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IDENTIFICATION OF WATER-SOLUBLE PROTEASES IN MYELIN PREPARATIONS

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Summary: Sodium chloride extracts obtained from purified bovine brain myelin were found to contain proteolytic activity capable of degrading isolated myelin basic protein as assessed by SDS gel electrophoresis. Using gels copolymerized with gelatin as substrate, two bands at about 54 and 117-125 KDa, respectively, were detected. Activity corresponding to the 54 KDa band was inhibited by zinc.
Data presented in this article suggest that proteolytic activity can be released from the myelin sheath in water-soluble form and recognize MBP as substrate.

Central nervous system myelin is known to be a relatively stable membrane. However, in spite of its low protein turnover and the low presence of enzymatic activity, myelin appears to contain a number of proteolytic enzymes (1-6) , the role of which is not yet clear both in myelinogenesis and in pathological demyelination.
All these enzymes appear to have as major target the myelin basic protein (MBP), (for a review see 7). MBP is known to be encephalitogenic also when hydrolyzed in smaller fragments and MBP fragments have been detected in the cerebrospinal fluid (CSF) of patients with multiple sclerosis (MS) (8), a disease in which myelin proteolytic activity appear to increase. (9). Therefore to know myelin associated proteases and their properties is of fundamental interest.
In this article we report on the identification of possibly novel proteases which can be extracted from myelin by increasing the ionic

Abbreviations: MBP, myelin basic protein; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; BSA, bovine serum albumine; 1.10 PA, 1.10 phenanthroline; EGTA, (oxyethylenenitrilo)-tetraacetic acid; KDa, kilodaltons.

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strength. The released water-soluble proteolytic activity is capable to degrade MBP and gelatin and can be inhibited by zinc.

MATERIALS AND METHODS

Preparation of myelin: Myelin was prepared from bovine brain white matter according to Norton (10) but using two cycles of sucrose gradient plus osmotic shock as described in (11). Material floating on 0.8 M sucrose after the second gradient centrifugation was washed twice and stored at - 70 °C.

Preparation of sodium chloride extract: Bovine brain myelin was resuspended in 5 volumes of 20 mM Tris-HCl/500 mM NaCl, pH 8.5. After homogenization in a Potter-Elvehjem the suspension was incubated for 10 min in ice and then centrifuged in a Beckman JA-20 rotor for 20 min at 15,000 rpm (27,000xg). The supernatant (10-15% myelin proteins) was concentrated about 10 fold by ultrafiltration on Amicon YM5, and then added to MBP to ascertain if proteolytic activity was present.

Purification of myelin basic protein (MBP): MBP was purified according to Deibler et al. (12,13).

Proteolytic cleavage of MBP: Lyophilized MBP was resuspended in Tris 20 mM, pH 8.5. For the assessement of water-soluble proteases, aliquotes of 20 ug of MBP were incubated with NaCl extract (20 ug) for 44 hours at 20 °C in the absence or in the presence of some protease inhibitors. Each sample was brought to a final volume of 150 ul by adding 20 mM Tris-HCl, pH 8.5 to the incubation mixture. The incubation was starting with the addition of NaCl extract.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE): SDS-PAGE was carried out according to Laemmli (14) as previously described (15). Briefly, aliquots of 150 ul of each sample containing 20 ug of proteins were incubated with 1 ml of cold acetone for 1 hour at -20° C and pellets obtained after removal of supernatants were solubilized with 10 ul of 4% SDS /1% B-mercaptoethanol/2 mM EDTA/10% glycerol/5% Bromophenol blue (pH 6.8) and applied on 15% running gels and 5.4% spacer gels. Following the run, proteins were stained for 2 hours in 0.2% Coomassie blue R-250 and 0.05% Coomassie blue G-250 in methanol/acetic acid/water (4:1:4, v/v) and excess of dye was removed with methanol/acetic acid/water (4:1:5, v/v).

Copolymerization of gelatin in SDS-PAGE gel: SDS-PAGE 5.4-10% gradient gels were copolymerized with 0.2% gelatin used as enzyme substrate, as already described by McKerrow et al. (16).

Aliquots of 40 ug of NaCl extract were solubilized in 10 ul of 4% SDS/10% glycerol/5% bromophenol blue, pH 6.8 and applied to SDS-PAGE. After the run, performed at 4°C, the gel was incubated in 2.5% Triton-X-100 at room temperature for 20 min with one change for additional 20 min to remove SDS. The gel then was incubated in Tris-HCl 100 mM, pH 7.4 for 15 min at room temperature and after replacement of the buffer, the gel was incubated overnight at room

temperature under gently stirring (in the presence or the absence of inhibitors). The gel was then stained and destained as described above. Proteolytic activity was detected as a white band on a blue background.
Standard proteins purchased from Pharmacia were: ovotransferrin (76-78 KDa), albumin (66.25 KDa), ovoalbumin (45 KDa), carbonic anhydrase (30 KDa), myoglobin (17.2 KDa), cytochrome c (12.3 KDa), mioglobin I (8.1 KDa), mioglobin II (6.2 KDa).
Ultrapure water was obtained with the Milli Q-plus apparatus of Millipore.

RESULTS

Myelin was treated with 0.5 M NaCl and separated in a water-soluble (the NaCl extract) and a tightly-bound fraction (the myelin residue) which on the other hand was the starting material for our preparation of MBP in the lipid-bound form (15). To test whether proteolytic activity towards MBP was present in NaCl extract, this was added to MBP and activity was detected by following the disappearance of the MBP band over a specific timespan using SDS gel electrophoresis. Short time incubation resulted in the formation of 16, 14.5, 11, 9.5, 8.0 and 6.5 KDa fragments (Fig. 1).
Fig. 2 shows the effect of the NaCl extract on MBP in the presence or in the absence of some inhibitors. Time was chosen to obtain total cleavage of MBP (Fig.2, lane c). It is important to note that MBP preparations, thanks to the denaturing conditions used for its purification and to the use of ion-exchange chromatography, are

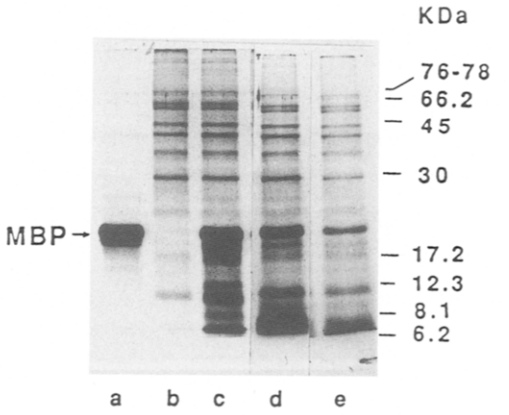
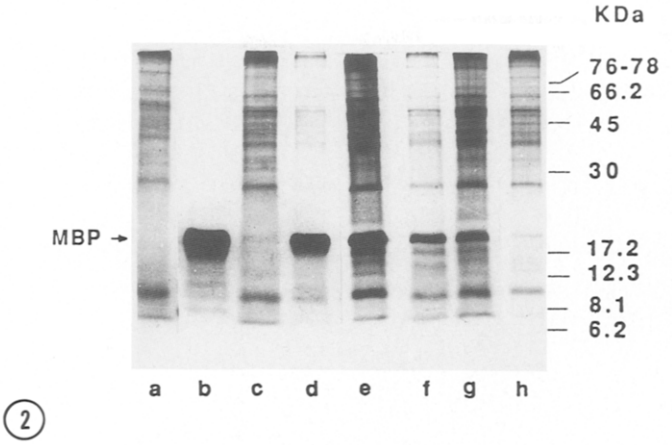


Fig. 1. Time course of MBP degradation by the NaCl extract . From left to right samples are: (a) MBP, 20 µg; (b) NaCl extract 20 µg protein; MBP + NaCl extract after: (c) 10 min; (d) 60 min; and (e) 120 min incubation at 20° C.



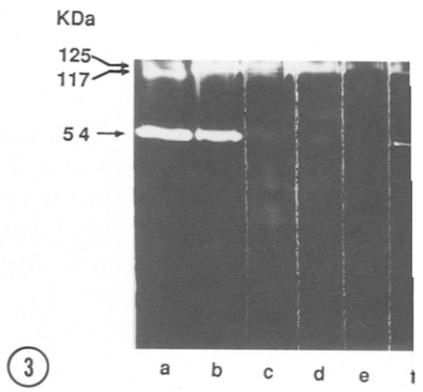


Fig.2. SDS-PAGE of MBP incubated for 20 hours at 20° C with NaCl extract. Samples are: (a) NaCl extract; (b) MBP; (c) MBP + NaCl extract; (d-h) MBP + NaCl extract in presence of: (d) 4 mM zinc acetate; (e) 1 mM B-mercaptoethanol + 1 mM EDTA; (f) 2 mM 1.10 PA; (g) 2 mM B-mercaptoethanol; (h) 2 mM EGTA .The same amount of protein was loaded in all cases, thus possible discrepancies should be ascribed to the inhibitor added.

Fig.3. SDS-PAGE of the NaCl extract in a gel copolymerized with 0.2% gelatin. Samples are: NaCl extract in the presence (a) or in the absence (b) of B-mercaptoethanol in the SDS medium; (c-f) NaCl extract as in (b) but in the presence of: (c) 4 mM zinc acetate; (d) 2 mM EGTA; (e) 1 mM EDTA + 1 mM B-mercaptoethanol; (f) 2 mM 1.10 PA.

devoid of proteolytic activity and are very stable. Thus, the proteolytic enzymes must be present in the NaCl extracts.

With regard to the effect of protease inhibitors, proteolytic activity was blocked by zinc ions (lane d) and in part by B-mercaptoethanol + EDTA (lane e), 1.10 phenanthroline (1.10 PA) (lane f) and B-mercaptoethanol (lane g) but not by (oxyethylenenitrilo)-tetraacetic acid (EGTA) (lane h). Other inhibitors, such as phenylmethylsulphonylfluoride (PMSF), iodoacetate, p-hydroxy mercuribenzoate, dithioerithrytol (DTE), N-ethylmaleimide (NEM), and leupeptin were not effective (not shown). NaCl extracts were not active towards BSA (not shown). As a rule MBP breakdown was limited in the cold and was found to increase with the temperature.

Using gels copolymerized with gelatin as substrate two bands were detected when the NaCl extract was applied to SDS-PAGE. The apparent molecular weight of the corresponding proteases, which can be observed as white bands on a dark background in fig. 3 lane b, was found to be about 54, and 125 KDa respectively. Both bands disappeared in the presence of 1 mM B-mercaptoethanol + 1 mM EDTA or (less efficiently) by 1.10 PA (Fig.3, lanes e and f

respectively), but only the proteolytic activity at 54 KDa was inhibited by zinc (lane c) or EGTA (lane d). We could not observe any activity when total myelin was extracted with detergents and then applied on gelatin-containing gels. Finally, when 1% B-mercaptoethanol was added to SDS incubation medium, the 125 KDa band shifted to 117 KDa, whereas the 54 KDa band did not change its mobility (Fig. 3, lane a).

DISCUSSION

In this article we report that water-soluble proteases are present in myelin preparations. Of course, one obvious interpretation for a water-soluble enzyme to be associated with a myelin membrane is that it has become artifactually associated with the membrane following tissue homogenization. However, other myelin proteins and in particular MBP have been already extracted by treatment of myelin with sodium chloride as well(17,18).

Here, the NaCl extract, which is capable of degrading MBP, appears to contain two gelatin-hydrolysing enzymes: a not better characterized 125 KDa protease, and a possibly zinc-dependent 54 KDa metalloprotease.The similar inhibition profile suggest that the same enzymes break down MBP. With regard to the 54 KDa protease, inhibition by zinc of zinc-dependent enzymes has been described by Larsen and Auld (19), whereas inhibition by 1.10 PA has been reported by Chantry et al. in the case of the metalloproteinase they purified from myelin (5) and from other tissues (20). On the other hand, inhibition by zinc ions of MBP breakdown was ascribed by Berlet et al. (21), and by Earl et al. (22) to the inhibitory effect of zinc on MBP release from the membrane rather than to inhibition of enzymes. In our present study, the use of myelin-free MBP as a substrate suggest that the effect of zinc is on the protease. On the other hand, since EGTA and zinc are effective only on the 54 KDa protease, and only zinc inhibits MBP breakdown, it may be suggested that Zn has the additional role of stabilizing MBP against proteolysis. In this respect, we have already shown that Zn is actually able to interact with MBP (23). Isolation and characterization of both proteolytic enzymes will be matter of further study in order to clarify their possible role in physiopathology.

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REFERENCES

1. Sato, S., Quarles, R.H., Brady, R.O., and Tourtellotte,W.W. (1984) Ann. Neurol. 15, 264-267.
2. Banik, N.L., McAlbaney,W.W., and Hogan, E.L. (1985) J. Neurochem. 45, 581-588.
3. Kerlero de Rosbo, N., Carnegie, P.R., and Bernard, C.A.A. (1986) J. Neurochem. 47, 1007-1012.
4. Berlet, H.H., Ilzenhofer, H., Schulz, R., and Gass, P. (1987) Neurochem. Pathol. 6, 195-211.
5. Chantry, A., Gregson, N.A., and Glynn, P. (1989) J. Biol. Chem. 21603-21607.
6. Yanagisawa, K., Sato, S., O'Shannessy, D.J., Quareles, R.H., Suzuki, K., and Miyatake, T. (1988) J. Neurochem. 51, 803-807.
7. Smith, M. E., and Benjamins, J. A. (1984) Myelin 2nd ed., pp. 441-447, Plenum Press, New York and London.
8. Whitaker, J.N. (1977) Neurology 27, 911-920.
9. Allen, I. V. (1983) Progress in Neuropathology, pp 1-17. Vol 5. Raven Press, New York.
10. Norton,W.T. (1974) Methods in Enzymol. 31, 435-444.
11. Cruz, T.F., and Moscarello, M.A. (1985) J. Neurochem. 44, 1411-1418.
12. Deibler, G.E., Martenson, R.E., and Kies, M.W. (1972) Prep. Biochem. 2, 139-165.
13. Deibler, G. E., Boyd, L. F., and Kies, M. W. (1984) EAE: A useful model for Multiple Sclerosis, pp. 249-256. Alan R. Liss, Inc., New York.
14. Laemmli, U.K. (1970) Nature 227, 680-685.
15. Riccio, P., Liuzzi, G.M., and Quagliariello, E. (1990) Mol. Chem. Neuropathol. 13, 185-194.
16. McKerrow, J.H., Pino-Heiss, S., Lindquist, R., and Werb, Z. (1985) J. Biol. Chem. 260, 3703-3707.
17. Berlet, H.H., Ilzenhofer, H., Gott, S., and Weinhardt, F.(1988) Biol. Chem. Hoppe-Seyler 369, 1195-1196.
18. Smith, R., and Braun, P.E. (1988) J. Neurochem. 50, 722-729.
19. Larsen, K.S., and Auld, J. (1988) FASEB J. 2, 4754.
20. Chantry, A., and Glynn, P. (1990) Biochem. J. 268, 245-248.
21. Berlet, H.H., Ilzenhofer, H., and Gass, P. (1988) Transact. Amer. Soc. Neurochem. 19, 270.
22. Earl, C., Chantry, A., Mohammad, N., and Glynn, P. (1988) J. Neurochem. 51, 718-724.
23. Cavatorta, P., Giovanelli, S., Bobba, A., Riccio, P., and Quagliariello, E. (1991) Acta Neurol. 13, 162-169.