DEGRADATION OF ENCEPHALITOGEN BY PURIFIED BRAIN ACID PROTEINASE

Elizabeth ROBOZ EINSTEIN and Judit CSEJTEY
Departments of Neurology and Biochemistry, University of California, School of Medicine, San Francisco, Calif., USA

and

Neville MARKS
The New York State Research Institute for Neurochemistry and Drug Addiction, Columbia University, New York City, N.Y., USA

Received 11 July 1968

1. Introduction

The expression "encephalitogen" in the title refers to a basic protein(s) of the myelin in the neural tissue. Injection of this protein into experimental animals produces an immunological reaction known as experimental allergic encephalitis (EAE) which results in paralysis.

Three encephalitogenic proteins with different mobilities and varying molecular weights have been isolated previously by Nakao, Davis and Einstein [1]. The question arose whether these encephalitogens are present endogenously in the neural tissue or whether the two smaller ones are enzymatic breakdown products of the larger one. There was some evidence to suggest that the smaller molecular weight encephalitogen may have derived from the larger one through action of a proteolytic enzyme present in the neural tissue [2].

The encephalitogen was incubated with a purified acid proteinase isolated from bovine brain [3]. After 5 hours, the substrate was degraded with the formation of three distinct protein (polypeptide) bands as monitored by acrylamide electrophoresis. One of these proteins corresponded to the protein reported earlier and was tested and found active.

In tracing the encephalitogen in the myelin, a novel use of solubilization of all the proteins and electrophoresis were employed. The electrophoretic pattern indicated that encephalitogen, after the lengthy process of purification, had the same position as originally present in the myelin. The present communication offers evidence that for the activity, only a portion of the protein molecule is needed.

2. Experimental methods and results

The encephalitogenic protein is a constituent of the myelin. The exact number of proteins present in the myelin was not known previously, because chloroform methanol, used for solubilization of the myelin, is not suitable for electrophoretic technique. Based on the method of Work [4] for ribosomal proteins, phenol-acetic acid-water was used for both extraction and electrophoretic separation of the myelin proteins. Fig. 1 shows that the encephalitogen, after a lengthy purification procedure, retains the same electrophoretic position as in the original myelin. (The myelin was prepared according to Autilio, Norton and Terry [5].)

Preparation of myelin on a large scale is a lengthy procedure, therefore, bovine spinal cord was used as a source for isolation of the encephalitogen (fig. 2). Details for isolation of the encephalitogen are reported elsewhere [6].

The acid proteinase was prepared from bovine brain cortex by the method of Marks and D'Monte [7] based on the technique of Barrett [8] worked out for liver proteinase , as summarized in fig. 3.

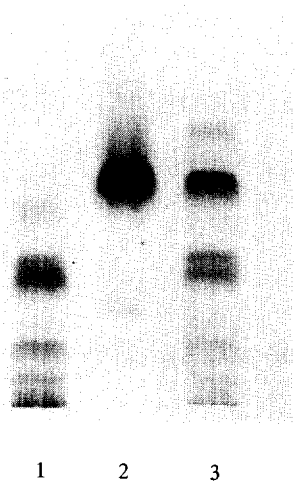

Fig. 1. Acrylamide electrophoresis of neural proteins. 1. Proteolipids. 2. Enceph. prep. 3. Myelin. Gel 10%, buffer phenol, acetic acid, water 2:1:1; *V* 250, length of run 24 hr; *T* 4°C; stain, 5% Amidoblack in acetic acid, methanol, water 1:4:5.

Brain proteinase activity was established with hemoglobin and then utilized for experiments with the encephalitogen. Three techniques for assay of the activity were employed: 1) pH Stat method, 2) ninhydrin reaction of liberated peptides, and 3) electrophoresis of the breakdown products on acrylamide gel. The results obtained with the three techniques proved that the encephalitogen is a substrate for the acid proteinase. Most relevant to this report is the electrophoretic experiment. Here 3 mg of encephalitogen were dissolved in 0.8 ml of Na acetate buffer pH 3.8 and reacted with 100 μg of the acid proteinase dissolved in 0.2 ml buffer and incubated at intervals from 10 min to 24 hr. As a control, the encephalitogen was incubated for 10 min and 24 hr. After incubation, the mixture was immersed in dry ice and acetone mixture and freeze-dried. The degradation products were electrophoresed on acrylamide gel with urea,

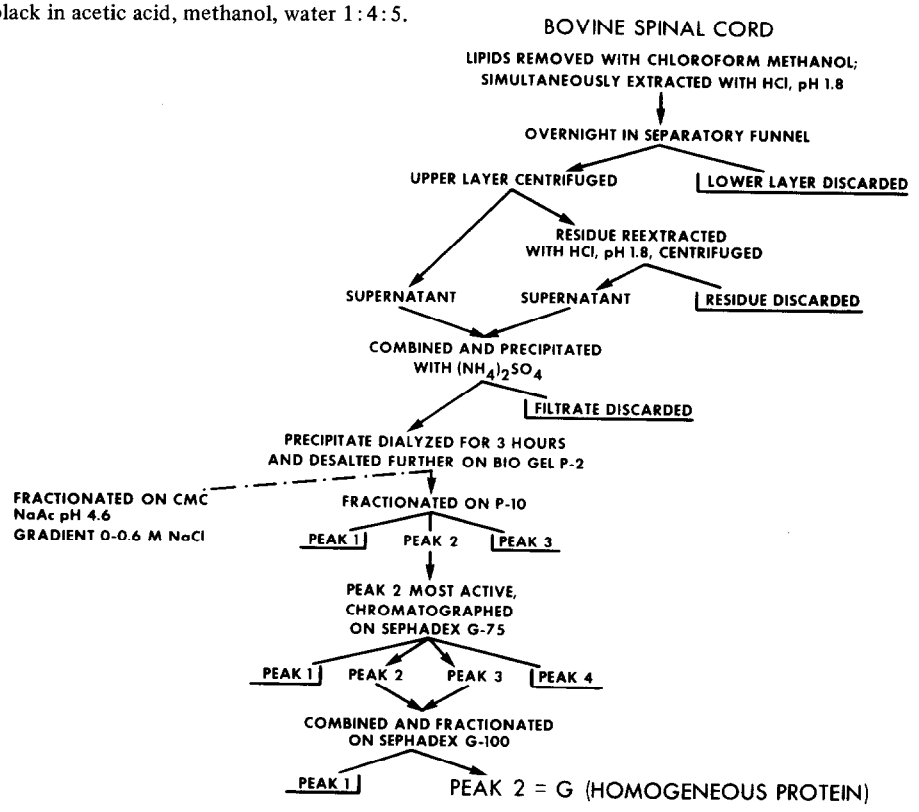

Fig. 2. The isolation of encephalitogen G.

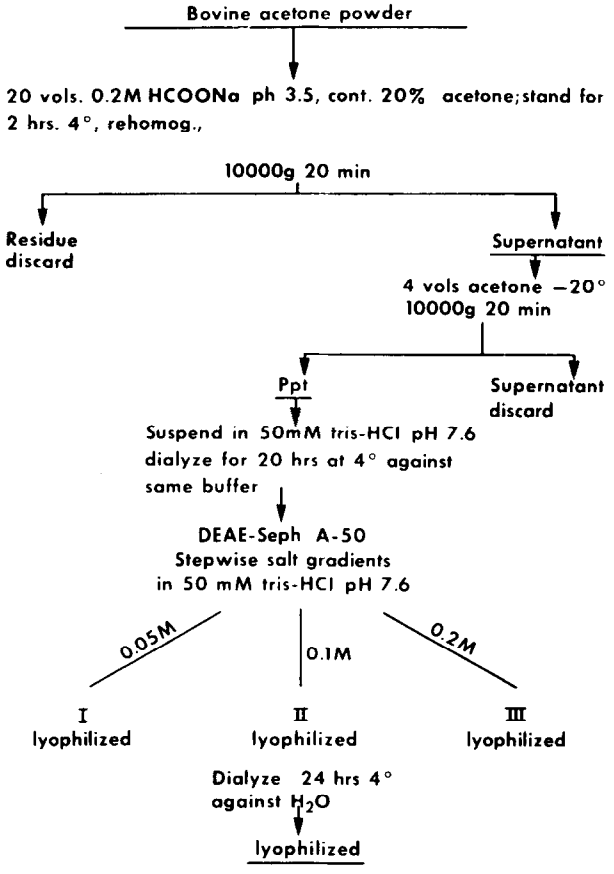

Fig. 3. Preparation of acid proteinase.

as in the method used for histones by Mauritsen et al. [9]. The formation of breakdown products with time is illustrated in fig. 5. The results demonstrate complete breakdown of the encephalitogen after 5 hours' incubation, yielding 3 smaller proteins (polypeptides). These degradation products retained EAE activity and one of the proteins corresponds to the smaller active protein previously isolated and described [1,2]. This polypeptide has now been isolated from the enzyme incubation mixture and was found to be encephalitogenic [10].

The pH dependence of hydrolysis of encephalitogen G and denatured hemoglobin was studied under conditions previously described for hemoglobin [3]. In the present study, incubation volume was 1 ml containing 100 μg purified acid proteinase with 5 mg denatured hemoglobin or 1 mg encephalitogen G. In-

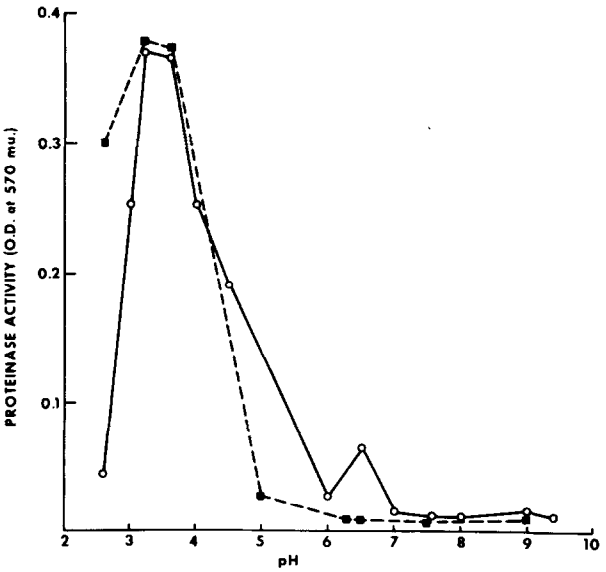

Fig. 4. pH dependence of protein breakdown. Encephalitogen G = –o–; Hemoglobin = ---▪---. The buffer system: 50 mM barbital-acetate (range pH 2.6–9.4).

cubations were continued for 2 hrs at 37° and 0.5 ml of 18% TCA was then added; the acid soluble ninhydrin positive materials were read at 570 mμ after appropriate dilution with 50% ethanol. With both proteins maximum breakdown occurred at pH 3.2, as shown in fig. 4. In the case of the encephalitogen, a second smaller peak was observed at pH 6.5.

3. Discussion

The various molecular sizes of encephalitogenic protein reported by different investigators [11–13] may be attributed in part to methodological differences. We obtained molecular weights of 12,400–20,000 by ultracentrifugation, 15,800 by calculation of the lysine and arginine content of peptides obtained by tryptic digestion and 37,000 by gel permeability measurements [1,14]. Similar differences in molecular weight determinations were found by Tomasi and Kornguth [15] who reported values of 20,000 by ultracentrifugation and 50,000 by gel filtration. The isolation of three encephalitogens with distinct molecular weights may indicate that they are native to the neural tissue or are aggregation products or result

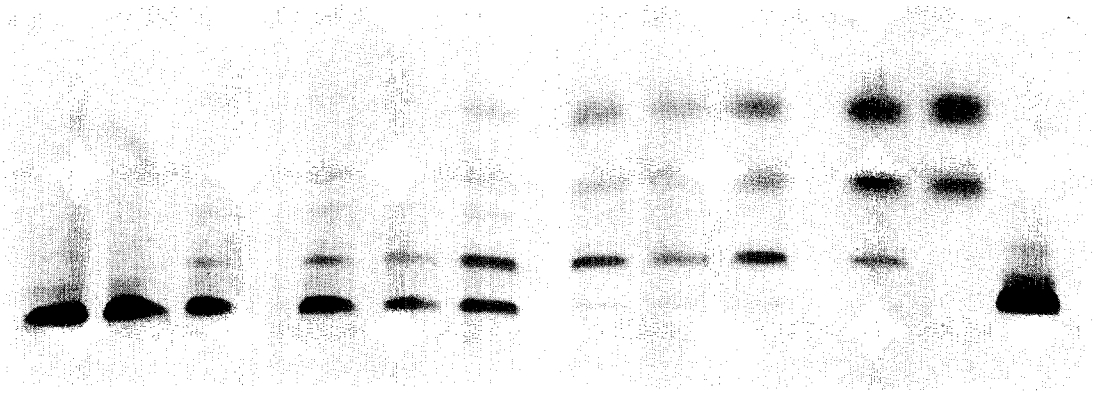

Fig. 5. Acid proteinase activity on encephalitogenic protein G. Acrylamide electrophoresis: gel 15%, soaked in buffer containing 4M urea; buffer: formic acid, acetic acid, water 1:4:45, current 50mA, length of run 7 hr, T 4°C, stain: 0.5% Amidoblack in acetic acid, methanol, water 1:4:5.

from enzymatic breakdown. Preliminary experiments indicated that enzymatic degradation was probable since extraction at pH 3.2, which is the pH optimum for the acid proteinase, resulted in three encephalitogens; extraction at pH 1.8, at which there is only minimal enzyme activity, resulted mainly in one component which represented the largest molecular species. The acid proteinase has been isolated from brain [3], with an optimal activity of pH 2.8–3.2. Our contention was that this enzyme is present in the pH 3.2 extracts and was responsible for the subsequent degradation. The availability of purified enzyme now provides conclusive evidence that the acid proteinase can degrade the encephalitogen into smaller active compounds and this could account for the transformation of the large to the smaller molecular weight components.

Studies on the pH dependence of encephalitogenic breakdown show maximum activity at pH 3.2 and this is identical to that previously reported for denatured hemoglobin as the substrate [3]. Purified acid proteinase preparations contained activity at pH 6.5 with encephalitogen G as the substrate. Since activity at this pH was absent with hemoglobin, it was concluded that typical neutral proteinases were absent. This finding suggests the interesting possibility that proteinase preparations contain additional enzymes which specifically hydrolyse encephalitogenic proteins at higher pH.

ACKNOWLEDGEMENT

These studies were supported by USPHS Grant NB 02545, the National Multiple Sclerosis Society Grant No. 457, and in part by the Hartford Foundation.

REFERENCES

[1] A.Nakao, W.J.Davis and E.R.Einstein, Biochim. Biophys. Acta 130, 163 Part I (1966).
[2] A.Nakao, W.J.Davis and E.R.Einstein, Biochim. Biophys. Acta 130, 171, Part II (1966).
[3] N.Marks and A.Lajtha, Biochem. J. 97 (1965) 74.
[4] T.S.Work, J. Molec. Biol. 10 (1964) 544.
[5] L.A.Autilio, W.T.Norton and R.D.Terry, J. Neurochem. 11 (1964) 17.
[6] E.R.Einstein, J.Csejtey, W.Davis, A.Lajtha and N. Marks, Int. Arch. Allergy (1968) in press.
[7] N.Marks and B.D'Monte, J. Neurochem. (1968) in press.
[8] A.J.Barrett, Biochem. J. 104 (1967) 601.
[9] C.M.Mauritzen, W.C.Starbuck, I.S.Saroja, C.W.Taylor and H.Busch, J. Biol. Chem. 242 (1967) 2240.
[10] P.Chao and E.R. Einstein, unpublished (1968).
[11] E.C.Alvord, S.Hruby, C.S.August and M.W.Kies, Fed. Proc. 25 (1966) 355.
[12] R.F.Kibler and R.Shapira, J. Biol. Chem. 243 (1968) 281.
[13] P.R.Carnegie, B.Bencina and G.Lamoureux, Biochem. J. 105 (1967) 559.
[14] E.R.Einstein, D.M.Robertson, J.M.DiCaprio and W. Moore, J. Neurochem. 9 (1962) 353.
[15] L.G.Tomasi and S.E.Kornguth, J. Biol. Chem. 242 (1967) 4933.