HYDROLYSIS OF MYELIN BASIC PROTEIN WITH BRAIN ACID PROTEINASE

N. Marks, M. Benuck, and G. Hashim
N.Y. State Research Institute for Neurochemistry and Drug Addiction, Ward's Island, N.Y. 10035
and St. Luke's Hospital Center, New York, N.Y. 10025

Received November 5, 1973

SUMMARY

The encephalitogenic basic protein from bovine spinal cord was treated with purified bovine brain proteinase (cathepsin D, EC 3.4.4.23) yielding 3-4 peptide fragments. An encephalitogenic fragment (peptide III-a, 8645 daltons) was purified by repetitive filtration on Sephadex gel. The purity of the peptide was established by polyacrylamide gel electrophoresis at acid and alkaline pH and by amino acid and end group analysis. The N- and C-terminal amino acid sequence of peptide III-a shows that the brain acid proteinase hydrolyzes the Phe-Phe linkage (residues 89-90) releasing the C-terminal 81 residues of the basic protein molecule.

Experimental allergic encephalomyelitis (EAE) is produced in animals following the administration of the intact myelin basic protein (BP) (1-4). Recent studies have shown that the BP is the major encephalitogen present in the central nervous system (CNS) (3, 4) whose complete amino acid sequence has been elucidated (5). Proteolysis in situ is a major problem in the preparation of pure BP from CNS tissues (3, 6). The breakdown of BP was attributed to the action of endogenous proteinases (6) active at acid pH and which is known to increase in animals with EAE (7-9). Further, a similar increase in acid proteinase activity was reported in CNS lesions of patients with multiple sclerosis accompanied by the disappearance of myelin BP (8-11).

Because of its unfolded conformation, the BP is susceptible to hydrolysis by proteolytic enzymes (12) including brain acid proteinases (6); however, the nature of the linkage hydrolyzed by brain proteinase has not been pre-

viously reported. This study describes the breakdown products of BP using purified brain acid proteinase and the nature of one of the susceptible bonds.

EXPERIMENTAL

Myelin BP was isolated from fresh frozen bovine spinal cord following established procedures (3). Acid proteinase (cathepsin D, EC 3.4.4.23) was prepared from bovine brain as described elsewhere (6,13). The BP was incubated with the enzyme (protein to enzyme ratio of 20:1) at 37° in 0.05 moles citrate buffer at pH 3.2. Preliminary experiments indicated that 3 hours of incubation with the enzyme resulted in complete hydrolysis of the BP as shown by polyacrylamide gel electrophoresis (14) at pH 2.4 and 10.6 (15). The enzyme was separated from the peptides on a column (3x100 cm) of Sephadex G-25 (upper portion) and G-75 (lower portion) in equal portions as described elsewhere (12). Peptide III-a was purified by repeated gel filtration on Sephadex G-75, G-100 and G-150 columns. In each case, the size of the column was 2x75 cm. The elution pattern was monitored at 280 nm, fractions common to each peak were combined and lyophilized. The amino acid composition was determined by the Technicon Autoanalyzer using the lithium citrate buffer procedure (16) following hydrolysis with 6N HCl. Tryptophan was quantitated by a modification (17) of the Spies and Chambers procedure (18). The N-terminal amino acid sequence was determined by aminopeptidase M (P-L Biochemicals, Inc., Milwaukee, Wis.) according to published procedures (19) and the C-terminal sequence was established with carboxypeptidase A and B (Worthington Biochemicals, Freehold, N.J.) following previously described methods (20). The EAE assay was performed in guinea pigs and the encephalitogenic activity was evaluated by clinical and histological criteria (21).

RESULTS and DISCUSSION

The basic protein undergoes rapid hydrolysis when incubated with extracellular proteases such as pepsin, trypsin, chymotrypsin and pronase (12), yielding encephalitogenic peptides (22) of known amino acid sequence (5, 23). The relationship between the in-vivo release of encephalitogenic fragments from the myelin basic protein to the etiology of EAE have been based upon the reported findings of acid proteinase in brain tissues and the susceptibility of the BP to degradation in situ (3, 10, 11). Although these studies constitute indirect proof for the hydrolysis of the BP in the native state, evidence for the release of a peptide by brain acid proteinase capable of inducing EAE has not been reported. The present study clearly shows that the BP undergoes proteolysis in the presence of a highly purified brain acid proteinase yielding 3-4 peptide fragments. Polyacrylamide gel electrophoresis of the digest (Fig. 1) shows the complete disappearance of a band corresponding to the basic protein and the simultaneous generation of 4 bands which migrate towards the cathode ahead of the BP. Repeated filtration on columns of Sephadex gels resulted in the isolation of peptide III-a in good yield to permit chemical and biological characterization. Approximately 25 mg of peptide III-a per 100 mg protein was isolated. This constitutes 57% of theoretical yield based on molecular weights of 8645 and 18, 200 for the peptide and the BP respectively. Amino acid analysis of peptide III-a gave values approximating whole integers based on 1 residue of valine, 2 residues of isoleucine and 2 residues of tyrosine giving a total of 81 residues and a calculated molecular weight of 8717 daltons (Table 1). The calculated molecular weight, 8717 daltons, is slightly higher than expected. This is attributed to the finding of 5 glutamic acid and 14 glycine residues by amino acid analysis compared to 4 and 15 residues respectively reported for the amino

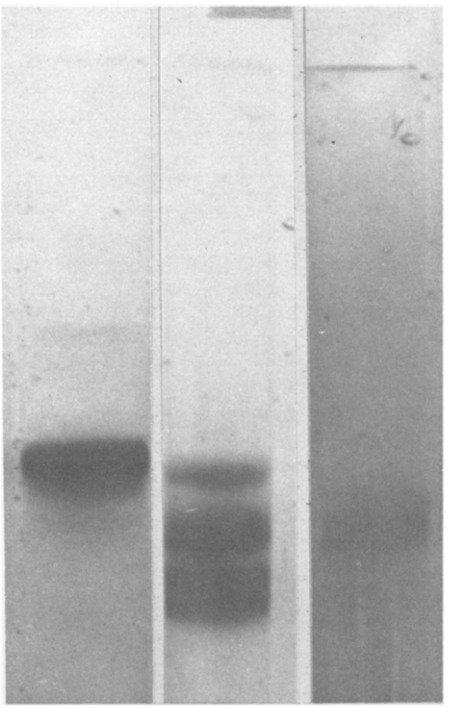

Figure 1. The polyacrylamide gel electrophoresis patterns (pH 2.4) of the purified basic protein (left gel), the enzyme digest (middle gel) and the purified peptide III-a (right gel). Approximately 50 ug protein or peptide was applied to each gel.

acid sequence of this region. Compared to the basic protein, peptide III-a is basic and contains the only tryptophan residue reported for the basic protein molecule (5). The presence of an intact tryptophan region was confirmed by the EAE assay. Injected at 25 and 100 ug doses, peptide III-a induced clinical and histological symptoms of EAE in all 10 animals tested.

The presence of tryptophan, methionine and methylarginine suggest that peptide III-a originates from the C-terminal region of the BP molecule. This was confirmed by N- and C-terminal amino acid sequence analysis. Approximately 1.7 moles of arginine, 0.5 moles of alanine and a trace of methionine per mole of peptide III-a were released within 3 minutes following the addition

Table 1. Amino Acid Composition Of Encephalitogenic Peptide III-a

Amino acid	Residues/Molecule		
	Peptide III-a		B. P.
	Found	Theoretical	
Aspartic acid	4	4	11
Threonine	3	3	7
Serine	10	10	19
Proline	6	6	12
Glutamic acid	5	4	10
Glycine	14	15	25
Alanine	5	5	14
Valine	1	1	3
Methionine	1	1	2
Isoleucine	2	2	3
Leucine	5	5	10
Tyrosine	2	2	4
Phenylalanine	4	4	8
Lysine	8	8	13
Histidine	2	2	10
Arginine and Methylarginine	8	8	18
Tryptophan	1	1	1

The number of amino acid residues per molecule of peptide III-a are computed by assuming one residue of valine, two residues of isoleucine and two residues of tyrosine Approximately 0.3-0.5 residues for methionine and 0.15 residues for methylarginine were found. The theoretical number of residues for both peptide III-a and the basic protein (BP) were computed from the amino acid sequence described in reference 5.

of carboxypeptidase A and B. The amount of alanine increased to 0.8 moles within 80 minutes and a slight increase in methionine (0.2 moles). Similar results were obtained when the BP was used as a substrate. These data are in agreement with previously published results (20) and establish a Met-Ala-Arg-Arg-OH sequence for peptide III-a identical to the C-terminal sequence reported for the BP. In contrast to the BP which has an N-terminal acetyl blocking group (20), a free N-terminal phenylalanine was established for peptide III-a. Aminopeptidase M released 0.1 and 0.05 moles of phenylalanine and lysine respectively per mole of peptide within 3 hours of incubation. Following 7 hours of incubation with the enzyme, the values increased to 0.4

and 0.2 moles respectively. In addition, the enzyme released 0.2 moles of asparagine and 0.1 mole of isoleucine establishing an N-terminal sequence of H-Phe-Lys-Asn-Ile. These results show that brain acid proteinase hydrolyzes the C-phenylalanine bond (residue #89) releasing encephalitogenic peptide III-a with an N-terminal phenylalanine and extending to the C-terminal arginine (residue #170) of the bovine myelin BP. Studies bearing on enzyme specificity using insulin B-chain for a substrate clearly show that cathepsin D catalyzes the hydrolysis of peptide bond preferentially formed between two aromatic amino acids such as Phe and Tyr. Similar conclusions may be drawn using the BP for a substrate. The enzyme catalyzes the hydrolysis of the peptide linkage between the two phenylalanine residues 89-90. The complete amino acid sequence of the BP reveals the presence of another Phe-Phe linkage formed between residue 43 and 44 which may have been hydrolyzed by the enzyme. The finding of more than two bands in the enzyme digest by polyacrylamide gel electrophoresis clearly indicates the presence of at least 3 peptides which are currently being investigated.

ACKNOWLEDGEMENT

Supported by The National Multiple Sclerosis Society Grant No. 876-A-1 and The Continental Corporation Foundation. We thank Mr. F. Stern for his assistance with the amino acid analyzer.

REFERENCES

1. Alvord, E.C., Jr., in The Central Nervous System, (Bailey, O.T. and Smith, D.E., editors), 1st edition, p. 52, Williams and Wilkins Co., Baltimore, M.D. (1968).
2. Nakao, A., Davis, W.J. and Einstein, E.R., Biochim. Biophys. Acta., 130, 163 (1966).
3. Eylar, E.H., Salk, J., Beveridge, G.C. and Brown, L.V., Arch.

Biochem. Biophys., 132, 34 (1969).
4. Adams, C.W.M., Research on Multiple Sclerosis, C. Thomas Co., Springfield, Ill. (1972).
5. Eylar, E.H., Brostoff, S., Hashim, G., Caccam, J. and Burnett, P., J. Biol. Chem., 246, 5770 (1971).
6. Einstein, E.R., Csejtey, J. and Marks, N., FEBS Letters, 1, 191 (1968).
7. Kuzulina, O.P., Terletska, Ya.T., and Syrovatska, L.P., Ukr. J. Biokhim. Zh., 44, 145 (1972).
8. Hallpike, J.F., Adams, C.W.M., Histochem. J., 1, 559 (1969).
9. Marks, N. and Lajtha, A. in Handbook of Neurochemistry, (Lajtha, A., ed.), Vol. 5A, p. 49, Plenum Press, N.Y. (1971).
10. Einstein, E.R., Dalai, K.B. and Csejtey, J., J. Neurol. Sci., 11, 109 (1970).
11. Riekkinen, P.J., Clausen, J., Frey, H.J., Fog, T. and Rinne, U.K., Acta. Neurol. Scandinav., 46, 349 (1970).
12. Hashim, G.A. and Eylar, E.H., Arch. Biochem. Biophys., 129, 635 (1969).
13. Marks, N. and Lajtha, A., Biochemistry (1974) in press.
14. Reisfeld, R.A., Lewis, U.J. and Williams, D.E., Nature, 195, 281 (1962).
15. Deibler, G.E., Martenson, R.E. and Kies, M.W., Prep. Biochem., 2, 139 (1972).
16. Perry, T.L., Stedman, D. and Hansen, S., J. Chromatogr., 38, 460 (1968).
17. Eylar, E.H. and Hashim, G.A., Arch. Biochem. Biophys., 131, 215 (1969).
18. Spies, J. and Chambers, D., Anal. Chem., 21, 1249 (1949).
19. Pfleiderer, G., Celliers, P.G., Stanulovic, M., Wachsmuth, E.D., Determann, H. and Brauntizer, G., Biochemische Zeitschrift, 340, 552 (1964).
20. Hashim, G.A. and Eylar, E.H., Arch. Biochem. Biophys., 135, 324 (1969).
21. Paterson, P.Y., Adv. Immunol., 5, 131 (1966).
22. Hashim, G.A. and Eylar, E.H., Arch. Biochem. Biophys., 129, 645 (1969).
23. Eylar, E.H. and Hashim, G.A., Proc. Natl. Acad. Sci. (U.S.A.), 61, 644 (1968).