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Brain Cathepsin B Cleaves a Caspase Substrate

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Abstract—We show that an enzyme exists in rat brain capable of cleaving the caspase-3 specific peptide substrate Ac-DEVD-AMC at low pH. The enzyme shows properties of a cysteine protease and is localized, predominantly, in lysosomes. We have purified this enzyme from rat brain and identified it by MALDI-TOF MS. The enzyme possessing "acidic" DEVDase activity in rat brain appears to be cathepsin B. It remains obscure, whether cathepsin B participates in cleavage of caspase-3 substrates *in vivo*. We suggest that under certain conditions (e.g. in hypoxia) cathepsin B participates in cleavage of caspase-3 substrates in brain cells.

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Special attention of many Russian and foreign groups is given to molecular and biochemical processes underlying apoptosis [1, 2]. Apoptotic cell death enables organisms to get rid of useless or potentially dangerous cells as well as of undesirable intracellular components [3]. Disturbances in mechanisms of apoptosis regulation may underlie development of some pathologies, including neurodegenerative diseases and cancer [4]. Most apoptotic scenarios depend on the activity of enzymes of the caspase family [5, 6]. All caspases are cysteine proteases cleaving polypeptide chains after an asparagine residue [5, 6].

Apoptotic cell death is of a specific importance in the brain. Apoptosis is essential for establishing inter-neuronal communications during ontogenesis, while some authors regard excessive apoptosis in the mature brain as a cause of neurodegeneration [4, 7]. Caspase-3 is believed to be the main executor of intracellular apoptotic program in the brain [8, 9].

A routine approach to caspase assay is based on the use of fluorogenic peptide substrates [10], mostly tripeptides with a covalently bound fluorophore. A free fluorophore is released from these substrates as a result of caspase cleavage. The method is reasonably sensitive, reliable, and reproducible. The synthetic tetrapeptide DEVD covalently bound to a fluorophore is the most popular substrate of caspase-3. The enzyme recognizes the DEVD sequence in its natural substrate, poly-(ADP-ribosyl)-polymerase, and cleaves the polypeptide chain of this protein after an aspartic acid residue [11]. It is believed that the synthetic substrate Ac-DEVD-AMC is highly specific for caspase-3 and caspase-7 [12], while other proteolytic enzymes, at least those not belonging to the caspase family, are unable to cleave this substrate with fluorophore release. All proteases belonging to caspase family are cysteine-dependent enzymes, and some need SH-reducing reagents for their activity. Maximal activity of caspases is detected at neutral pH values [13].

On one hand, the enzymes of caspase family represent a relatively specific protease group fulfilling their functions in the brain at strictly fixed conditions, while, on the other hand, there are many proteolytic enzymes belonging to other families in the brain. Interactions of caspases with other intracellular proteolytic enzymes have been demonstrated using animal models of different

Abbreviations: AMC) 7-amino-4-methylcoumarin; CA-074) N-[L-3-trans-(propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline; DTT) dithiothreitol; MALDI-TOF MS) matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry.

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pathologies [14-17]. However, it remains unclear whether other proteases are able to cleave caspase substrates, and, in case they are, which conditions are necessary for this. The aim of this study was to determine, using a simple *in vitro* system, whether enzymes able to cleave the caspase-3 substrate Ac-DEVD-AMC exist in the brain.

MATERIALS AND METHODS

Isolation of cathepsins from rat brain. Cathepsin-containing fraction was isolated as described in [18] with minor modifications. Rat brain supernatant was acidified to pH 4.0, and ammonium sulfate was added up to 20% saturation. After 1 h, the pellet was removed by centrifugation at 12,000*g* for 20 min. The supernatant was desalted using Sephadex G-25 and put on S-Sepharose Fast Flow. All fractions were spin desalted on Bio-Spin 6 columns (BioRad, USA). In the fractions, DEVDase activity was measured at pH 4.0, and activity-containing fractions were isolated using SDS-PAGE. Polypeptide bands were cut out and subjected to trypsinolysis. The masses of polypeptides obtained were assessed using MALDI-TOF MS. The results were analyzed using Mascot software (http://www.matrixscience.com).

Denaturing SDS-PAGE. Polypeptides were separated by electrophoresis as described earlier [19]. Twelve percent denaturing polyacrylamide gels (0.75 mm thick) were used. The gels were stained by silver nitrate [20].

Caspase-3 assay. Rats were decapitated and brain (without brainstem and cerebellum) was isolated and quickly frozen in liquid nitrogen. The tissue was homogenized in the isolation medium (10 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 0.5 mM EDTA, pH 7.5; all reagents from Sigma (USA)) using a Potter S homogenizer (Teflon–glass; Braun, Germany) at 1500 rpm. Homogenates were centrifuged at 14,000*g* for 30 min at 4°C, and supernatants were used for caspase-3 assay. All procedures were carried out on ice. Caspase-3 activity was measured by a fluorometric method described earlier [21]. Supernatants were incubated for 60 min at 37°C in the 0.1 M reaction buffer with different pH (100 mM Mes-NaOH, 10 mM DTT, 1 mM EDTA, 100 μM Ac-DEVD-AMC; Biomol, USA). The fluorescence at 380 nm (excitation) and 440 nm (emission) was measured using a Wallac fluorometer (Perkin Elmer, USA). 7-Amino-4-methylcoumarin (AMC) (Sigma) was used as a fluorescence standard. Cathepsin B activity was measured in a similar way except for the substrate used—Z-RR-AMC. Protein concentration was measured according to Bradford [22].

Intracellular localization. The isolation of subcellular fractions was performed as described before [23]. Rats were decapitated and brains (without brainstem and cerebellum) isolated. The tissue was immediately homogenized in four volumes of 0.32 M sucrose containing 0.1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride. All procedures were carried out on ice. Washed nuclear and lysosomal fractions were homogenized and assayed for DEVDase as described above.

RESULTS

The first experiment gave evidence that at least one enzyme is present in rat brain that cleaves the synthetic caspase-3 substrate Ac-DEVD-AMC at low pH (Fig. 1, results of a typical experiment are presented). A similar pH profile for DEVDase activity was also revealed in rat neuroblastoma cells (data not shown). To exclude the effects of buffer constituents on DEVDase activity, we performed similar experiments using citrate-phosphate and acetate buffers. The pH dependence of brain DEVDase activity was similar to that in our usual Mes buffer (data not shown). Thus, an enzyme exists in the brain able of cleaving a specific caspase-3 substrate under conditions far from optimal for caspase-3 functioning.

In the next experiment, an attempt was made to check specificity of brain supernatant DEVDase activity at low pH. One of the approaches to check the specificity of the activity is to evaluate the fraction of the activity inhibited by a specific caspase-3 inhibitor, Ac-DEVD-CHO. The dependence of brain DEVDase activity on Ac-DEVD-CHO concentration at two pH values is presented in Fig. 2. Our results suggest that Ac-DEVD-CHO in the micromolar concentration range fully blocks “acid” DEVDase activity. Thus, an enzyme exists in the brain capable of cleaving caspase-3 substrate Ac-DEVD-AMC at low pH, this activity being totally inhibited by the caspase-3 inhibitor Ac-DEVD-CHO.

To characterize this enzyme, we studied effects of a series of functionally relevant substances on its activity. Zinc ions inhibit enzymes of the caspase family, while

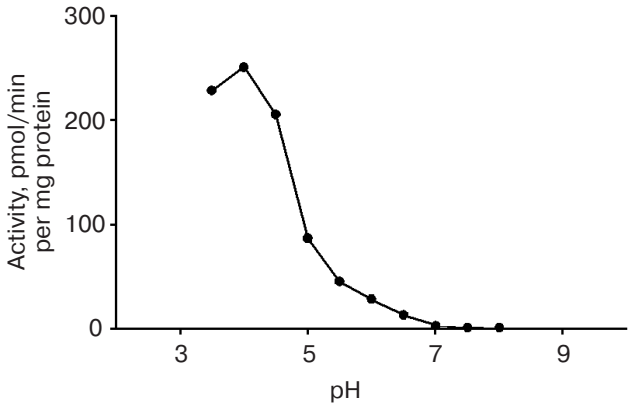

Fig. 1. Dependence of brain DEVDase activity on pH.

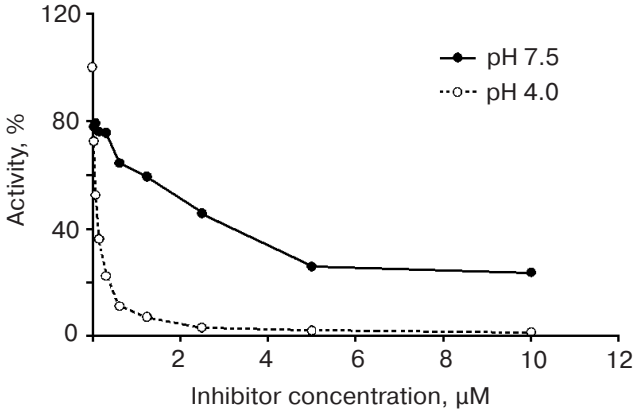

Fig. 2. Inhibition of brain DEVDase activity by the specific caspase-3 inhibitor Ac-DEVD-CHO at neutral and acid pH values. 100% is activity in the absence of the inhibitor.

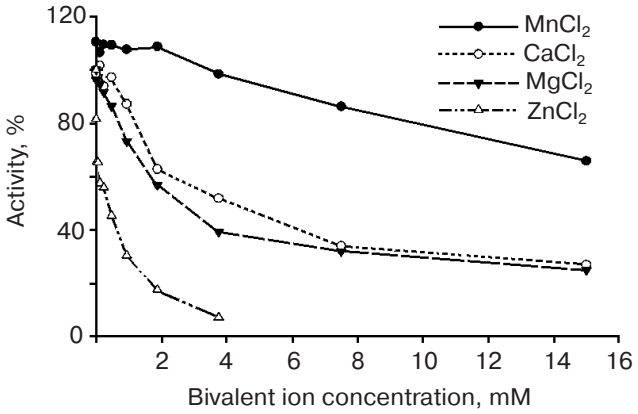

Fig. 3. Dependence of brain DEVDase activity on concentration of bivalent ions.

other bivalent cations are without effect [13]. The dependence of brain DEVDase activity at pH 4.0 on bivalent ions was obviously different (Fig. 3). Besides inhibition by zinc ions, this activity was also partially inhibited by magnesium and calcium ions. Brain DEVDase activity at low pH was dependent on SH-reducing reagents (Fig. 4). The presence of DTT or reduced glutathione at concentrations >10 mM significantly increased DEVDase activity at pH 4.0. Moreover, "acid" DEVDase activity dropped in the presence of SH-oxidizing reagents (Fig. 5). Typically for cysteine-dependent proteases, SH-oxidizing reagents at concentrations >10 mM significantly decreased "acid" DEVDase activity.

The data presented above suggest that the brain enzyme with "acid" DEVDase activity is an acid cysteine protease. Such proteases are believed to be localized predominantly in the lysosomal compartment. To examine the intracellular localization of the enzyme responsible for DEVDase activity at pH 4.0 we performed subcellular fractionating of rat brain homogenate. Figure 6 represents the distribution of "acid" DEVDase activity in intracellular compartments of the brain. The results show that this activity predominantly belongs to lysosomal fraction of brain cells.

Biochemical properties and intracellular localization of "acid" DEVDase activity suggest that the enzyme responsible for this activity belongs to the cathepsin family. The purification of brain homogenates [18] yielded a fraction containing an enzyme with DEVDase activity at pH 4.0. This "acid" DEVDase-containing fraction was studied using SDS-PAGE with subsequent silver nitrate staining (Fig. 7). We suggest that the two protein bands belonged to the target enzyme. Indeed, in both bands rat cathepsin B was identified by MALDI-TOF MS, Mascot score being 107 and 86 for the upper and lower band,

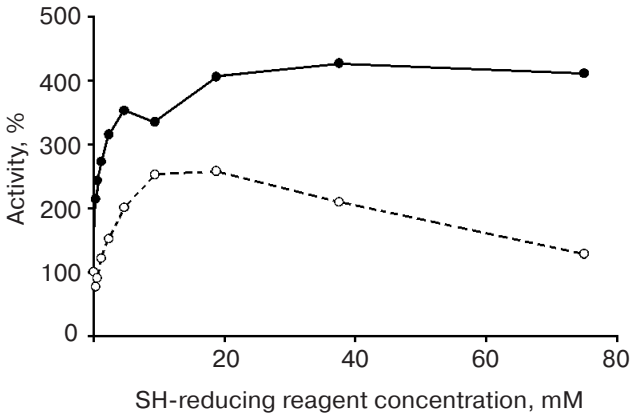

Fig. 4. Dependence of brain DEVDase activity on concentration of SH-reducing reagents. Closed circles, DTT; open circles, reduced glutathione.

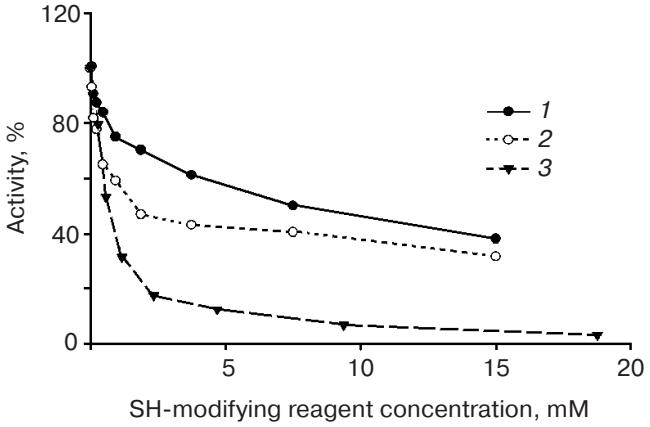

Fig. 5. Dependence of brain DEVDase activity on concentration of SH-oxidizing reagents: *1-3*) N-ethylmaleimide, iodoacetamide, and oxidized glutathione, respectively.

respectively. The results suggest that brain "acid" DEVDase activity belongs to cathepsin B.

The enzyme possessing "acid" DEVDase activity was able to cleave a cathepsin B substrate at pH 4.0 (Fig. 8). Moreover, both DEVDase and cathepsin B activities could be inhibited by both caspase-3 inhibitor Ac-DEVD-CHO and cathepsin inhibitor CA-074 (N-[L-3-trans-(propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline). At pH 7.5, the enzyme from the isolated fraction did not cleave caspase-3 substrate Ac-DEVD-AMC (data not shown). These results confirm our conclusion that "acid" DEVDase and cathepsin B is one and the same enzyme in the brain.

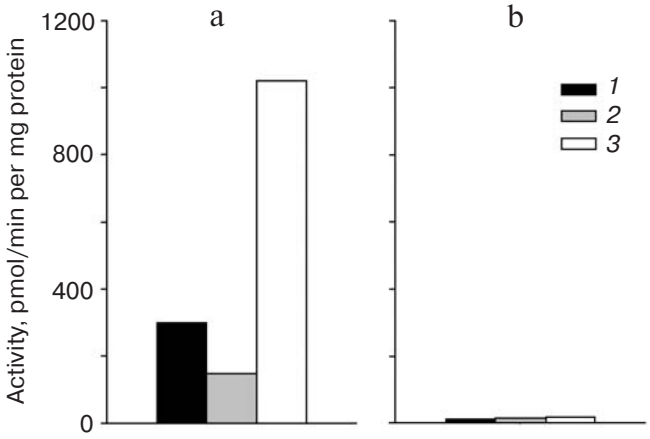

Fig. 6. Distribution of brain DEVDase activity at pH 4.0 (a) and 7.5 (b) in intracellular fractions: *1-3*) nuclear, cytoplasmic, and lysosomal fraction, respectively.

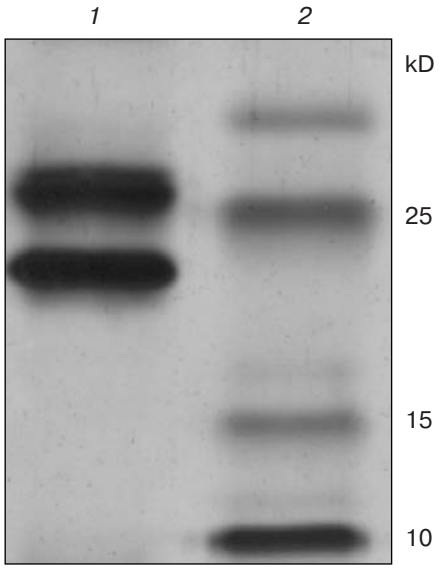

Fig. 7. SDS-PAGE of the fraction containing DEVDase activity at pH 4.0; staining with silver nitrate: *1*) purified enzyme from rat brain; *2*) molecular mass markers.

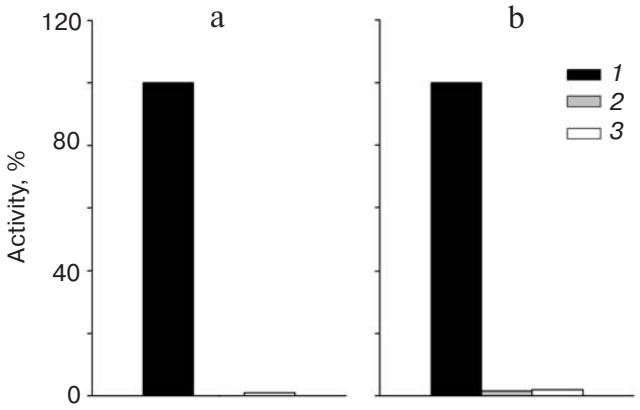

Fig. 8. Ability of the purified fraction to cleave cathepsin B (a) and caspase-3 (b) substrates at pH 4.0 and inhibition of these activities by specific inhibitor of these proteases: *1*) no inhibitor; *2*) cathepsin B inhibitor CA-074; *3*) caspase-3 inhibitor Ac-DEVD-CHO.

DISCUSSION

In this study, we have demonstrated that cathepsin B from rat brain is able to cleave a synthetic peptide substrate of caspase-3. Peptidase activity of cathepsin B towards this substrate is expressed at pH 4.0 and is fully inhibited by Ac-DEVD-CHO, a specific caspase-3 inhibitor. However, this enzyme does not cleave caspase-3 substrate at neutral pH. It remains unclear whether cathepsin B is able to cleave natural caspase-3 substrates in brain cells. However, it is known that in some physiological and pathological situations significant pH shifts may take place in cells and their compartments. Specifically, a prominent acidification of neuronal and glial cells has been reported under ischemia [24]. Indeed, in all animal models of ischemia, the pH of brain cells is <7. Depending on experimental conditions of ischemia, intracellular pH may decrease to 6, and in some cellular compartments even lower.

Caspase-3 activation and subsequent apoptotic cell death in ischemia are now widely accepted [25]. It may be of particular interest that in ischemic neurons a necrotic scenario of cell death mediated by cathepsin B may take place along with an apoptotic program mediated by caspase-3 [26]. In this experimental model, lysosomal membrane is damaged with subsequent release of cathepsin B into the cytoplasm and accumulation of caspase-specific products of protein degradation [26]. We suggest that in a pathological situation like ischemia, cathepsin B may be involved in the degradation of caspase-3 substrates. In general, we can suggest a putative scenario of ischemic neuronal cell death in the following way. Oxygen and glucose deficits induce the neuronal program of caspase-3-mediated apoptotic cell death. A prolonged deficit in nutrients causes acidification of neuronal intracellular space, damage to lysosomal membranes, and the release

of cathepsin B into the cytoplasm. Taking into account the results of our study about the specificity of cathepsin B to caspase-3 substrates at low pH, cathepsin B cleaves intracellular caspase-3 substrates, worsening ischemic neuronal damage. During a prolonged ischemia, other proteases cleaving other substrate sets are being recruited, and neuronal cell death loses apoptotic characteristics, turning to necrosis.

Deterioration of lysosomal membrane integrity is characteristic not only for ischemia. Specifically, for different cell types rupture of lysosomal membrane and release of lysosomal contents into the cytoplasm has been demonstrated as a result of oxidative stress [27-29]. As a rule, release of lysosomal contents results in cell death. Oxidative stress was also shown to decrease intracellular pH [30]. Thus, in oxidative stress cathepsin B may be released from lysosomes into cytoplasm with lowered pH and cleave cytoplasmic proteins, including caspase-3 substrates.

So far there is no evidence that cathepsin B cleaves intracellular caspase-3 substrates *in vivo*. Moreover, it is not fully clear which consequences for cell functioning this cleavage may have. However, our data and the results published by other groups suggest that such substrate specificity of two proteases underlies one of the mechanisms of switching from apoptotic to necrotic program of cell death.

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