

## Identification and Isolation of a 155-kDa Protein with Neuropathy Target Esterase Activity<sup>1</sup>

CHRISTOPHER E. MACKAY,\* BRUCE D. HAMMOCK,† AND BARRY W. WILSON\*

\*Departments of Avian Science and Environmental Toxicology and †Entomology, University of California, Davis, California 95616

Received December 12, 1994; accepted September 19, 1995

Identification and Isolation of a 155-kDa Protein with Neuropathy Target Esterase Activity. MACKAY, C. E., HAMMOCK, B. D., AND WILSON, B. W. (1995). *Fundam. Appl. Toxicol.* 30, 23-30.

A method is presented for the isolation of a 155-kDa protein that possesses phenyl valerate hydrolysis activity in the presence of paraoxon but is inhibited by mipafox; the functional definition of neuropathy target esterase (neurotoxic esterase; NTE). Microsomes, isolated from 18-day-old chicken embryos were treated with phospholipase A<sub>2</sub> to solubilize the NTE activity. The extract was then combined with polyoxyethylene W1 detergent and resolved by gel filtration chromatography to yield an active fraction with an approximate mass of 200 kDa. This fraction was further purified by preparative isoelectric focusing and native electrophoresis to yield two separate bands possessing NTE activity. The slower migrating band was highly enriched in a 155-kDa protein that was identified as a source of the NTE activity by affinity chromatography using 3-(9'-mercaptiononylthio)-1,1,1-trifluoropropan-2-one bound to Sepharose CL6B. This represents the first report of the isolation of NTE in its active form and aids in the confirmation of the 155-kDa protein as the most likely candidate for NTE. © 1996 Society of Toxicology

The study of organophosphorus-induced delayed neuropathy (OPIDN) has been hindered because of the lack of a clear site of action for the causative agents. The concept that the inhibition of an esterase was responsible for OPIDN dates back to the work of Aldridge and Barnes (1966). Johnson proposed that this esterase was that activity manifest in the presence of paraoxon, a nonneuropathic organophosphorus agent, and absent in the presence of the neuropathic mipafox (Johnson, 1969a). He termed this activity neurotoxic esterase (neuropathy target esterase; NTE).

A likely candidate for NTE was identified by Johnson (1969b) when he discovered a series of proteins that radiolabeled with [<sup>3</sup>H]diisofluorophosphate (DFP) in the absence of mipafox, but not in its presence. Later work using differential inhibitors indicated that NTE was a 155-kDa protein (Wil-

liams and Johnson, 1981). However, corroboration based on esterase activity was not forthcoming, since the enrichment procedure used involved the inactivation of the enzyme. The first successful attempt at affinity purification of the 155-kDa protein was accomplished by Thomas *et al.* (1993) using 3-(9'-mercaptiononylthio)-1,1,1-trifluoropropan-2-one (MNTFP) bound to Sepharose. Recently, Glynn *et al.* (1994), reported the isolation of the 155-kDa fragment using a biotinylated saligenin phosphate analog as an active site ligand. Neither method resulted in an active purified enzyme. Attempts to isolate the active enzyme by gel filtration chromatography studies have yielded a wide range of estimated sizes for the protein from approximately 850 to 1800 kDa (Thomas *et al.*, 1990; Pope and Padilla, 1989; Chemnitius *et al.*, 1984). The problem of identification has been further compounded by numerous reports of multiple isoforms of NTE (Davis *et al.*, 1980; Olajos and Rosenblum, 1981; Chemnitius and Zech, 1984; Chemnitius *et al.*, 1984; Carrington and Abou-Donia, 1985; Tormo *et al.*, 1993).

The purpose of this line of investigation was twofold: first, to discover the sources of the proposed NTE enzymatic activity, and second, to develop a method of isolation of these sources for further study.

### MATERIALS AND METHODS

**Microsomal preparations.** Microsomes were derived from 18-day-old chicken embryos. Embryos were decapitated and the whole brains placed on ice, washed with 0.33 M sucrose, and homogenized with a Polytron in 50 mM Tris-HCl (pH 8.0) using 3 ml solvent per gram fresh tissue. The homogenate was centrifuged at 10,000g for 20 min. The supernatant was removed and the pellet ground once again in the same buffer and proportional volume. The microsomes were extracted from the supernatant by centrifugation at 100,000g for 60 min.

**NTE solubilization.** NTE activity was isolated from the microsomal pellet by a modification of the method of Seifert and Wilson (1994). The pellets were suspended in 50 mM Tris-HCl buffer, pH 8.8, using a glass homogenizer and 0.4 ml of buffer per gram initial brain fresh tissue weight. To this suspension, the equivalent of 12.5 U/ml phospholipase A<sub>2</sub> (isolated from *Apis mellifera*<sup>2</sup>) was added and the microsomal suspension incubated at 37°C for 30 min. After incubation, the suspension was placed on ice and

<sup>1</sup> Portions of this research were presented at the 34th Society of Toxicology Meetings (1995) and the 7th International Toxicology Congress (1995).

<sup>2</sup> Sigma Chemical Co., St. Louis, MO.

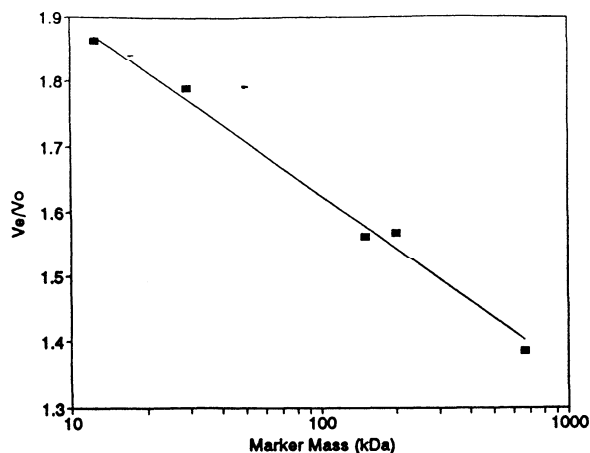


FIG. 1. Calibration curve of the gel filtration chromatography column. The column used was a  $5 \times 100$ -cm S-400 HR column<sup>3</sup> with a reported molecular cut-off of 2000 kDa. Flow was maintained using a peristaltic pump at a constant output of 5 ml/min. The column's total exclusion was determined to be 220 ml using dextran blue (average mass, 2000 kDa). The selective permeation range was standardized using known commercial protein standards<sup>2</sup>: thyroglobulin (663 kDa),  $\beta$ -amylase (200 kDa), alcohol dehydrogenase (150 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.5 kDa). Values are the mean of three determinations resolved to the closest milliliter.

EDTA, EGTA, and DTT were added to yield final concentrations of 1 mM, 1 mM, and 250  $\mu$ M, respectively. This suspension was then centrifuged at 100,000g for 60 min and the resulting supernatant collected and tested for NTE activity. The final supernatants were routinely stored in liquid nitrogen prior to further purification.

**Gel filtration chromatography.** Gel filtration chromatography was performed with a  $5 \times 100$ -cm S-400 HR column<sup>3</sup> (molecular cut-off of 2000 kDa) using a peristaltic pump-based Econoline liquid chromatography system.<sup>4</sup> The column's exclusion volume was determined using dextran blue (average mass, 2000 kDa) and standardized using known commercial protein standards<sup>2</sup> with a range from 663 kDa (thyroglobulin) to 12.5 kDa (cytochrome c; Fig. 1). The exclusion volume was found to be 220 ml with a void volume of 475 ml.

The basic buffer used in the gel filtration chromatography consisted of 50 mM Tris/HCl, 1 mM EDTA, and 1 mM EGTA, pH 8.2 (buffer 1), to which various detergents and adjuncts were added. In the testing, 2-ml samples of phospholipase A<sub>2</sub>-solubilized NTE extracts were combined with the agent/s in buffer 1 and incubated at 4°C for 1 hr. An aliquot of 100  $\mu$ l was retained for NTE activity determinations and the rest applied to the column. The samples were eluted with the same buffer. Eluates were collected in 5-ml aliquots that were analyzed for NTE activity. Eluates containing chaotropic agent which adversely affected the NTE enzyme assay were dialyzed 1:40,000 against buffer 1 containing the indicated detergent prior to analysis.

The most successful method utilized buffer 1 containing 0.1% Polyoxyethylene W1,<sup>2</sup> and 500 mM NaCl, at pH 8.2 (buffer 2). W1 is a combination of 64% PEG(20) cetyl alcohol (Brij 58) and 36% PEG(10) cetyl alcohol (Brij 56). The 2-ml preparations were injected onto the column and eluted with the same buffer 2. Fractions were collected in 1- to 5-ml aliquots

and analyzed for NTE activity. Chromatography was performed at room temperature because of the high cloud point of the W1 detergent.

**Preparative isoelectric focusing.** Isoelectric focusing was performed in a fluid matrix using a Rotofor system.<sup>4</sup> The 200-kDa fraction with the highest NTE activity was collected from several gel filtration runs and concentrated by ultrafiltration to an NTE activity of 300 nmol/min·ml. Samples of 50 ml with 5% ampholyte (Biolyte 3/10<sup>4</sup>) added were applied to the chamber and focused under a constant power output of 12 W. The temperature was maintained at 10°C with a circulating refrigerated water/glycol bath. Focusing was deemed complete when a constant potential across the cell was attained (approximately 10 hr). The samples were harvested in 20 fractions and the pH determined with a temperature-compensated pH meter standardized against 50 mM potassium biphthalate buffer (pH 4.0), 50 mM potassium phosphate monobasic-sodium hydroxide buffer (pH 7.0), and 50 mM potassium carbonate-potassium borate-potassium hydroxide buffer (pH 10.0). The approximate pI ranges reported were those that bracketed the fraction containing the protein of interest.

**Native electrophoresis.** NTE active fractions from the preparative IEF were solubilized in buffer 1 containing 0.1% W1 and 250 mM sucrose, pH 8.2 (buffer 3). These samples were applied to a  $7 \times 8$ -cm 0.75% agarose gel made from a 6.8 pH Tris/glycine buffer containing 1% W1 and 250 mM sucrose. A running buffer of 25 mM Tris, 192 mM glycine, 250 mM sucrose, and 0.1% W1, pH 8.3, was used. A constant field of 200 V was applied to the agarose gel until the solvent front ran to within 0.5 cm of the bottom of the gel. Typical running times were approximately 60 min. The gel was sectioned into 5-mm lateral sections, macerated by passing through an 18-gauge syringe needle, combined with either 1 ml of buffer 2 for NTE activity analysis and affinity chromatography or 1 ml Laemmli buffer for SDS-PAGE, and centrifuged at 10,000g for 10 min.

**Affinity chromatography.** Affinity chromatography was performed using MNITFP bound to Sepharose CL6B (MNITFP was shown to be an inhibitor of NTE activity by Thomas *et al.*, 1993). The inhibitor was prepared in accordance to the procedure of Szekacs *et al.* (1989). Aliquots of 1 ml containing approximately 100 nmol/min NTE activity in buffer 3 were applied to a 20% (w/v) slurry of prewashed gel in a ratio of 1:1 and incubated with gentle agitation at 4°C for 12 hr. The fluid was removed from the gel suspension by 10,000g centrifugation and the gel washed five times with equal volumes of buffer 1. The bound protein was eluted from the gel in Laemmli buffer after incubation at 100°C for 5 min. When the same procedure was performed on Sepharose CL6B, no significant nonselective binding was observed.

**SDS-PAGE electrophoresis.** Sample purity was monitored by SDS-PAGE. Samples were desalted when necessary by ultrafiltration using a Minicon concentrator<sup>5</sup> and then taken up in Laemmli buffer. Low salt samples were simply combined 1:1 with 2 $\times$  Laemmli buffer. After the addition of the Laemmli buffer, the samples were incubated at 100°C for 3 min, loaded onto a  $7 \times 8$ -cm 4–20% polyacrylamide linear gradient gel and electrophoresed at a constant voltage of 200 V until the sample front was within 0.5 cm of the bottom of the gel. Typical running times were approximately 40 min. Molecular equivalent masses were determined by comparing retention times to known standards<sup>2</sup> run concurrent with the samples.

**Inhibitor profile and NTE activity.** NTE activity was determined by a method modified from Correll and Ehrlich (1991) based on phenol evolution from phenyl valerate hydrolysis. Total phenol valerate activity was determined as enzymatic hydrolysis with no inhibitors added. NTE activity was defined as that portion of enzymatic phenol valerate hydrolysis present after pretreatment with 40  $\mu$ M paraoxon but inhibited after pretreatment with 50  $\mu$ M mipafox. Analyses were performed in 96-well plates. Samples of varying volume were combined with buffer 2 to yield a final volume of 100  $\mu$ l per well. To these samples either paraoxon or a combination of paraoxon

<sup>3</sup> Pharmacia Biotech Inc., Piscataway NJ.

<sup>4</sup> Bio Rad Laboratories, Hercules, CA.

<sup>5</sup> Amicon Inc., Beverly, MA.

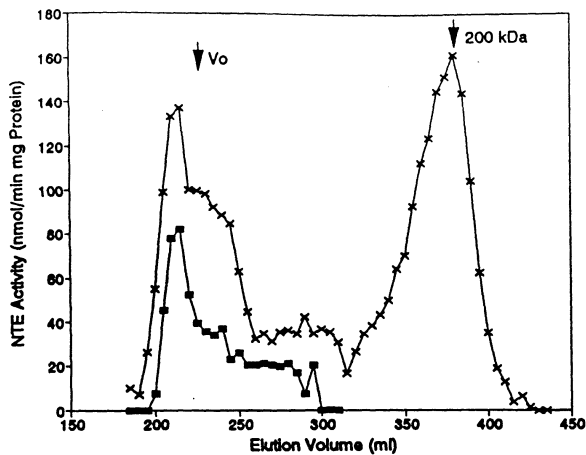


FIG. 2. Gel filtration profile of total esterase activity for phenyl valerate (X) and NTE activity (■) eluted in a Triton X-100 buffer. Chick brain extracts of 1 ml were combined 1:1 with buffer 2 containing 1 M NaCl and 0.6% Triton X-100. After 30 min, the samples were applied to a  $3 \times 100$ -cm S-400 high-resolution gel filtration column and eluted with the same buffer. The arrows represent the exclusion volume and the mean elution volumes based on known standards.

and mipafox were added to yield final concentrations of 40  $\mu$ M paraoxon and 50  $\mu$ M mipafox and the plates incubated at 37°C for 15 min. After incubation, 50  $\mu$ l of 6.7 mM phenyl valerate was added to a final concentration of 1.9 mM per well and the plates incubated at 37°C for 15 to 60 min. The reaction was stopped by the addition of 50  $\mu$ l of 10% aqueous SDS solution containing 0.04% aminoantipyrine. The chromophore was resolved by the addition of 50  $\mu$ l of 0.5% potassium ferricyanide. The absorbances were determined at 490 nm after 5 min incubation at room temperature using a Bio-Tek 96-well plate reader.<sup>6</sup> Phenol concentration was determined from a phenol standard curve. NTE activity was expressed as the amount of phenol evolved per unit time. In the determinations of mipafox inhibition curves, the same procedure was used varying the concentration of the inhibitor. In all cases mipafox was applied for 30 min at 37°C and pH 8.2.

Protein concentrations were determined by the Bradford method using a commercial Coomassie reagent.<sup>4</sup> In buffers containing W1, which interferes with the Bradford test, protein concentrations were determined using the Lowry method (Lowry *et al.*, 1951). In all determinations, the protein concentrations were determined by comparison to a standard curve based on bovine serum albumin fraction V.<sup>2</sup>

## RESULTS

### NTE Solubilization

Solubilizing up to 95% of NTE activity in the supernatant was accomplished by scaling up the methods of Seifert and Wilson (1994) utilizing phospholipase A<sub>2</sub>. However, the resulting activity, like that from detergent-solubilized preparations, tended to be unstable even at low temperatures. Incorporation of 1 mM EDTA, 1 mM EGTA, and 250  $\mu$ M DTT greatly increased the longevity of the NTE activity, permit-

ting storage of the extracts at 4°C for up to 5 days without significant loss of activity.

### Gel Filtration Chromatography

When using either phospholipase A<sub>2</sub> pretreatment (buffer 1) or Triton X-100 as a solubilizing detergent, NTE activity consistently appeared in the exclusion volume of the column, indicating a mass greater than 2000 kDa (Fig. 2). The addition of chaotropic agents such as NaCl, sucrose, KSCN, urea, and guanethidine either had no effect on the elution profile or resulted in a total loss of NTE activity (Table 1). Similar results occurred when zwitterionic detergents such as *N*-alkyl-*N,N*-dimethyl-3-ammonio-1-propanesulfonate (Zwittergent) 3-10 and 3-12 and 3-[(3-chloramidopropyl)-dimethylammonio]-1-propanesulfonate (Chaps) or nonionic detergents such as *n*-octylglucoside or *N,N*-dimethyldodecylamino-*N*-oxide (LDAO) were used to increase the protein's solubility.

Resolution of an active NTE fraction of  $200 \pm 30$  kDa by gel filtration was accomplished by the addition of 0.1% W1 and 500 mM NaCl to the phospholipase solubilized mi-

TABLE 1  
Effects of Various Detergents and Chaotropic Agents on the Gel Exclusion Profile of Neuropathy Target Esterase (NTE)

Treatment	NTE activity <sup>a</sup> (%)	Gel filtration profile
0.3% Triton X-100	100	Exclusion volume <sup>b</sup>
0.5 M NaCl	100	Exclusion volume <sup>c</sup>
1 M Sucrose	100	Exclusion volume
2 M Urea	20 $\pm$ 3	No NTE activity detected
4 M Urea	0	No NTE activity detected
2 M Guanidine HCl	0	No NTE activity detected
1 M KSCN	0	No NTE activity detected
0.1% Zwittergent 3-10	0	Not tested
0.1% Zwittergent 3-12	0	Not tested
0.1% <i>N</i> -octylglucoside	84 $\pm$ 7	Exclusion volume
0.3% Chaps	100	Exclusion volume
0.1% LDAO	37 $\pm$ 2	Exclusion volume
0.3% LDAO	0	Not tested
0.1% Polyoxyethylene W1	100	Elution 1000–200 kDa
0.1% Polyoxyethylene W1 with 0.5 M NaCl	100	Elution at approx. 200 kDa

<sup>a</sup> All solutions were made in buffer 1 (see text) containing 30 nmol/min · ml NTE activity from phospholipase A<sub>2</sub> solubilized extracts. Specific activity compared to a solution compared to a concomitant suspension made up in 0.3% Triton X-100.

<sup>b</sup> All NTE activity appears in the exclusion volume (see Fig. 2) unless otherwise indicated.

<sup>c</sup> There was a shoulder on the descending region of the exclusion volume peak. Its exact size was unknown since it was outside the range of the gel filtration standards.

<sup>6</sup> Bio-Tek Instruments, Winooski, VT.

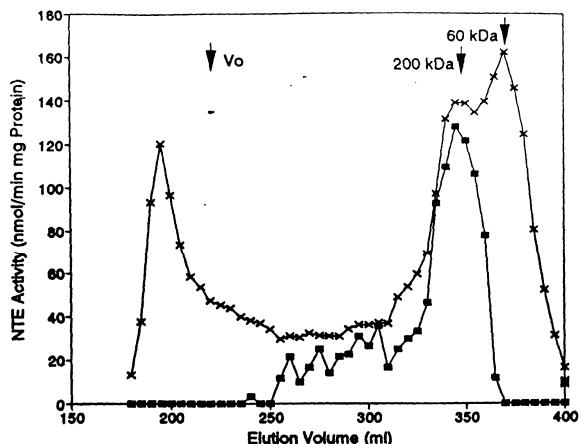


FIG. 3. Gel filtration profile of total esterase activity for phenyl valerate hydrolysis (X) and NTE activity (■) eluted in a polyoxyethylene W1 buffer. Phospholipase  $A_2$ -solubilized chick brain extracts of 1 ml were combined 1:1 with buffer 2 containing 1 M NaCl and 0.2% polyoxyethylene. After 30 min, the samples were applied to a  $3 \times 100$ -cm S-400 HR gel filtration column and eluted with the same buffer. Arrows represent the exclusion volume and 200 kDa volume based on the mean elution volumes of known standards.

rosomal preparation (Fig. 3). Both phospholipase treatment and 0.1% W1/500 mM NaCl were necessary to get this resolution. Lower concentrations of W1 resulted in poor migration, and higher W1 concentrations tended to precipitate even at room temperature. The yield of soluble enzyme dropped to only 40% of the total when the microsomes were solubilized directly with W1/NaCl treatment. The gel exclusion profile of NTE activity first appeared in the exclusion volume with highly prolonged tailing. Furthermore, W1/NaCl was necessary in both the sample and the elution buffers to prevent the majority of the activity eluting in the exclusion volume. Analysis of mipafox inhibition curves suggested that the NTE activity obtained was the same enzyme responsible for the NTE activity in the initial extract (Table 2). Unfortunately, SDS-PAGE showed numerous proteins coeluting with the NTE activity (data not shown). Although all mipafox-resistant phenyl valerate esterase activity was removed, there was a residual paraoxon-sensitive activity that accounted for about 50% of the total phenyl valerate hydrolase activity.

To determine whether the enzyme eluting at 200 kDa was a constituent of the 2000+ kDa fraction, phospholipase-treated extract in 0.5 M NaCl and 0.3% Triton X-100 was run on the column using buffer 2 as an elution buffer. Again, the activity was recovered from the exclusion volume. When this fraction was combined with 0.1% W1 and passed over the same column again using buffer 2 as the elution buffer, the majority of the activity eluted at 200 kDa (Fig. 4).

There was no significant increase in the specific activity

of the enzyme obtained by gel filtration. However, when the protein removed from the active fraction was accounted for, calculations indicated that an apparent purification of 29-fold had occurred. This suggests there was an appreciable deactivation of the enzyme during purification. This trend was seen throughout the purification process; large amounts of protein without NTE activity were removed from the active fraction with no appreciable increase in NTE specific activity.

#### Preparative Isoelectric Focusing

NTE activity migrated in a pH band of  $4.5 \pm 0.6$  when the enriched 200-kDa fraction from the gel filtration was focused in the Rotofor. No other NTE activity was found. A precipitate was formed within the cell containing approximately 80% of the NTE activity. This precipitate and associated activity could be resolubilized in buffer 2.

The NTE activity isolated in the 200-kDa fraction showed the same mipafox inhibition curve as the initial extract (Table 2). Based on specific activity, the IEF procedure resulted in a fivefold concentration over gel exclusion and a fourfold increase over the initial extract. However, it did not remove the residual paraoxon sensitive esterase activity which still accounted for approximately 50% of the total.

Reversing the order of the gel filtration/IEF method resulted in poor chromatographic resolution. The gel filtration of the solubilized NTE fraction after IEF tended to spread the activity between the cut-off and the approximate 200-kDa elution volume with no consistent single peaks (data not shown).

#### Native Electrophoresis

Native electrophoresis of NTE has been unsuccessful in the past; the NTE enzyme activity precipitated at the gel interface (perhaps because of local increases of protein concentration relative to detergent concentration). To get active NTE to migrate into a 0.75% agarose gel, it was necessary to use W1 and 250 mM sucrose in the sample, gel, and running buffers. Omission from any of these buffers resulted in either the lack of enzyme migrating into the gel or a total loss in resolution. Two peaks of activity were found when the IEF fraction containing the NTE activity was resolved in this manner; a minor one that migrated with the solvent front, and a major one that remained near the top of the gel (Fig. 5). All of the activity in the upper band met the definition of NTE activity. Only 12% of the activity in the lower band was paraoxon-resistant and mipafox sensitive. SDS-PAGE of the upper fraction showed a prominent band of approximately 155 kDa that accounted for about 43% of the total protein on the SDS-PAGE gel. SDS-PAGE of the lower migration band did not show an obvious 155-kDa band, but high contamination made it difficult to make any inferences. The most interesting difference between the two

TABLE 2  
Activity, Protein Concentrations Recovery, and Mipafox Inhibition Values for NTE throughout the Enzyme Purification Process

Treatment	NTE activity		[Protein] ( $\mu\text{g/ml}$ )	% Recoverable <sup>a</sup> NTE activity	Protein enrichment factor <sup>b</sup>	$I_{50}$ <sup>c</sup> ( $\mu\text{M}$ Mipafox)
	nmol/mg Protein	nmol/ml				
Phospholipase A <sub>2</sub> -solubilized extract	155 $\pm$ 68	380 $\pm$ 167	2450 $\pm$ 485	100	1	1.93 $\pm$ 0.12
NTE activity isolated in the 200- kDa fraction	121 $\pm$ 54	4.63 $\pm$ 2.1	32.3 $\pm$ 6.43	78	64	1.89 $\pm$ 0.17
NTE activity isolated after IEF	594 $\pm$ 105	4.53 $\pm$ 0.8	7.63 $\pm$ 0.73	383	321	1.77 $\pm$ 0.16
Total NTE from native electrophoresis	182 $\pm$ 21	0.597 $\pm$ 0.1	3.27 $\pm$ 0.29	118	749	—
Slow migrating NTE from native electrophoresis	280 $\pm$ 24	0.572 $\pm$ 0.1	2.04 $\pm$ 0.19	181	1200	0.94 $\pm$ 0.11
Fast migrating NTE from native electrophoresis	85 $\pm$ 10	0.423 $\pm$ 0.1	4.98 $\pm$ 0.52	42	492	3.3 $\pm$ 0.29

<sup>a</sup> Percentage ratio of the specific activity of the fraction over the specific activity of the initial extract.

<sup>b</sup> Estimate of the total protein to NTE ratio based on the amount of protein removed from the indicated active fraction.

<sup>c</sup> The estimates of  $I_{50}$  are based on a 30-min incubation with mipafox at 37°C.

was a significant change in the mipafox inhibition curve; The  $I_{50}$  of the lower band was 3.3  $\mu\text{M}$  while the  $I_{50}$  of the upper band was 0.94  $\mu\text{M}$  (Table 2; Fig. 6).

#### Affinity Chromatography

Final identification and purification of the 155-kDa band as the source of the NTE activity was performed by selective

binding to the MNTFP-Sepharose gel. This trifluoroketone has been shown to be a potent inhibitor of NTE as well as being able to selectively bind the 155-kDa protein in affinity chromatography (Thomas *et al.*, 1990, 1993). The NTE activity was lost from the soluble fraction when the upper NTE-active band from the native electrophoresis was combined with the affinity media and incubated at 4°C for 12 hr (Fig. 7). Furthermore, there was a loss of the 155-kDa

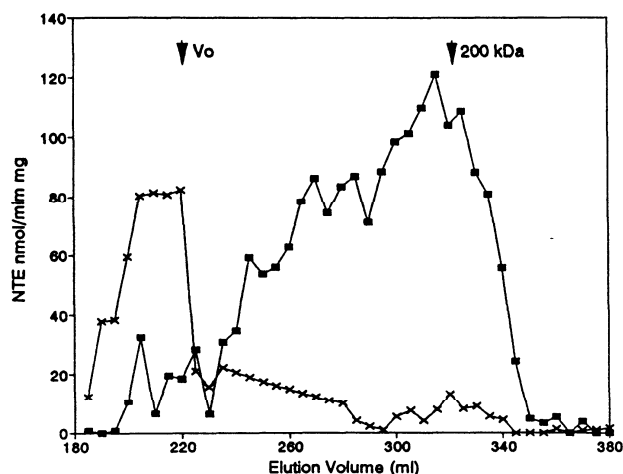


FIG. 4. Comparison of gel filtration profile of phospholipase A<sub>2</sub>-solubilized NTE. Samples of 2 ml containing approximately 150 nmol/min · mg protein in buffer 1 were combined with 0.3% Triton X-100 and 500 mM NaCl and eluted with the same buffer (X). The fractions with the highest specific NTE activity (225–235 ml) were concentrated to 2 ml, resuspended in buffer 1 containing 0.1% W1 and 500 mM NaCl, and again applied to the column (■).

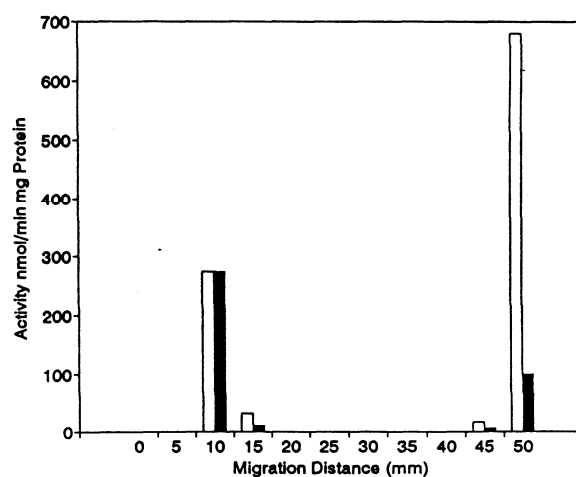


FIG. 5. Activity profile of phenyl valerate hydrolysis (□) and NTE (■) from the native electrophoretic gel. Solubilized aliquots were applied to a 0.75% agarose gel in 0.1% polyoxyethylene W1 and 250 mM sucrose. A constant potential of 200 V was applied until the dye front was within 0.5 cm from the bottom. The gel was then cut into 5-mm slices, the contents extracted with buffer 3, and the eluates examined for enzyme activity and protein content.

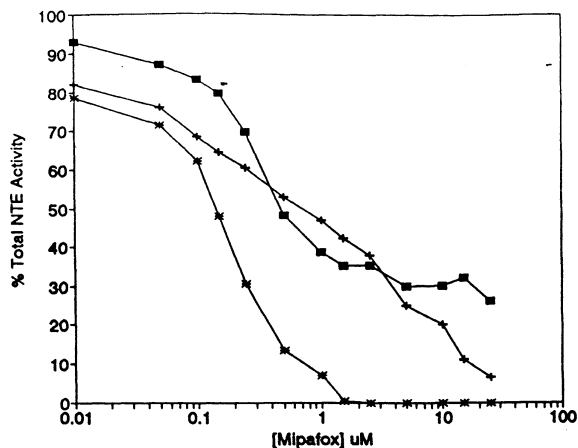


FIG. 6. Mipafox inhibition curves for the initial phospholipase  $A_2$ -solubilized brain extracts (■) and the two NTE activity bands from the native electrophoretic gel, the slow migrating [\*] and fast migrating (+) bands.

band from the gel (Fig. 8) with SDS-PAGE of the same fraction. The 155-kDa fragment was retrieved when the affinity media was eluted with Lamelli buffer. Pretreating the native electrophoretic eluate with 350  $\mu$ M paraoxon did not affect binding of the 155-kDa protein, while it was inhibited by pretreatment with 50  $\mu$ M mipafox. Protein concentrations retrieved from the SDS-PAGE of the 155-kDa protein were equivalent to 65 ng/g brain fresh weight. There was no significant loss in NTE activity when the lower band was subjected to the same treatment, suggesting that it had no affinity for the MNTFP ligand.

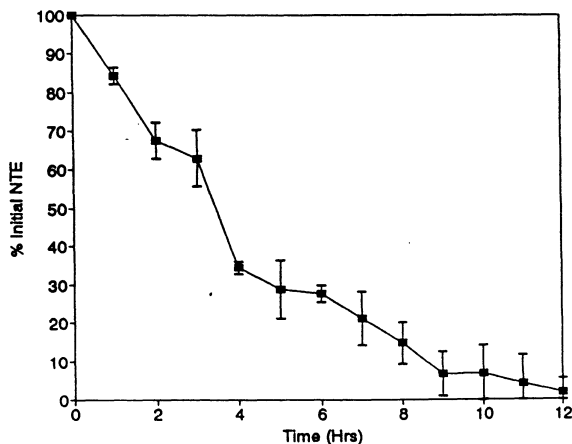


FIG. 7. Time course of NTE activity loss in the supernatant during incubation with MNTFP-Sepharose affinity binding gel compared to a similar concurrent incubation with Sepharose alone. All incubations were performed at 4°C. Values represent the mean of three repetitions  $\pm$  SE.

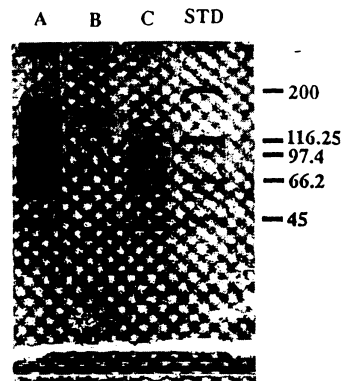


FIG. 8. SDS-PAGE of native electrophoretic and affinity chromatographic eluates. The figure is a composite of three silver stained gels. Lane A represents the eluate from the native gel of the slow migrating NTE activity band. Lane B represents the protein eluted in Lamelli buffer from the MNTFP-Sepharose after incubation with the eluate from the slowly migrating NTE active region of the native electrophoresis gel. Note the highly prominent single band of 138–157 kDa. Lane C represents the proteins remaining in solution after 12 hr. incubation with the MNTFP affinity gel which lacks the 150-kDa band manifest in lane B.

## DISCUSSION

The basic paradigm of NTE research was established in 1969 (b) when Johnson defined NTE as a 155-kDa protein that was labeled with [ $^3$ H]DFP in the presence of paraoxon, but inhibited by pretreatment with mipafox, and that the phenyl valerate hydrolysis activity that was likewise inhibited by mipafox but active in the presence of paraoxon was due to NTE activity (Johnson, 1969a, 1977). However, over the past 24 years, a molecular basis for this definition has not been forthcoming, and attempts at isolating NTE in an active form have not been successful.

There have been several attempts at isolating NTE by gel filtration. Estimates of the mass of NTE have ranged from 1800 kDa (Chemnitiu *et al.*, 1984) to 970 kDa (Pope and Padilla, 1989) to 850 kDa (Thomas *et al.*, 1990). Pope and Padilla (1989) reported that the 970-kDa active NTE fraction contained a 148- to 160-kDa protein that demonstrated the proper inhibition properties with regard to [ $^3$ H]DFP binding but had no NTE activity. They hypothesized that the previously identified 155-kDa protein was a subfragment of this larger NTE molecule.

One explanation for these conflicting results is that NTE may be unstable in an aqueous medium. This would explain why detergents such as the zwittergent series inactivated the enzyme. Success in the dissociation of the 850+ kDa superform may result directly in the inactivation of the enzyme. Exceptions are the reports of Ishikawa *et al.* (1983) and Thomas *et al.* (1989), who utilized sucrose gradient centrifugation to get NTE activity to migrate into the S9

fraction that correlated with a mass of 155–178 kDa. This suggested that if the ambient conditions were appropriate, the 155-kDa protein could be isolated in an active form. However, no effect on the 850+ aggregate form was seen when high sucrose concentrations were used in conjunction with gel exclusion. Furthermore, with the exception of high NaCl or sucrose concentrations, which had no effect on dissociating the aggregate form, all the chaotropic agents tested here resulted in the inactivation of NTE (Table 1).

The use of the polyoxyethylene W1 detergent may have protected the NTE by providing a highly lipophilic environment. W1 is a commercial detergent consisting of 64% of the relatively hydrophilic Brij 58 that possesses a hydrophile–lipophile balance (HLB) of 15.7 and a critical micellar concentration (CMC) of 77  $\mu\text{M}$ . The remaining 36% consists of the relatively hydrophobic Brij 56 that possess a HLB of 12.9 and a CMC of only 2  $\mu\text{M}$ . Both have an aggregate number of 40. As a comparison, Triton X-100 has a HLB of 13.5, a CMC of 250  $\mu\text{M}$  and an aggregate number of 100–155 (Chambers and Rickwood, 1993). Solubilization of the enzyme with W1 may create a lipophilic cocoon around the NTE permitting it to remain active in the 155-kDa form thus allowing it to migrate in the 200-kDa fraction in the gel filtration column. A similar effect was reported by Davis and Richardson (1987). They reported that it was necessary to add exogenous phosphatidylcholine in order to maintain NTE activity in solution. The phosphatidylcholine, itself being amphopathic, likely mimicked the same effect as the Brij 56 constituent of the W1. This adjunct was tried in earlier isolation attempts but was rejected due to its interference in the gel filtration chromatography. It is not clear why a high concentration of NaCl was necessary in the elution buffer. Since its absence resulted in a smearing of the NTE activity across a broad band, it could be hypothesized that the 0.5 M NaCl inhibited the aggregation of the solubilized protein/detergent complexes by increasing the ionic concentration of the mobile phase.

The specific activity attained throughout the purification process appeared to indicate there was no substantial increase in purity. Furthermore, the ratio of recovery indicated that there was a 50-fold loss in the NTE activity and that the slow migrating band accounted for only 0.0113% of the total initial activity. When the amount of protein removed from the active fractions was back-calculated, assuming none of it was NTE, there was an apparent 1200-fold enrichment of the NTE. However, none of these indicators is an accurate estimate of the NTE purification since NTE, being a lipophilic enzyme, is unstable in an aqueous environment. This was supported by preliminary studies that indicate that: (a) NTE activity does not increase proportionally with microsome concentration, and (b) NTE kinetics can be greatly affected by the composition and concentration of adjuncts and detergents in the preparation. Therefore, it would be

prudent in this case to consider activity calculations as only qualitative markers for the chromatographic and electrophoretic properties of the 155-kDa protein and not a quantitative measure of purification efficiency.

Twenty years of research has shown that NTE is not a classical, aqueous soluble protein and that its activities are not quantitatively interpretable as if it were G6PDH. For example, if we use specific activity as a measure of yield efficiency we obtain unrealistic estimates of initial NTE content in tissue. Given a yield of 0.0113% (Table 2) of the 155-kDa protein based on NTE activity, the final yield of 1.3  $\mu\text{g}$  NTE per 20 g wet weight of embryo in the 155-kDa band would represent an unrealistic concentration of 11.5 mg per gram fresh weight; at least three orders of magnitude above that found by Williams and Johnson (1981). The most likely outcome of the purification process was a loss in catalytic activity without necessarily a loss in NTE protein. The activity is most useful therefore as a qualitative marker at this stage in the isolation process.

An unexpected event was the separation of two apparently independent bands of NTE activity on the native electrophoretic gel. The slowly migrating one contained the 155-kDa band and accounted for 43% of the total protein eluted. Determination of the activity showed that this fragment demonstrated no hydrolytic activity with phenyl valerate other than that defined as NTE. Analysis of the gel elution (Fig. 8, lane A) showed six predominant protein bands with the highest density between 150 and 160 kDa. This band was the only one that demonstrated an affinity for MNTFP: a proven NTE affinity ligand (Thomas *et al.* 1993). Furthermore, the binding of this protein to the affinity ligand was concomitant with the loss of NTE activity from the batch chromatographic suspension. Therefore, it could be concluded that the 155-kDa peptide was responsible for the NTE enzyme activity.

The rapid migrating NTE activity band comigrated with the residual paraoxon-sensitive esterase activity to phenyl valerate. SDS–PAGE of this fraction showed numerous proteins. When the eluate was subjected to MNTFP affinity chromatography, the NTE activity did not bind under conditions that bound the 155-kDa protein from the slower migrating fraction. Furthermore, the activity showed a different mipafox inhibition curve compared to the slower migrating NTE activity. Past studies have suggested that there may be more than one source of NTE activity with differing inhibitor  $I_{50}\text{S}$  (Vilanova *et al.*, 1990, 1993; Tormo *et al.*, 1993). The second band of NTE activity may represent another and independent source of NTE activity. However, since this second source comigrated with the larger one through both the gel filtration and the IEF procedures, it is more likely that it is an active subfragment of the larger NTE form. Further examination into the nature of this second source of NTE activity is ongoing. Regardless, the results of this study,

using three sequential methods of purification based on three separate chemical properties and confirming it with an affinity ligand, show that the vast majority of the NTE activity is associated with the 155-kDa protein.

#### ACKNOWLEDGMENTS

Supported in part by NIOSH U.C. Agricultural Health and Safety Center at Davis (U07-CCU906162-02), U.S. Air Force (AFOSR-91-0226), NIEHS Center for Environmental Health Sciences (P30-ES-05707), a NIEHS Training Grant (5-T32-ES07059), and the U.S. EPA (R814709) Center for Ecological Health Research at U.C. Davis. Although the information in this document has been wholly or in part funded by the United States Environmental Protection Agency, it does not necessarily reflect the views of the Agency and no official endorsement should be inferred.

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