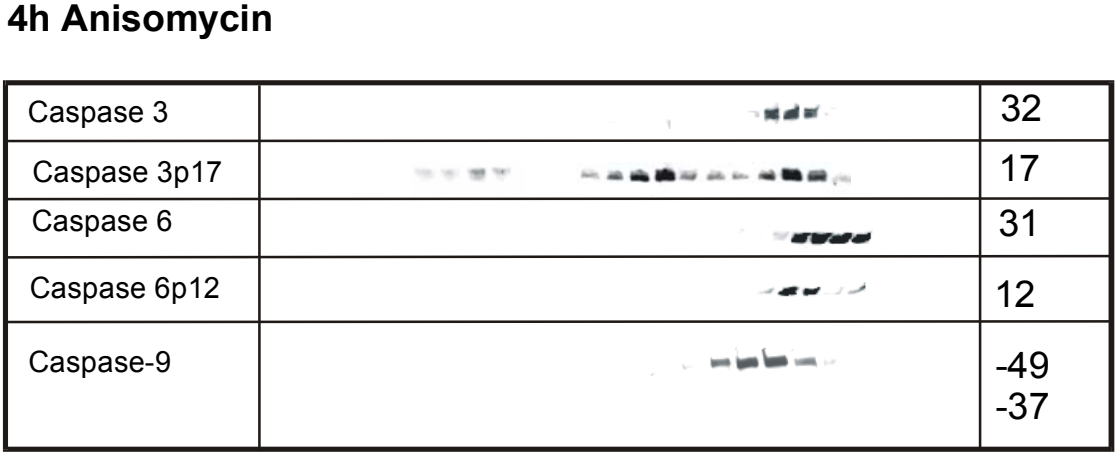


Fig.4.3.5. Analysis of complex formation by Western blotting. Extracts from either Anisomycin or non treated cells were seperated by S-300 sephacryl coloumn

To further characterization of the complexes especially to find out the molecular speciation/composition of the complexes, fractions of Sephacryl S-300 coloumn fractions were analyzed by SDS-PAGE and Western blottings. From the control (non treated cells),

same results were obtained by western blotting as detected from non starved extracts previously. From extracts of Anisomycin treated cells, the zymogenes of caspases were still detectable in the free enzyme region. The p17 subunit of activated caspase-3 was mainly detected as uncomplexed enzyme which was inline with the above DEVDase activity data and in a lesser extent in the 250 kDa microapoptosome and 600 kDa apoptosome, respectively. Active caspase-6p12 was detected only as uncomplexed free enzymeCaspase-9 was again detected only as a zymogene as in the control (non treated). Activation of caspase-12 was also examined by Western blotting. From control extracts, the zymogene of caspase-12 (49kDa) was detected at very high molecular weight region and totaly released as free enzyme and cleaved in to the fragments (37-25 kDa) by Anisomycin treatment, as well Thus these results showed that in response to Anisomycin treatment, caspases were activated in a very similar manner to starvation.