TRYPTIC HYDROLYSIS of BRAIN PROTEOLIPID

M. B. Lees, B. F. Messinger and J. D. Burnham

Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, N. H.; Research Laboratory, McLean Hospital, Belmont, Mass.; and Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts.

Received June 12, 1967

A distinguishing characteristic of the protein moiety of the brain white matter proteolipids isolated by Folch and Lees (1951) has been its complete resistance to the action of proteolytic enzymes under conditions which would be suitable for the digestion of most other proteins. This situation has limited the studies which could be carried out on the structure of the protein moiety and its relationship to the lipids. The present study was undertaken to systematically investigate possible requirements for the tryptic hydrolysis of proteolipids. It has been found that proteolipids can be digested by trypsin in the presence of the non-ionic detergent Triton X-100. Evidence is presented to support the conclusion that tryptic hydrolysis occurs under the conditions described.

METHODS

Crude bovine white matter proteolipid (Folch, 1963) consisting of 30 to 50 percent protein was dissolved in 2:1 chloroform-methanol and an aliquot evaporated to dryness. To the dry proteolipid was added an aqueous solution containing 0.6 percent Triton X-100 and either 0.05 M Tris buffer (pH 8.0 at 37°) or 0.01 M phosphate buffer (pH 8.0). In some experiments 0.001 M EDTA was added. A freshly prepared trypsin solution (Sigma Type I: twice recrystallized ethanol precipitate) in buffered Triton was added to give a final trypsin to protein concentration of 1:100 and a total volume of 0.5 ml solution per mg protein. The mixture was incubated at 37° for twenty-four hours with gentle shaking. Suitable proteolipid, enzyme and buffer controls were included with each experiment.

The degree of proteolytic digestion was followed by determining the release of free amino groups on an aliquot of the digest by either the ninhydrin procedure of Moore and Stein (1948) or a modification of the trinitrobenzensulfonate (TNBS) procedure of Satake, et al. (1960) in which the color reaction was allowed to proceed for 15 minutes at 60°. Values were converted to micromoles of amino groups released using a standard curve based on leucine.

RESULTS AND DISCUSSION

Figure 1 shows the release of free amino groups from the proteolipids over a twenty-four hour period. Although experiments were routinely carried out for eighteen to twenty-four hours, at least eighty percent of the digestion had occurred by the end of three hours. The presence of the non-ionic detergent, Triton X-100 was essential for hydrolysis, with maximal digestion occurring at a final detergent concentration of 0.6 percent (Figure 2A). Significantly lower detergent concentrations limited hydrolysis whereas higher concentrations were apparently inhibitory. No tryptic hydrolysis of proteolipid occurred in the presence of various concentrations of either sodium dodecyl sulfate (an anionic detergent) or cetyl trimethylammonium bromide (Cetavlon,^{R} a cationic detergent). Inhibition of tryptic hydrolysis of proteolipid occurred when inorganic salts were added to the digestion medium. Divalent cations (Ca^{++} and Mg^{++}) at a concentration of 0.01 M inhibited the digestion by 60 percent whereas monovalent cations (Na^{+}) caused comparable inhibition at 0.1 M concentration.

The low arginine and lysine content of the proteolipid protein accounts for only 2 and 4% of the total α-amino acid nitrogen respectively (Folch and Lees, 1951; Folch, 1963; Wolfgram and Rose, 1961). As a consequence of the known specificity of trypsin for these residues, a maximum release of 0.5 µmoles of amino groups per mg protein can be expected. In a series of fifteen separate experiments carried out under the same conditions, the proteolipid control and the trypsin control amounted to 0.43 ± 0.11 and $0.14 \pm$

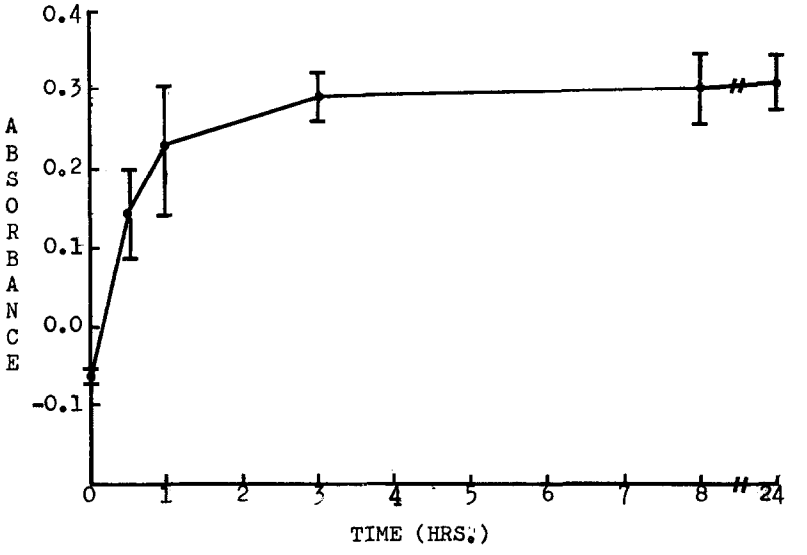

Figure 1: Time course of tryptic hydrolysis of proteolipids: Absorbance readings are for the TNBS procedure and have been corrected for proteolipid and trypsin controls.

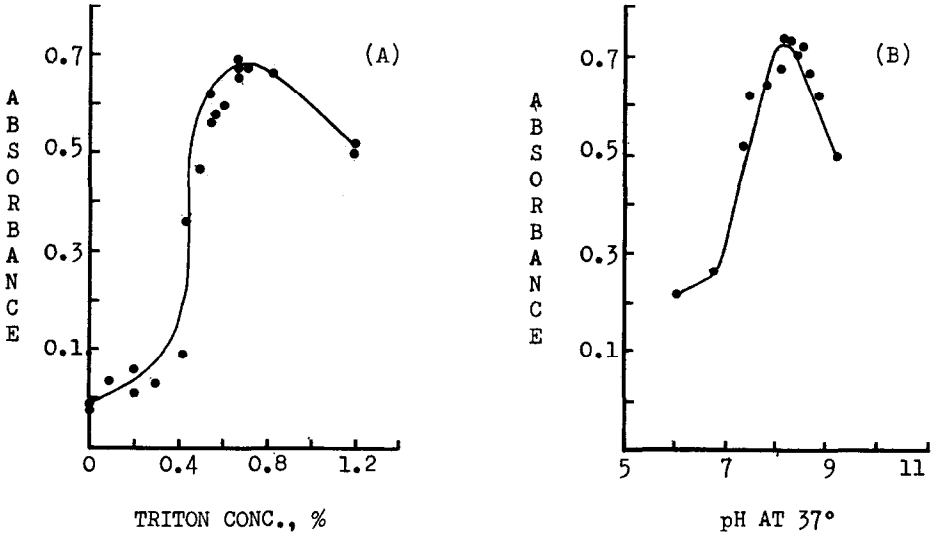

Figure 2: Tryptic hydrolysis of proteolipids under various conditions: A) Effect of Triton X-100 concentration. B) Effect of pH Absorbance readings are for the TNBS procedure and are uncorrected for control values.

0.04 µmoles free amino groups per mg protein, respectively, whereas the digested protein amounted to 0.98 µmoles ± 0.08. The corrected value of 0.41 µmoles free amino groups per mg protein thus corresponds to a release of approximately 80% of the expected groups.

The release of amino groups shown in Figure 1 is compatible with the conclusion that proteolytic digestion has actually occurred. Further evidence for proteolytic digestion is shown by the following: 1) the pH optimum is the same as for tryptic digestion of other proteins (Figure 2B); 2) the release of amino groups is essentially completely inhibited in the presence of soybean trypsin inhibitor; 3) a decreased release of free amino groups occurrs when the incubation is carried out at 60°; this is consistent with enzyme destruction at higher temperatures; 4) there is a fourfold increase in the number of amino groups released by crude trypsin as compared with purified trypsin; 5) an increasing release of free amino groups is observed with chymotrypsin, papain and pronase, in that order. The greater release of free amino groups with less specific enzymes is consistent with the conclusion that proteolytic digestion is occurring.

The use of many conventional techniques for the identification of the products obtained was made difficult by the insolubility in aqueous solutions of both the original proteolipid and a portion of the tryptic residues, by the presence of amino-containing lipids and by the presence of Triton, which is a phenol derivative and reacts to a greater or lesser degree in many procedures for the analysis of proteins. In order to eliminate these problems and obtain further evidence for proteolytic digestion, the tryptic digest was partitioned between a chloroform lower phase and a methanol-water upper phase (Folch, et al; 1957). All of the Triton and the lipids partitioned into the chloroform phase, a large amount of insoluble material accumulated at the interface and peptide fragments were detected in the upper phase, accounting for between one-fourth and one-half of the amino groups freed during tryptic hydrolysis.

Table 1
ANALYSIS OF UPPER PHASE OBTAINED AFTER
$CHCl_3$:CH_3OH:H_2O PARTITION OF TRYPSIN DIGESTED PROTEOLIPID

	Control (- trypsin)	Digest (+ trypsin)
Total protein (Lowry, et al., 1951)	2.8 mg	17.9 mg
Free amino groups (TNBS procedure)	3.3 μM	12.8 μM
Absorbance at 275 mμ	0.067	0.601
Paper electrophoresis (1M acetic acid, pH 2)	0	+++
Thin layer chromatography of amino acids after 6 N HCl hydrolysis	0	+++
Phosphorus	5.5 μg	11.3 μg

120 mg of crude proteolipid (30% protein) was incubated overnight at 37° in 30 ml of 0.6% Triton made up in 0.05 M Tris buffer (pH 8) and containing 670 μg trypsin. $CHCl_3$:CH_3OH 2:1 v/v (114 ml) was added and, after mixing and centrifugation, the upper phase (55ml) was collected and analyzed.

The analysis of the upper phase is given in Table 1 and it is apparent that amino acids and/or peptides are present. There is a sixfold increase in the amount of protein in the upper phase from the tryptic digest as compared with the proteolipid control and a fourfold increase in the number of free amino groups. The trypsin control gave essentially nothing. The high absorbance at 275 mμ compared with the negligible absorbance in the control corresponds to $A^{1cm}_{1\%\ protein} = 18.4$. At least eight protein fragments were detected after paper electrophoresis of the upper phase solutes of the tryptic digest. Hydrolysis of this phase with 6 N HCL produced a five to sixfold increase in the number of free amino groups as measured by the TNBS procedure and these were shown to be a mixture of free amino acids by thin layer chromatography. The significance of the increase in total phosphorous in the tryptic digest upper phase remains to be investigated.

The observation that proteolipids can be attacked by a proteolytic

enzyme of high specificity provides a method which is potentially useful for studying the amino acid sequence of the proteolipid protein, for comparing proteolipids obtained from various tissues, and for understanding the role of proteolipids in membrane structure. In addition, the breakdown of proteolipids in the presence of a surface-active agent may be pertinent to certain pathological conditions of the nervous system. Adams and Tuqan (1961) demonstrated increased proteinase activity and a dissociation of protein and lipid during the course of Wallerian degeneration. In other abnormal conditions characterized by myelin breakdown, proteolytic digestion may be involved.

ACKNOWLEDGEMENTS

This investigation was supported by grants NB 04419 and NB 07136 from the National Institute of Neurological Diseases and Blindness, U. S. Public Health Service. Preliminary studies on various aspects of the effects of proteolytic enzymes on proteolipids were carried out by Mr. David S. Zamierowski while participating in a Dartmouth Medical School student fellowship program.

REFERENCES

Adams, C.W.M. and Tuqan, N.A. J. Neurochem. 6, 334 (1961).
Folch-Pi, J. in J. Folch-Pi and H. Bauer (editors), Brain Lipids and Lipoproteins, and the leucodystrophies, Elsevier, Amsterdam 1963, p. 18.
Folch, J. and Lees, M. J. Biol. Chem. 191, 807 (1951).
Folch, J., Lees, M. and Sloane Stanley, G.H. J. Biol. Chem. 226, 497 (1957).
Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R. J. J. Biol. Chem. 193, 265 (1951).
Moore, S. and Stein, W.H. J. Biol.Chem. 176, 367 (1948).
Satake, K., Okuyama, T., Ohashi, M. and Shinoda, T. J. Biochem. 47, 654 (1960).
Wolfgram, F. and Rose, A.S. J.Neurochem. 8, 161 (1961).