Elucidation of cathepsin B-like activity associated with extracts of human myelin basic protein

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Myelin basic protein (MBP) extracted from human delipidated white matter was found to be degraded at pH 3.0 by endogenous proteolytic activities of extracts. Electrophoretic peptide patterns were consistent with limited proteolysis of MBP. Based on pH, activation by EDTA and DTE, and inhibition by *p*-CMPS, E-64 and, in particular, by leupeptin, the protease involved was tentatively identified as cathepsin B or a cathepsin B-like enzyme. As pepstatin failed to inhibit acid proteolysis of MBP cathepsin D was ruled out.

Human myelin basic protein *Endogenous limited proteolysis* *Cathepsin B* *Activator* *Inhibitor* *Electrophoresis*

1. INTRODUCTION

Delipidated white matter of the central nervous system usually serves as a source for the preparative extraction of myelin basic protein (MBP) under mildly acidic conditions [1]. Neutral proteolytic activities were found to be relevant contaminants of crude extracts in causing a marked degradation of MBP on prolonged incubation at elevated temperatures [2]. Recently, acidic proteolytic activities both of human and bovine MBP extracts were observed as well [3,4], but were of much lower potency than neutral activity. Since proteolytic rates were enhanced upon eliminating both atmospheric and dissolved oxygen, as well as trace metal impurities from the incubation assay, it was suspected that sulfhydryl-dependent proteases might be involved. Besides, neutral protease activities were found to be inhibited by *N*-ethylmaleimide [1,4]. The effects of appropriate activators and inhibitors of cysteine proteases on the endogenous degradation of human MBP at neutral and acid pH were therefore investigated.

Abbreviations: *p*-CMPS, *p*-chloromercuriphenylsulfonic acid; DTE, dithioerythritol; E-64, L-*trans*-epoxysuccinylleucylamido-(4-guanidino)butane; MBP, myelin basic protein; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Materials*

EDTA and other common chemicals of analytical grade were purchased from E. Merck (Darmstadt). DTE (Cleland's reagent) and chemicals for SDS-PAGE were obtained from Serva (Heidelberg) and *p*-CMPS, E-64 and leupeptin from Sigma Chemie GmbH (München). The Coomassie dye reagent for protein assays was supplied by Bio-Rad Laboratories GmbH (Munich). Ultrapure water deionized on a Milli-Q unit (Millipore Corp.) was degassed by boiling and saturated with nitrogen during cooling [5].

2.2. *Preparation and incubation of MBP extracts*

Crude extracts of MBP of delipidated white human matter [1] were prepared as in [3], lyophilised and stored at −75°C. Samples for the assessment of endogenous proteolysis were prepared by dissolving 0.5 mg MBP lyophilisate in

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1 ml of 0.05 M glycine-sodium chloride buffer (pH 3.0) or 0.05 M Tris buffer (pH 7.5). Individual samples were transferred to acid-washed 1.5-ml disposable reaction tubes, purged with nitrogen, tightly capped and incubated at 37°C in a thermostatted metal block. After the desired time periods, samples were frozen in liquid nitrogen and temporarily stored at −25°C, permanently at −75°C.

2.3. *Analytical methods*

A modified Coomassie dye-binding assay [6] was used for the determination of total protein and for monitoring the degradation of MBP [3]. Bovine serum albumin served as protein standard. Discontinuous SDS-PAGE was carried out on 15% polyacrylamide slab gels as in [3] using Laemmli's buffer system [7]. Gels were stained with Coomassie brilliant blue R-250. Aliquots for electrophoresis were first lyophilised, then redissolved in a reducing SDS-containing sample buffer and heated at 56°C for 5 min.

3. RESULTS

Acid proteolysis of MBP total protein was strongly stimulated by EDTA (table 1). This effect was further potentiated when DTE was added, whereas the latter alone exerted little effect. Neither treatment significantly stimulated the degradation of protein at pH 7.5, nor did pro-

Table 1

Activation of endogenous acid proteolysis of MBP

Treatment		Degradation of total protein (μg/ml)[a]	
		pH 3.0	pH 7.5
Control	I	91 ± 4	105 ± 1
	II	153 ± 5	95 ± 1
EDTA, 2 mM	I	178 ± 2	106 ± 9
	II	247 ± 1	109 ± 1
DTE, 10 mM	I	157 ± 3	138 ± 7
	II	161 ± 1	102 ± 3
EDTA, 2 mM + DTE, 10 mM	I	315 ± 2	101 ± 1
	II	365 ± 6	101 ± 2

[a] Samples were incubated at 37°C for 12 h

Results are means ± ranges of duplicate assays with a fresh extract of MBP (I), and one stored at −75°C and, intermittently, at −25°C (II)

Table 2

Inhibition of endogenous acid and neutral proteolysis of MBP

Inhibitor	Concentration		Degradation of total protein in % of controls[a]	
			pH 3.0	pH 7.5
p-Chloromercurisulfonic acid	100 μM	I	15.9 ± 0.1	91.3 ± 0.7
		II	25.2 ± 0.9	87.8 ± 1.6
Leupeptin	2 μg/ml	I	12.3 ± 4.2	106.7 ± 0.3
		II	14.4 ± 0.1	109.4 ± 0.5
E-64[b]	5 μM	I	4.1 ± 0.4	130.6 ± 1.0
		II	27.2 ± 1.1	98.7 ± 5.4
Pepstatin[b]	1 μg/ml	I	92.2 ± 1.3	48.7 ± 5.5
		II	98.3 ± 1.0	65.7 ± 0.1

[a] Samples were incubated at 37°C for 24 h

[b] Stock solutions of pepstatin, 100 μg/ml (w/v), in absolute ethanol, and of E-64, 10 mg/ml (w/v), in dimethyl sulfoxide were diluted with appropriate buffer solutions immediately prior to use

Results are means ± ranges of duplicate assays with two different extracts of human MBP I and II

longed storage of MBP lyophilisates seem to affect the control rates or prevent the activation of acid proteases by the agents tested.

Incubations were also carried out in the presence of typical protease inhibitors used to differentiate major classes of proteases [8]. E-64, *p*-CMPS and leupeptin all resulted in marked inhibitions of acid proteolysis (table 2). Pepstatin, in contrast, was ineffective at acid pH. At pH 7.5 inhibitory patterns were virtually reversed in that the cysteine protease inhibitors were ineffective while pepstatin entailed a moderate degree of inhibition.

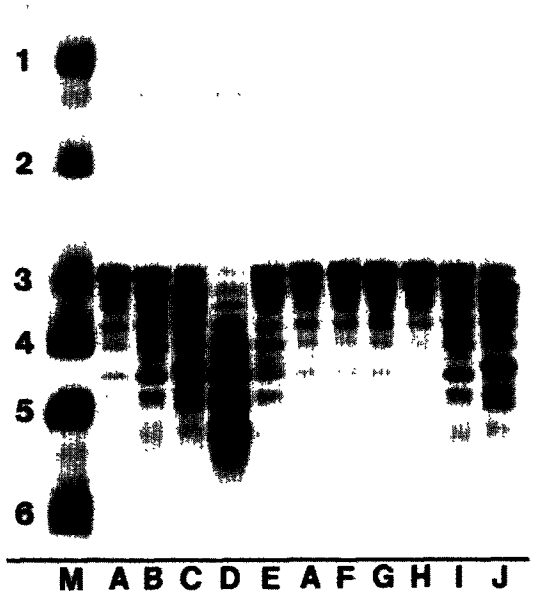

Fig.1. SDS-PAGE of selected samples of MBP extracts treated with activators and inhibitors of proteases as described in the text. Controls prior to incubation are shown in lane A. Controls incubated for 24 h at pH 3.0 and 7.5 were run in lanes B and C, respectively. Additional aliquots were incubated in the presence of 2 mM EDTA and 10 mM DTE, either at pH 3.0 (D) or pH 7.5 (E). Because of the rapid cleavage at pH 3.0, aliquots were incubated for 12 h only and 15 μg total protein were applied instead of 10 μg, as for the other samples. Samples shown in lanes F–J were incubated at pH 3.0 for 24 h in the presence of the following inhibitors: *p*-CMPS, 0.1 mM (F); leupeptin, 2 μg/ml (G); E-64, 5 μM (H); and pepstatin, 1 μg/ml (I). E-64 was also employed at pH 7.5 (J). A set of prestained marker proteins (Bethesda Research Laboratories) is shown to the left and includes ovalbumin, 43.0 kDa (1), α-chymotrypsinogen, 25.7 kDa (2), β-lactoglobulin, 18.4 kDa (3), lysozyme, 14.3 kDa (4), bovine trypsin inhibitor, 6.2 kDa (5), and insulin, A and B chains, approximately 3 kDa (6).

The electrophoretic peptide patterns of representative samples (fig.1) conform to those typically seen in limited proteolysis, the overall peptide patterns being essentially similar at pH 3.0 and 7.5. The differential activation of acid and neutral proteolysis by EDTA and DTE combined is clearly evident from electrophoresis, especially if the amounts of residual MBP are considered. In turn, E-64, *p*-CMPS and leupeptin largely prevented the initial degradation of MBP at acid pH while pepstatin failed to do so. Neither of the cysteine protease inhibitors interfered with the degradation of primary MBP or of intermediate proteolytic peptides at neutral pH (not shown).

4. DISCUSSION

The results of this study strongly indicate that acid proteolysis of MBP at pH 3.0 was accounted for by a protease other than cathepsin D, the most abundant acid protease of the brain [9]. Its properties conform to those of cathepsin B (EC 3.4.22.1) in several respects. Like cathepsin B (review [10,11]), it appears to be a cysteine protease as may be inferred from the stimulatory effects of a reducing agent. Since its effect only became apparent when combined with EDTA, a high metal sensitivity of the enzyme is suggested that would also point to an essential sulfhydryl group of the enzyme. The inhibitory effect of *p*-CMPS also serves to substantiate this conclusion as does that of E-64, a rather specific inhibitor of cathepsin B [14,15], and of leupeptin, a potent inhibitor of both serine and cysteine proteases, yet with some preference of and specificity toward the latter [12]. Accordingly, inhibitors of serine proteases, including phenylmethylsulfonyl fluoride and soybean trypsin inhibitor, were only moderately effective (not shown), as was also described by others for cathepsin B [13]. The unusual inhibition of neutral proteolysis of MBP by pepstatin confirms previous observations [3].

Because of its pH optimum of about 5, cathepsin B is considered a lysosomal hydrolase [10,11] which is, however, also involved in the processing of peptide prohormones and precursor proteins

[11]. The reason why cathepsin B or a related enzyme withstood the harsh conditions applied in preparing MBP extracts, but cathepsin D did not, is unclear. Cathepsin B may either be a more resistant enzyme, or, during handling, part of it may become selectively bound to particulate matter, not discounting the possibility that this activity is due to a genuine membrane-bound enzyme to begin with. The electrophoretic peptide patterns of acid proteolysis of MBP are essentially in line with what has been observed when cathepsin B was used [16]. Patterns are also similar to those obtained by limited proteolysis at neutral pH of human and bovine MBP [3,4], although the minor differences found herein also suggest that acid and neutral proteases associated with MBP extracts are different enzymes.

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