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Recombinant Caspase-3 Expressed in *Pichia pastoris* Is Fully Activated and Kinetically Indistinguishable from the Native Enzyme

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Intracellular cysteine proteinases (caspases) play key roles in inflammation and apoptosis. Recombinant caspases are typically produced in *Escherichia coli* expression systems with the attendant problems of solubilization, re-folding and activation of the protease. Here we describe the expression of hexahistidine-tagged caspase-3 (CPP32/Yama/Apopain) in the methylotropic yeast *Pichia pastoris*, and the purification of soluble enzyme from yeast lysates using cobalt affinity chromatography. The recombinant protease is fully activated, stable, and cleaves the synthetic substrate DEVD-AFC (Km 16.8 μM) but not YVAD-AFC. It mediates the cleavage of the apoptotic death substrate poly(ADP-ribose) polymerase in cell extracts, but does not cleave pro-interleukin-1β. It is inhibited by the peptide DEVD-CHO (K_i 2.2 nM), far less efficiently by YVAD-CMK (K_i 0.3 μM), and not detectably by CrmA. By these criteria, recombinant caspase-3 is indistinguishable from native caspase-3 purified from apoptotic cell extracts. Activation of recombinant caspase-3 occurs in yeast in the absence of any intrinsic caspase activity, suggesting that caspase-3 can auto-activate. However, the purified enzyme was incapable of cleaving pro-caspase-3 indicating that autoactivation of caspase-3 *in vivo* is not likely to occur unless very high concentrations are achieved. © 1997 Academic Press

Apopotosis is crucial to the development and homeostasis of higher eukaryotes. This process is specific and consists of a death signal that activates intracellular effector machinery leading to the distinctive program of cell death that comprises membrane blebbing, cytoplasmic condensation and nuclear distintegration (for review see (1)). Genetic analyses of apoptosis occurring during the development of the nematode *Caenorhabditis elegans* first identified the cell death gene *ced-3* as a component of the effector machinery, and subsequent studies have shown that this gene encodes an intracellular cysteine proteinase that has ten or more mammalian homologues (for review, see (2)). Collectively these proteinases are now known as caspases, and it is thought that most function as apoptotic effector molecules, although there is increasing evidence that the primary role of caspase-1 (interleukin-1β converting enzyme or ICE) is in cytokine maturation (3,4).

Due to their central roles in apoptosis and inflammation there is great interest in understanding the structure and function of the caspases. To date there have been two structures (5,6) and two gene knockouts (4,7) described. A key requirement for biochemical and structural studies of these enzymes is access to practical quantities of pure protein. This is usually achieved via recombinant protein production systems, typically those based on *Escherichia coli* (for example, see (8-11). As caspases are tetrameric complexes composed of two proteolytically generated subunits, preparation of active enzyme from bacterial extracts requires a precise means of cleaving the polypeptide precursor and assembling the subunits. For some caspases, this can be achieved by auto-activation in the lysates (9), while others may require separate expression and purification of the subunits with subsequent reconstitution of the active complex (8). In many situations purification is hampered by aggregation of the recombinant protein, necessitating extra solubilization and re-folding steps.

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Abbreviations: CrmA, cytokine response modifier A; DEVD-AFC, z-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin; PARP, poly-(ADP-ribose) polymerase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; YVAD-AFC, z-Tyr-Val-Ala-Asp-7-amino-4-trifluoromethyl coumarin.

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Caspase-3 (CPP32/Yama/apopain) was first identified on the basis of sequence homology to caspase-1 (12,13). It was subsequently shown to be activated in apoptotic cell extracts, and to mediate the hallmark cleavage of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) during apoptosis (14). Caspase-3 is activated in response to most cell death signals, including granzyme B (15,16), and is one of the two most abundant caspases present in apoptotic cells (17). Gene targeting experiments have demonstrated a critical role for caspase-3 in brain development (7). Recombinant caspase-3 is commonly produced in *E.coli* expression systems (5,9,12). For example, production of pure enzyme for crystallographic analysis involved independent expression of the two subunits, solubilization of each chain from inclusion bodies, mixing in the presence of denaturant, re-folding and subsequent purification of the tetrameric enzyme (5).

Here we show that practical quantities of pure, soluble and active caspase-3 can be obtained using a yeast expression system followed by one-step affinity chromatography. The biological and kinetic properties of the recombinant enzyme are comparable to those of the native enzyme purified from apoptotic cell extracts. This simple system should facilitate the production of caspase-3 and variants for structure and function analysis.

MATERIALS AND METHODS

Production of recombinant caspase-3. Hexahistidine-tagged caspase-3 was produced in the methylotropic yeast, *Pichia pastoris* and purified following methods described previously (18). Briefly, the mature caspase-3 coding sequence was amplified from a plasmid template (a kind gift of Dr. J. Trapani) by PCR using the primers 5′-TCTGCCATCATGCATCATCATCATCATTCTGGAATATCCCTGGAC-3′ and 5′-TTAGTGATAAAAATAGAGTTCTTTTG-3′. The PCR used 20 pmole of each primer and 1 ng of template in Vent polymerase reaction buffer (New England Biolabs) containing 200 μM dNTP and 1 unit of Vent polymerase (New England Biolabs). 30 cycles of 95 C for 90 s, 57 C for 45 s and 72 C for 60 s were performed. Amplified fragments were separated by 1 % agarose gel electrophoresis, purified from the gels and cloned into pCRII (Invitrogen) for sequence analysis. A clone with an in-frame fusion of the His-tag and no second site mutations was chosen for the subsequent steps. The modified caspase-3 cDNA was released from pCRII by *Eco*RI digestion, cloned into the vector pHIL-D2, linearized with *Sal*I, and used to transform *P.pastoris* as previously described (18). Transformants were grown and analyzed as described (18), except that induction was for 3 days. Preparation of His-tagged protein was as described (18) except that caspase-3 was eluted from a cobalt affinity column (Talon, CLONTECH) in 50 mM imidazole following washes in 0.1 M NaCl. Purified caspase-3 was stored in 25 mM imidazole, 5 mM DTT, 50 % glycerol at −20 C.

Enzyme assays. Protease activity was assessed using the fluorogenic substrates z-Tyr-Val-Ala-Asp-7-amino-4-trifluoromethyl coumarin (YVAD-AFC) or z-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD-AFC) from Enzyme Systems Products (Dublin, CA). The tetrapeptide caspase inhibitors Ac-DEVD-CHO and Ac-YVAD-CMK were from Bachem (King of Prussia, PA). Recombinant CrmA was produced in the *P.pastoris* expression system, and was assessed for inhibitory activity against caspase-1 (J. Sun and P. Bird, unpublished results).

Assays were performed in 100 mM Hepes pH 7.5, 10 % sucrose, 0.1 % CHAPS, 10 mM DTT at 25 C. Hydrolysis of each substrate was monitored by measuring the fluorescence λ_{em} = 500 nm and λ_{ex} = 420 nm, 5 nm slit widths in a Perkin-Elmer spectrofluorometer (LS50B). A temperature-controlled cuvette containing 0.5 ml of the substrate solution was allowed to equilibrate to 25 C, then the enzyme solution was added and the change in fluorescence measured. K_m values were derived by non-linear curve fitting of initial velocities to the Michaelis-Menton equation. Determination of K_i values was as described (19).

In vitro translation. An expression plasmid containing a cDNA encoding proIL-1β was constructed by subcloning a 1.5 kb *Eco*RI-*Pst*I fragment from a pSP65 derivative (20) into pSVTf (21). A pcDNA3 derivative containing pro-caspase 3 has been described (13). Plasmids were linearized with *Bam*HI and used to program 50 μl of TNT Coupled Wheat Germ Extract system, according to the manufacturer's instructions (Promega). 4 μl of [^{35}S]-methionine (1000 Ci / mmol at 10 mCi / ml; DuPont NEN) was included to label nascent proteins. Cleavage of translation products by 5 ng of recombinant caspase-1 (G. Parasivam and S. Kumar, unpublished), recombinant caspase-3 (this study) or granzyme B (22) was carried out for 30 min at 37 C. Products were visualized following 15 % SDS-PAGE and fluorography (Amplify, Amersham).

Cell extracts. Cytosolic extracts from the human T cell leukemia line, Jurkat, were prepared and stored as described (16).

SDS-PAGE. SDS-PAGE was performed according to Laemmli (23) followed by silver staining. Immunoblotting was carried out using standard procedures. Blots were developed using an enhanced chemiluminescence (ECL) kit (DuPont). Anti-PARP antibodies were purchased from Boehringer Mannheim.

RESULTS AND DISCUSSION

Active caspase-3 is generated from a zymogen that consists of an N-terminal prodomain followed by a larger subunit (p17) containing the active site and then a smaller subunit (p12) (14). The enzyme is produced by proteolytic cleavage of pro-caspase-3 between Asp28-Ser29 that removes the prodomain, and between Asp175-Ser176 that yields the 17 kDa and 12 kDa subunits. To simplify the generation and purification of active recombinant enzyme from *P.pastoris*, we used PCR to ampify the coding sequence of caspase-3 from Ser29, effectively deleting the prodomain. At the same time we inserted a new initiation codon and six histidine codons immediately upstream of Ser29 to allow expression and affinity purification.

The modified caspase-3 cDNA was cloned into the expression vector pHIL-D2 and transformed into the *P. pastoris* strain, GS115, following methods previously described (18). In this system, the cDNA is under the control of the alcohol oxidase 5′ and 3′ regulatory regions, and the transcriptional unit is silent until methanol is added to the cultures as the sole carbon source (24). Growth and induction conditions were optimized empirically. We noted that following methanol induction, growth rates of the caspase-3 - expressing yeast declined markedly, implying that active protease was being produced in these cells and inhibiting growth. This suggested that either caspase-3 was auto-activat-

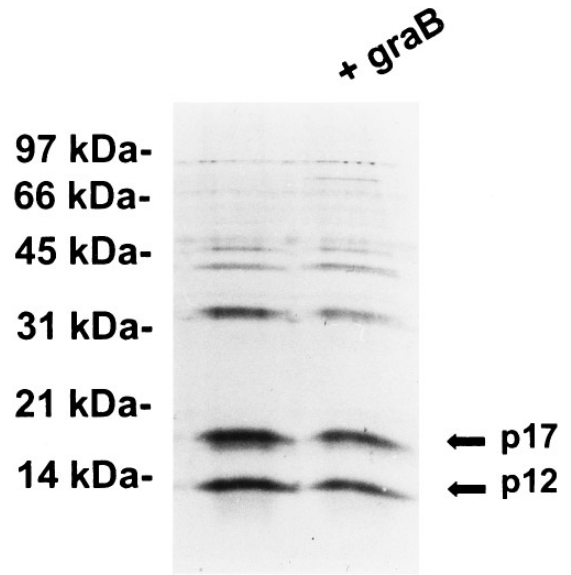

FIG. 1. Purification of recombinant caspase-3 from *P.pastoris* lysates. Lysates from methanol-induced yeast were subjected to cobalt affinity chromatography, and His-tagged proteins were eluted in 50 mM imidazole. Samples of the eluate were incubated for 30 min at 37 C in the absence or presence of granzyme B (graB), and were then analyzed by 15 % SDS-PAGE and silver staining. The p17 and p12 subunits of caspase-3 are indicated by arrows.

ing, or that *P.pastoris* possesses a protease capable of cleaving pro-caspase-3. We favour the former possibility because there is no evidence for caspases in yeast, and we were unable to detect any activity in untransformed, induced GS115 lysates capable of cleaving the synthetic caspase substrates YVAD-AFC or DEVD-AFC (data not shown).

Due to growth inhibition the caspase-3 - producing cultures did not reach the same biomass as the controls after the usual 5 day induction period. Yields were optimal after a shorter induction time (3 days). His-tagged caspase-3 was purified from yeast lysates by cobalt affinity chromatography. Non-specifically bound protein was removed by washing with 0.1 M NaCl, and the remaining protein was eluted in the presence of 50 mM imidazole. As shown in Fig. 1, analysis of the eluate on silver-stained SDS polyacrylamide gels after this simple purification procedure revealed two major bands of 17 kDa and 12 kDa, which correspond in size to the size of the subunits of active capase-3 (14). It is possible that the 30 kDa species present in the samples may represent unprocessed caspase-3. To test this we added granzyme B, which is known to cleave pro-caspase-3 (15,16). No alteration in the banding pattern was noted, suggesting that this and other higher MW species are yeast proteins, which could be removed by ion-exchange or gel filtration chromatography. We estimate a yield of 50 μg purified caspase-3 from 50 g (wet weight) of induced yeast.

Analysis of the properties of the purified caspase-3 demonstrated that it efficiently cleaves the synthetic substrate DEVD-AFC with a K_m of 16.8 μM (Fig. 2). This compares favourably with the activity reported for the native enzyme purified from apoptotic cell extracts on a related substrate DEVD-AMC (K_m of 10 μM (14)). By contrast, recombinant caspase-3 cleavage of the caspase-1 substrate YVAD-AFC was undetectable. This is also consistent with previous studies (14).

Addition of recombinant caspase-3 to a cytosolic extract prepared from human Jurkat cells resulted in proteolysis of PARP (Fig. 3), indicating that the recombinant enzyme can cleave the same substrates as the native enzyme. Although incomplete, this pattern was indistinguishable from that observed by others (15,25-27), and suggests that a proportion of PARP is inaccessible or resistant to caspase-3. This contrasts with the complete PARP cleavage mediated by caspase-1 and granzyme B (Fig. 3 and (16,28)). Presumably these proteases activate other caspases in the extracts that are able to more efficiently proteolyse PARP. Finally, the ability of recombinant caspase-3 to cleave the caspase-1 substrate, proIL-1β, was tested (Fig.3). As expected (13,14), no cleavage was observed.

Inhibition of caspase-3 activity by various inhibitors was also investigated. As expected, caspase-3 was efficiently by the tetrapeptide inhibitor Ac-DEVD-CHO (K_i 2.2 nM) but inefficiently by Ac-YVAD-cmk (K_i 0.3 μM). The cowpox virus serpin, CrmA, did not detectably inhibit recombinant caspase-3 when used in equimolar amounts (data not shown). This is consonant with previous studies on the interaction of caspase-3 and CrmA (14).

Assuming that *P.pastoris* does not contain a protease capable of activating caspase-3, our results suggest that caspase-3 can fully auto-activate. This is consis-

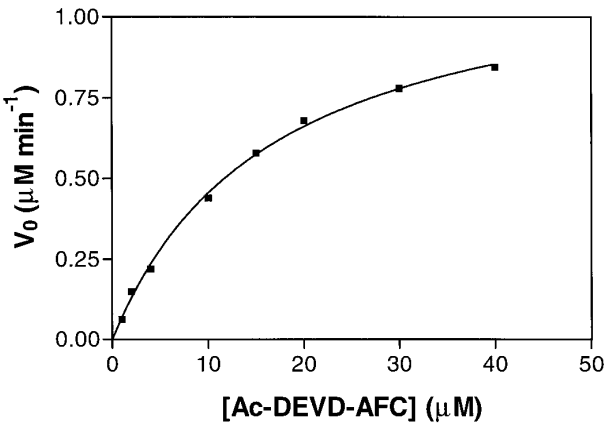

FIG. 2. Determination of K_m for recombinant caspase-3 catalysis of Ac-DEVD-AFC. Reactions were at 25 C and monitored continously in a spectrofluorometer. Initial velocities and substrate concentrations were fit by non-linear regression to the Michaelis-Menton equation.

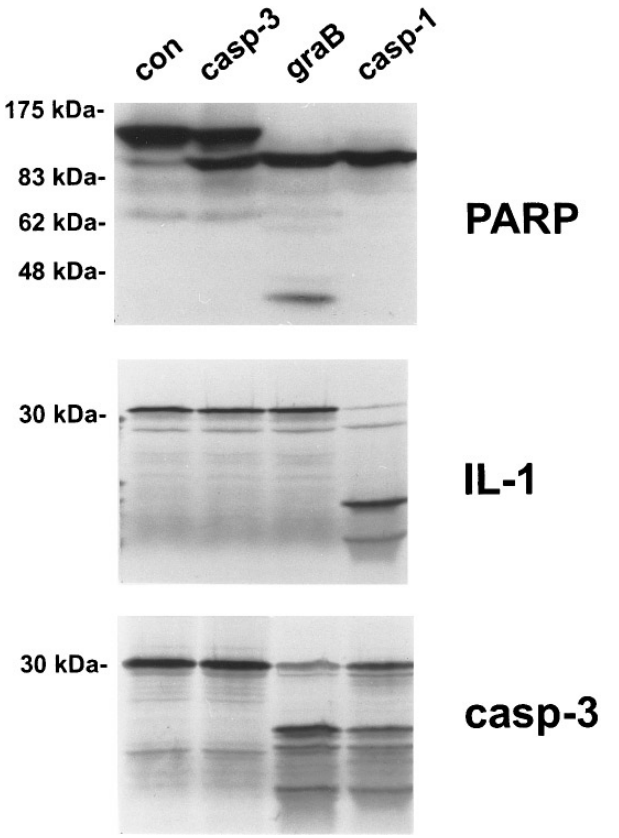

FIG. 3. Effect of recombinant caspase-3 on protein substrates. *Top panel:* Cleavage of PARP in Jurkat cell cytosolic extracts. 50 μg of extract was incubated with 5 ng of each of the indicated proteases for 2 h at 37 C. Samples were analyzed by 7.5 % SDS-PAGE followed by immunoblotting with rabbit anti-PARP antiserum (1:1000) and detection via ECL. *Middle panel: In vitro* translated pro-IL-1β was indicated with 5 ng of the indicated proteases for 30 min at 37 C. Samples were analyzed by 15 % SDS-PAGE and fluorography. *Lower panel: In vitro* translated pro-caspase-3 was treated and analyzed as described for middle panel samples.

tent with previous observations of caspase-3 auto-activation in bacterial lysates (for example, see (9)). However, a number of studies suggest that *in vivo* caspase-3 can only cleave itself to remove the prodomain, and that this occurs after another protease has released the p12 subunit (13,16,29-31). In keeping with this currently-held model for caspase-3 activation, we were unable to demonstrate cleavage of *in vitro* translated pro-caspase-3 by our purified enzyme (Fig. 3.). No cleavage was observed even after adding recombinant caspase-3 to an enzyme to substrate ratio of approximately 100 to 1 (data not shown). This suggests that autoactivation probably only occurs when pro-caspase-3 reaches a very high concentration, as it might in the confines of fully-induced yeast or bacterial cells. In the context of apoptosis this property might serve to amplify the initial caspase-3 activating signal in a positive feedback loop that functions in the later stages of apoptosis when high levels of active caspase-3 are present.

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