COMPARISON OF SOME PROPERTIES OF A PROTEOLYTIC ENZYME ISOLATED FROM ANIMAL MYELIN AND FROM BODY FLUIDS OF PATIENTS WITH MULTIPLE SCLEROSIS

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The problem of the metabolic inertia of myelin is now being reexamined [7]. A number of enzymes which evidently play a direct part in metabolism of the structural components of the biological membrane have been found in myelin taken from the central nervous system (CNS) [5]. The presence of at least two proteolytic enzymes has been demonstrated in myelin – leucine aminopeptidase (LAP) and a neutral protease [8] with pH-optimum between 7.2 and 7.6.

However, protease activity from the myelin of healthy animals is comparatively low and does not correspond to the half-life of animal myelin proteins, which is 20-40 days [12]. It has accordingly been suggested that myelin also contains other enzymes with proteolytic activity.

The aim of this investigation was to look for a new proteolytic enzyme in myelin, to isolate it, and to study its role in demyelination.

EXPERIMENTAL METHOD

Myelin was removed from the rabbit brain immediately before use by the method of Norton and Poduslo. All operations were performed in the cold at 0-4°C [1].

The enzyme was removed from the purified myelin preparation by an original method. For this purpose a suspension of myelin (5-8 mg protein) in a dilution of 1:14 was applied to a chromatographic column measuring 5 × 1 cm, with facilities for centrifugation, and packed with Sephadex G-75, then centrifuged for 2 min at 700 g, washed with physiological saline, freed from ballast proteins, and recentrifuged. The column was then kept for 4-5 days at 0-4°C. During this time, as a result of auto-oxidation, rupture of the chemical bonds evidently took place between the enzyme and the binding agent, which has high affinity for Sephadex. The enzyme was then eluted with 5 ml of physiological saline.

The eluate was concentrated with Ficoll 400 to a volume of 0.4-0.6 ml. Activity of the enzyme thus isolated was determined by the method in [6], using an LAP test kit (from Fermognost, East Germany). The results were expressed in activity units: 1 U denotes the quantity of enzyme which hydrolyzes 1 μ mole of leucine hydrazide in 1 min at 37°C.

Protein was determiend by Lowry's method and specific activity of the enzyme was expressed in units/mg protein.

Enzyme isolation by the method described above also was undertaken from the cerebrospinal fluid (CSF) and blood serum of 76 patients with multiple sclerosis (MS), of whom 36 were in a stage of remission and 40 in a stage of exacerbation. In this case, 1-2 ml of the body fluid was applied to the column packed with Sephadex, or 3-5 ml for electrophoretic investigations.

The electrophoretic mobility of the enzyme was studied with the aid of cellulose acetate plates (from Beckman, USA) by a modified method [10]. The sample in a volume of 1 μl

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was applied to the plate which was placed in an electrophoresis chamber filled with 0.05 M Tris-borate buffer, pH 9.2. Electrophoresis was carried out with a voltage of 250 V and a current of 5 mA for 30 min. Next the samples were treated with a 3 M solution of ammonium sulfate in 0.1 M Tris-HCl buffer, pH 7.0, immersed in a bath with the substrate (18 mmoles of leucine hydrazide in 5 ml of 0.1 M ethanolamine buffer, pH 9.7) for 60 min, and developed with a solution of dimethylaminobenzaldehyde. The gels after electrophoresis were fixed with a mixture containing 40% methanol and 10% formalin in the ratio of 2:1.

EXPERIMENTAL RESULTS

Specific activity of the enzyme isolated by the method described above was 2.6 U/mg protein, which is higher than the total activity of the known proteases of myelin [9]. It was found that enzyme activity could be substantially increased by eluting the enzyme with formaldehyde solution in one stage. Under these circumstances the ballast proteins were removed with a 2-4% solution, and the enzyme with a 6-8% solution of formaldehyde. Activity of the enzyme, purified in this way, varied from 8 to 26 U/mg protein, with a degree of purity 650 times higher than initially. Meanwhile analysis showed that the properties of the enzyme, isolated by different methods, were identical.

The isolated enzyme differed from the known proteases of myelin, first, in having a pH-optimum at 9.6-9.8 (the pH-optimum of known myelin proteases is 7.2-7.8), and second, by substrate specificity: the enzyme did not catalyze hydrolysis of the classical arylamidase substrates, which are most frequently used to determine activity of myelin proteases (leucine-p-nitranilide, leucine-β-naphthylamide [11]), but readily hydrolyzed leucine hydrazide and leucinamide – substrates which are usually used to determine LAP activity of somatic organs. The results suggested that the isolated enzyme is a multiple form of LAP.

Since one of the conditions of the method is that the enzyme must be isolated immediately before use (not later than 30 min after decapitation of the animal) investigation of myeline from human brain tissue, obtained from the victim of an accident, could not yield positive results. To confirm the presence of the test enzyme in myelin from human brain tissue, investigations were accordingly undertaken to study its activity in the body fluids of patients with a primary demyelinating disease. Activity of the enzyme in blood serum from patients with MS was found to be significnatly ($P < 0.01$) higher, by 2.35 ± 0.28 U/liter, than in the control group. No changes in LAP activity in the CSF were found compared with the control. However, after treatment of the samples by our suggested method of purification of the enzyme, additional (bound) activity could be detected. Under these circumstances, activity of the enzyme in the serum from patients with MS in a stage of remission was increased from 4.2 ± 0.6 to 17.3 ± 2.7 U/liter, and in the exacerbation stage from 3.6 ± 0.4 to 28.8 ± 1.3 U/liter. This dependence was even more marked for the CSF: increased from 0.65 ± 0.16 to 70.5 ± 8.3 U/liter in the stage of remission and from 0.40 ± 0.09 to 99.4 ± 5.1 U/liter in the stage of exacerbation.

It could thus be concluded from the significant increase in activity of the enzyme in the stage of exacerbation compared with its level in the remission stage ($P < 0.01$) and also from the higher value of bound enzyme activity in the CSF, that the enzyme enters the body fluids from myeline of the CNS, possibly in the form of a complex with its inhibitor.

The substrate specificity and pH-optimum of the enzyme isolated from the body fluids of patients with MS were found to be similar to properties of the enzyme from animal brain myelin. To prove that the enzyme isolated form different sources is identical, a comparative electrophoretic investigation was undertaken.

The results confirmed this hypothesis: it will be clear from the results of electrophoresis (Fig. 1) that the enzyme from animal myelin and from the serum and CSF of patients with MS stays at the starting line, whereas LAP in the serum of patients with acute hepatitis (the hepatic form) and nephritis (the renal form) possesses considerably electrophoretic mobility.

The results of this comparative study of the properties of the enzyme isolated from blood serum and CSF of patients with MS and from animal myelin led to the conclusion that an analogous enzyme (possibly a multiple form of LAP) is present in myelin of the human CNS.

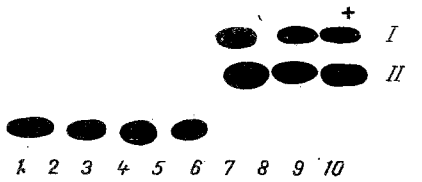

Fig. 1. Comparative study of electrophoretic mobility of enzyme preparations isolated from various sources on cellulose acetate plates: 1, 2) enzyme from myelin; 4, 5) enzyme from blood serum of patients with MS; I (7, 9, 10) LAP from blood serum of patients with acute nephritis (renal form); II (7, 9, 10) the same from patients with acute hepatitis (hepatic form).

These results confirmed the hypothesis that proteolytic enzymes play a leading role in the mechanism of the demyelination process. Under the influence of a pathogenic factor the enzyme–inhibitor complex is evidently destroyed with release of the active enzyme which, in turn, destroys the protein stroma of the myelin. Under these circumstances the inhibitor passes into the body fluids, where it binds the serum and hepatic forms of LAP [3]. Ivanova [2] has drawn attention to the possible outflow of an inhibitor, specific for LAP, from foci of disintegration of myelin.

As regards the immediate causes of destruction of the inhibitor–enzyme complex, this may be associated with the intensification of lipid peroxidation in patients with MS [4]. The results now obtained warrant a study of the trigger mechanisms of the demyelination process at the molecular level.

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