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The separation and identification of enolase isozymes of brain and sciatic nerve by high-pressure liquid anion-exchange chromatography

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A rapid technique for separating and quantitating the three enolase isozymes present in rodent brain and sciatic nerve was developed using high-pressure liquid anion-exchange chromatograhy. At pH 7.9, one cationic and two anionic enzyme forms were separated with baseline resolution in an imidazole buffer containing ethylenediaminetetraacetic acid (EDTA) and magnesium. The recovery of enolase activity was 90% or greater for brain and 85% for sciatic nerve. Chromatography of liver and axon-free (degenerated) sciatic nerve allowed the identification of non-neuronal, hybrid, and neuron-specific enolase isozymes. These enzyme forms, respectively, constituted 40%, 29% and 19% of total activity in brain, and 63%, 13% and 4% of total activity in normal sciatic nerve.

Pathogenetic theories of axon degeneration in certain peripheral neuropathies have stressed a defect in the cellular machinery of energy production as the critical event[11]. Experiments designed to test this hypothesis have been complicated by an inability to separate metabolic enzymes of Schwann cell and axonal origin in nerve homogenates. The identification and characterization of a single enolase isozyme (2-phospho-D-glycerate hydrolase; EC 4.2.1.11) restricted to neurons and neuroendocrine cells[5,7,10] has provided a system which can be utilized to circumvent these difficulties. Neuron-specific enolase (NSE) has been shown to be the most heat-stable of the enolase isozymes[6], and heat inactivation of non-neuronal isozymes has been utilized to measure NSE in brain and nerve supernatant solutions[2,3]. Antibodies directed against the NSE isozyme do not cross-react with oligodendroglial enolase and have provided another technique to estimate the quantity of NSE[4,8]. Low-pressure chromatography by DEAE-anion exchange has been used to separate, purify and quantitate the enolase isozymes of rat brain[1,13] and the tissue concentrations reported in these studies agree with those established by immunoassay[1]. This paper presents a rapid and direct micro-technique to separate enolase isozymes with high yield and to quantity axonal enolase activity.

All tissues were dissected from male Sprague–Dawley rats sacrificed by decapitation. Prior to sacrifice, rats were housed on corn-cob bedding with free access to Purina rat chow and water. Brain and liver homogenates were prepared using a Polytron tissue homogenizer (Brinkman). Organs were disrupted in 4 vols. of ice-cold 25 mM imidazole-HCl buffer at pH 7.9, containing 1 mM EDTA and 5 mM $MgSO_4$ (buffer A). Homogenates of sciatic nerve were prepared in the same manner except that 10 vols. of buffer A was used. Brain and nerve preparations were centrifuged at 105,000 *g* for 60 min. Liver homogenate required an initial low-speed centrifugation (10,000 *g* for 20 min) in addition to ultracentrifugation. The clear supernatant solutions were rapidly frozen on dry-ice and stored at −80 °C until chromatography. The enolase activity of these preparations was stable for several weeks when stored in this manner.

The soluble cationic and anionic proteins of brain or nerve supernatant were separated at room tem-

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perature on a 10-cm diethylaminoethyl (DEAE) 5PW protein pak column (Waters Associates). The high-pressure liquid chromatography (HPLC) apparatus consisted of a Beckman Model 332 high-pressure gradient liquid chromatograph, an LKB Uvicord S absorbance monitor with HPLC flow cell and strip chart recorder, and an LKB Ultrorac II fraction collector. The column was equilibrated prior to a chromatographic run with multiple volumes of water, buffer A with 1 M sodium chloride, and buffer A. Samples were loaded onto the column, and proteins were eluted in multiple steps by a microprocessor-controlled salt gradient. This was accomplished by an initial 10-min isocratic wash with buffer A followed by a two-step linear application of buffer A containing sodium chloride. In the first salt step, the ionic strength was increased from 0 to 450 mM over 8 min, followed by a 5-min isocratic period and then a long shallow linear gradient which increased the ionic strength from 450 mM to 500 mM over 40 min. The column void was collected in 1-ml fractions while active enolase fractions in the gradient were collected in 0.5 or 0.4 ml volumes.

The enolase (EC 4.2.1.11) activity of the eluted fractions was measured by the method of Marangos and colleagues[5] with minor modifications. The increase in absorbance at 240 nm was measured at 25 °C in a final assay volume of 1 ml.

The high-pressure liquid-anion-exchange chromatography technique described above was effective in rapidly separating the three enolase isozymes present in rodent neuronal tissue. Two-hundred μl samples of rat brain supernatant solution were chromatographed to establish the procedure. Enolase isozyme I (numbers assigned in the order of elution from the DEAE column) was easily separated from forms II and III due to its cationic nature at pH 7.9. Anionic enolase isozymes II and III were separated with baseline resolution (peak separation of 2.8 min) by the programmed gradient described above. The com-

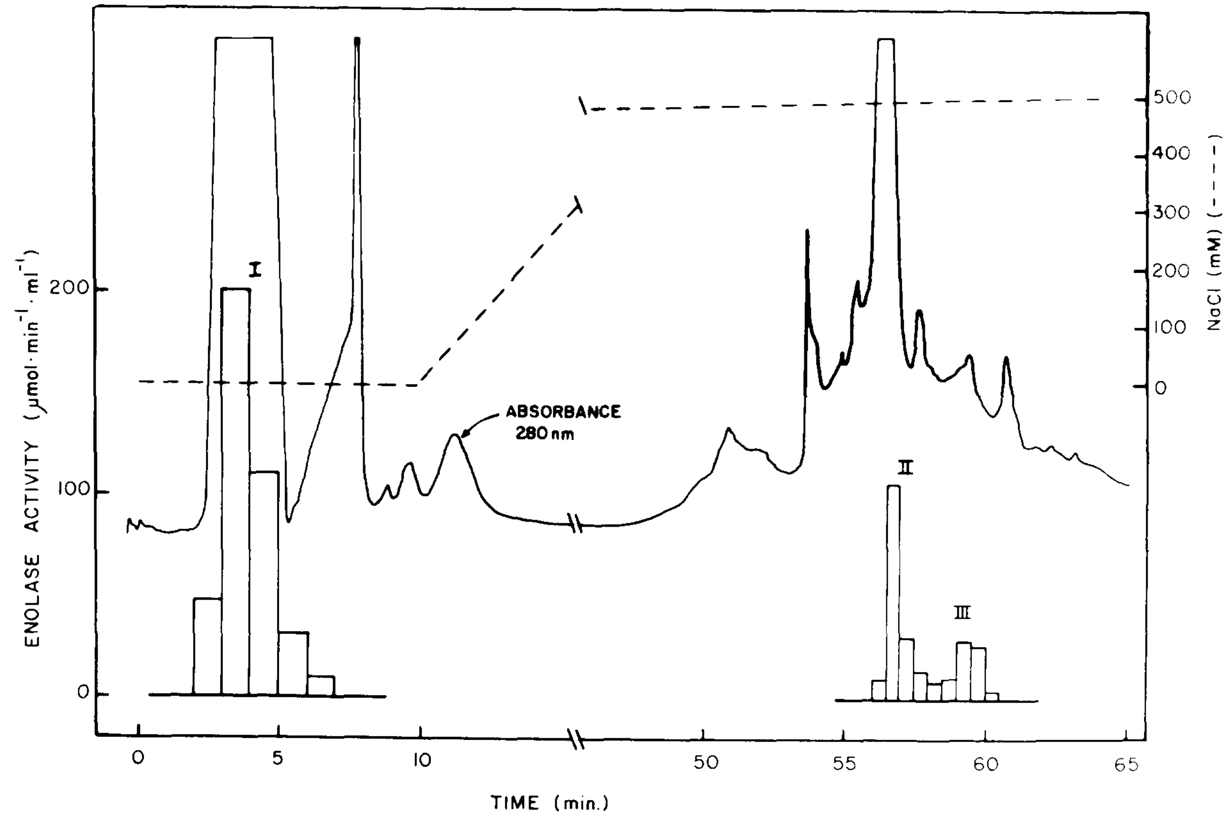

Fig. 1. High-pressure liquid anion-exchange chromatography of the enolase isozymes of sciatic nerve. Eight-hundred μl of sciatic nerve supernatant solution was chromatographed at room temperature. Vertical bars represent the enolase activity of eluted fractions. The solid line shows the profile of protein elution as measured by continuously monitoring the absorbance at 280 nm. The dashed line indicates the sodium chloride concentration. Enolase isozymes are identified by the roman numerals I, II and III.

plete resolution of all three enolase isozymes was achieved in 60 min with a 90% recovery of activity. Isozymes I, II and III contributed 40%, 29% and 19% of the total enolase activity of this preparation, respectively.

The results of a typical separation of an 800-μl sample of rat sciatic nerve supernatant solution are shown in Fig. 1. The sodium chloride gradient used for an optimal separation of brain isozymes was also effective in separating analogous enzyme forms from sciatic nerve. Anionic enolase isozymes (II and III) were separated by 2.0 min as measured from peak to peak. The recovery of enolase activity was 85%. Isozymes I, II and III, respectively, contributed 63%, 13% and 4% of total enolase activity in normal nerve.

Experiments were conducted to identify the various enolase isozymes observed in our system. A 200-μl sample of liver supernatant solution was chromatographed. Ninety-nine percent of the applied enolase activity was found under peak I, indicating that form I is non-neuronal in origin. No hepatic enolase activity could be detected in the region where peaks II and III were eluted (data not shown).

To identify enolase isozymes of neuronal origin axon-free sciatic nerve was prepared in a manner analogous to previously described methods[9,12]. Rats were anesthetized with sodium pentobarbital and bilateral sciatic nerves were surgically exposed from the notch to the trifurcation of tibial, sural and peroneal nerves. Exposed nerves were bilaterally transected at a point 1–1.5 cm below the sciatic notch. Proximal and distal sections of nerve were reflected 180° from the point of transection and sutured into muscle. The incisions were closed and the animals were kept warm to facilitate recovery. After 17 days, the incisions were reopened, and the distal stump of each sciatic nerve located and recut to ensure an absence of reinnervation. Axon-free nerves enriched in Schwann cells were harvested 6 weeks after the initial transection and were homogenized in an identical manner to normal sciatic nerve.

The distribution of enolase activity observed in the 6-week degenerated preparation of sciatic nerve is shown in Fig. 2. In axon-free nerve, isozyme I was significantly increased. Peak II was markedly attenuated, while peak III was absent in this nerve preparation. Isozymes I, II and III contributed 86%, 4% and 0% of the total enolase activity, respectively. These observations strongly suggest that enzyme form III is neuron-specific enolase. Thus, peak II can be identified as the hybrid enzyme form.

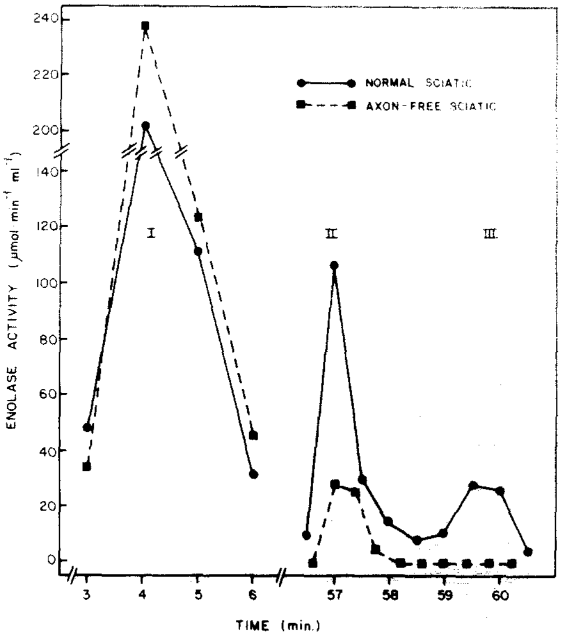

Fig. 2. High-pressure liquid anion-exchange chromatography of the enolase isozymes of normal (●—●) and axon-free (degenerated) (■- - -■) sciatic nerve. Enolase isozymes are indicated by the roman numerals I, II and III.

Early studies which measured neuron-specific enolase as the activity remaining after heat inactivation of the non-neuronal isozymes reported values of 37% and 15% for the contribution of the neuron-specific enzyme to the total enolase activity of rodent brain and sciatic nerve, respectively[2]. Measurements from feline brain and sciatic nerve estimated the neuron-specific isozyme at 16% and 6% of total enolase activity[3]. Surprisingly, the values from the cat nervous system agree more closely with the present observations of rodent brain and sciatic nerve. Low-pressure chromatographic separations of the enolase isozymes from whole rodent brain are in agreement with our observations, neuron-specific enolase representing approximately 18% of total enolase activity[13].

The distribution of enolase isozymes in rodent peripheral nerve differs from that observed in brain. As one might expect, a greater percentage of total activity is non-neuronal or of Schwann cell origin in peripheral nerve. In normal sciatic nerve, the Schwann cell compartment contributes approximately 60% of the total enolase activity. This is increased to 86% of total activity in denervated distal stump enriched in Schwann cells (Fig. 2). The difference in enolase content between normal and axon-free nerves provides the physiological basis of our assignment of tissue origin for enolases I, II and III. The loss of peak III in axon-free nerve indicates that this enzyme form corresponds to NSE. The hybrid form (II) is significantly reduced but still present in axon-free nerves. Since neuron-specific enolase is absent from the degenerated preparation, it is unlikely that the hybrid arises as an artifact of the homogenization of forms I and III as has been suggested[13]. Whether the reduction in the activity of the hybrid form (II) indicates a contribution to axonal enolase is presently unclear.

The direct measurement of axonal enolase activity has been demonstrated in rodent sciatic nerve. The technique takes advantage of the rapid separation and high resolution achievable with HPLC systems. Typical separations require as little as 1.5 mg of protein from either brain or sciatic supernatant solution. A high yield of magnesium-stabilized neuronal isozyme is recovered in approximately 60 min. These data provide a basis to reinvestigate the action of selected neurotoxins (e.g. acrylamide) which have been suggested to inhibit neuron-specific enolase and thereby precipitate axonal degeneration.

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