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Short communication

Purification of human brain metallothionein by organic and reversed-phase high-performance liquid chromatography under acidic conditions

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Abstract

A simplified high-performance liquid chromatographic method for the detection of metallothioneins, notably metallothionein-III, has been developed. In order to purify metallothionein, differential acetone precipitation at 50% (v/v) and at 80% (v/v) was employed on a 20% normal human brain homogenate. The reconstituted pellet was injected into a C_{18} microbore reversed-phase HPLC column, equilibrated with 0.1% trifluoroacetic acid, and developed at a flow-rate of 800 μl/min with a linear gradient from 0% to 60% acetonitrile in 0.094% trifluoroacetic acid for 60 min. Western blots indicated that metallothioneins-I and II eluted at 16% acetonitrile and metallothionein-III eluted at 37% acetonitrile.

Keywords: Metallothioneins

1. Introduction

Initial tissue culture experiments with Alzheimer's disease (AD) homogenates on neuron-enriched cultures indicated that neuronal survival was increased 5-fold with the addition of extracts from AD brains with a high degree of tangle formation but not with AD brains with a high degree of amlyloid cores. It was subsequently found that growth inhibitory factor (GIF), or metallothionein III (MT-III) [1], is severely decreased in areas of the brain where there is tangle formation [2]. The principle morphological substrate of neurofibrillary tangles are paired helical filaments (PHFs) [3]. PHFs are composed of isoforms of the abnormally phosphorylated microtubule-associated protein, tau, co-localized with elevated concentrations of aluminum [4]. It is important to note that MT-III is the first glial protein to be implicated in the pathogenesis of AD which has traditionally been viewed as a neuron-specific disease.

MT-III is localized in astrocytes in the grey matter. The molecular layer in the dentate gyrus, the pyramidal cell layer in the hippocampus, and neocortex layers 2–6 are specifically stained by anti-MT-III antibodies and are also sites of tangle formation [2]. MT-III is expressed in mature cells but is down-regulated during fetal development [5] where there is extensive neuritic growth.

MT-III belongs to the metallothionein protein

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family and, as such, is approximately 7 kDa, cysteine rich, contains no aromatic residues and exhibits high affinity for divalent metals [6]. The N-terminal domain of MT of residues 1–29, termed β, contains 9 cysteines and forms a cluster of 3 Cd or Zn liganded by cysteine ligands; the C-terminal domain of residues 33–61, termed α, contains 11 cysteines and forms a cluster of 4 Cd or Zn liganded by cysteines ligands [7]. Residues 30–32 serve as a linker between the two domains of MT. MT-III is similar to other metallothioneins except for two inserts: a 6 amino acid glutamic acid rich insert EAAEAE in the C-terminal region and an threonine insert in the N-terminal domain [2]. As a consequence, the net charge on the MT-III apoprotein is −4 whereas the net charge on other apo-MT are in the range of +2 to +5, at neutral pH.

A previously published technique to purify MT-III from human brain used a cumbersome combination of DEAE-anion exchange, size-exclusion chromatography and C_{18} reversed-phase chromatography. A bioassay was used to detect MT-III after each of the chromatographic procedures [2]. Attempts at repeating the purification procedure, with subsequent microbore C_{18} reversed-phase HPLC, demonstrated significant protein contamination in the MT-III fraction. Therefore, a simpler and faster purification procedure for MT-III was devised, and is presented, which utilizes differential acetone precipitation and C_{18} microbore reversed-phase HPLC. The same procedure will also permit detection of MT-I and MT-II.

2. Experimental

2.1. Generation of anti-MT-III antibodies

A 12 amino acid peptide was synthesized which included the unique 6 amino acid insert in MT-III, EAAEAE. The synthetic peptide GGEAAEAEAEKC was used for antibody production [8]. Briefly, peptide (4 mg) was reduced in 15 mM Tris-HCl pH 8.8, 1 mM EDTA, 100 mM dithiothreitol (DTT) for 16 h at room temperature and desalted by reversed-phase HPLC. Reversed-phase HPLC was performed on a Waters binary gradient HPLC. Peptides were chromatographed on a 250×4.6 mm I.D. C_{18} column, with a particle size of 5 μm/100 nm (E.S. Industries, Berlin, NJ, USA), using a 1-h gradient from 3% acetonitrile to 60% acetonitrile at 0.8 ml/min. Peptide elution was monitored at 214 nm. Reduced peptide was recovered from the HPLC eluent by drying under vacuum and 2 mg was employed in coupling to 2 mg each of maleimide activated bovine serum albumin (BSA) and maleimide activated keyhole–limpet haemocyanin (KLH) (Pierce, Rockford, IL, USA) according to manufacturer's instructions except that post-coupling desalting was not performed. Three New Zealand white rabbits were immunized by subcutaneous injection of 200 μg peptide–KLH conjugate emulsified with complete Freunds adjuvant (Difco, Detroit, MI, USA) on day 1 followed by further injections containing 200 μg (day 14) or 100 μg (days 21 and 28) peptide–KLH conjugate emulsified with incomplete Freunds Adjuvant (Difco). Aliquots of the peptide–KLH conjugate in coupling buffer (83 mM sodium phosphate pH 7.2, 900 mM NaCl, 100 mM EDTA) were adjusted to a final volume of 500 μl with distilled water prior to emulsification with 500 μl of the appropriate adjuvant. Blood was collected on day 38 and allowed to clot for 2 h at room temperature. Serum was prepared by centrifugation at 2000 g for 20 min after clot retraction at 4°C for 16 h. Antibodies specific for the peptide were then isolated by affinity chromatography. Reduced peptide (4 mg), prepared as above, was coupled to 2 ml of iodoalkylagarose (SulfoLink Gel, Pierce) according to the manufacture's instructions. Crude immunoglobulin (prepared by three rounds of 50% ammonium sulphate precipitation of 120 ml pooled serum) in 60 ml 20 mM Tris-HCl pH 7.4, 154 mM NaCl, 1 mM EDTA (TBSE) was applied to the affinity column at 4 ml/h at 4°C. The column was then washed sequentially with 5 volumes of TBSE, 5 volumes of TBSE containing 1 M NaCl and 5 volumes of TBSE at 4°C. Bound antibody was then eluted with 0.1 M glycine-HCl pH 2.2 and immediately

neutralized with sufficient 1 *M* Tris base pH 11.2 to give a final pH of 7.4 prior to desalting on TSK HW40F (Supelco, Rohm and Haas, Bellefonte, PA, USA) using TBSE as eluent. The concentration of affinity-purified desalted antibody was estimated by absorbance at 280 nm assuming 1 $A_{280}=0.71$ mg/ml rabbit IgG.

2.2. Organic precipitation and chromatography of human brain proteins

One gram of temporal cortex from normal human brain was homogenized in 4 ml of 10 m*M* Tris-HCl (pH 8.6), 1 m*M* phenylmethylsulfonylfluoride, and 5 m*M* EDTA and centrifuged at 20 000 *g* for 1 h at 4°C. A 400-μl aliquot of the brain homogenate was reserved for chromatographic purposes. Chilled acetone was added to the supernatant to give 50% (v/v) acetone and placed in a −20°C freezer for 1 h and then centrifuged at 11 500 *g* for 5 min. The supernatant was made up to 80% (v/v) acetone and placed in a −20°C freezer for 1 h before centrifugation at 11 500 *g* for 5 min. The pellet was reconstituted in 10 m*M* Tris-HCl pH 8.6. Reversed-phase HPLC was performed on a Beckman-System Gold HPLC (Mississauga, Ont., Canada) equipped with an injector with a 5-ml loop. A 100-μl aliquot of the protein solution was injected into a C_{18} microbore reversed-phase HPLC column (250×2 mm I.D. with a particle size of 5 μm/100 nm, ES Industries) which was equilibrated with 0.1% TFA. The column was developed at a rate of 800 μl/min with a linear gradient from 0% to 60% acetonitrile in 0.094% TFA for 60 min. Peptide elution was monitored at 214 nm. Eluates were collected at 1-min intervals, aliquoted, and dried down in a speed-vacuum.

2.3. Preparation of protein samples for gel electrophoresis and silver staining

SDS-PAGE [9] was performed using a Tricine 10–20% gradient polyacrylamide gel in a mini-gel apparatus (Novex, Helix, Scarborough, Ont., Canada). Protein samples from the HPLC column fractions were dissolved in 20 μl of sample buffer (10 m*M* Tris-HCl pH 7.5, 10 m*M* EDTA, 20% (v/v) glycerol, 1.0% (w/v) SDS, 0.005% (w/v) bromophenol blue, 100 m*M* DTT) were heated in boiling water for 5 min before loading. Control proteins, 0.5 μg equine kidney MT (Sigma, St. Louis, MO, USA) and 1.5 μg MT-III peptide–BSA conjugate, were also dissolved in 20 μl of sample buffer and heated in boiling water for 5 min before loading. Electrophoresis was performed at 200 V at room temperature until the bromophenol blue tracking dye reached the bottom of the gel. Gels to be stained were then immersed in Coomassie Brilliant Blue (CBR) R-250 (BioRad, Richmond, CA, USA) staining solution (0.22% CBR, 50% methanol, 10% acetic acid) for 3–24 h prior to destaining in 50% methanol, 10% acetic acid.

2.4. Silver staining of proteins

Silver staining of proteins [10] was performed on a 10–20% gradient tricine gel (Novex) of the fractions collected from the reversed-phase chromatographic procedure. The gel was prefixed in 50% CH_3OH, 10% CH_3COOH for 30 min and fixed in 10% glutaraldehyde overnight. The gel was washed in an overflow tank for 4 h and then rinsed 10 times in deionized water to remove contaminant ions resultant from tap water in the overflow tank. The gel was soaked in 50 μg/ml (DTT) for 30 min and then soaked immediately in 0.1% $AgNO_3$ for 30 min. The gel was subsequently soaked in developer (500 μl HCHO, 30 g Na_2CO_2, H_2O to 1 l) until the desired staining was achieved. The reaction was stopped by the addition of 12.5 ml of 2.5 *M* citric acid per 250 ml of developer. After 30 min, the gel was washed in an overflow tank for 30 min and then removed for photography. MT and GIF positive fractions were identified and aliquots of the respective metallothionein fractions were pooled separately.

2.5. Electrophoretic transfer

Gels to be transferred [11] were equilibrated with transfer buffer for 20 min prior to assembly in transfer cassettes. Transfer of proteins was

performed in a mini-gel blotting apparatus (BioRad) at 40 V for 3 h using transfer buffer that had been cooled to 4°C with further cooling provided by the ice-block component of this apparatus. Nitrocellulose membranes with 0.45-μm diameter pores (Schleicher and Schuell, Keene, NH, USA) were used in transfer. Secondary or backup membranes were stained for total protein with copper phthalocyanine 3,4′,4″,4‴-tetrasulfonic acid (Aldrich, Milwaukee, WI, USA) [12] to monitor migration of proteins through the primary membrane. Transferred gels were stained with CBR to monitor protein transfer efficiency. Under the optimal protocol for metallothionein detection, gels were incubated for 20 min in CAPS buffer, pH 10.8, in 10% methanol containing 2 m*M* $CaCl_2$, prior to transfer in that buffer at 40 V for 3 h. Following transfer, primary membranes were incubated in 2.5% glutaraldehyde in water for 1 h and then washed for 5 min repeated 3 times in phosphate buffer (8.1 m*M* Na_2HPO_4, 1.2 m*M* KH_2PO_4, 2.7 m*M* KCl, pH 7.4) with 50 m*M* monoethanolamine (J.T. Baker, Toronto, Ont., Canada) added to the third wash solution.

2.6. Immunodetection

All primary membranes were blocked in 3% BSA in TBS (20 m*M* Tris-HCl pH 7.4, 154 m*M* NaCl) for 2 h at room temperature. Membranes were then incubated overnight in primary antibody diluted in 3% BSA in TBS at room temperature. Affinity purified polyclonal antibody to MT-III was used at 0.75 μg/ml. A monoclonal antibody to polymerized equine renal MT-1 and MT-2 (Dako, Dimension, Mississauga, Ont., Canada) was diluted 1:1000. Membranes were then washed for 5 min 5 times in 150 ml TBS containing 0.05% Tween-20 (Sigma). Affinity purified secondary antibody horse radish peroxidase conjugates [goat anti-rabbit IgG (Sigma) or goat anti-mouse IgG (BioRad)] were applied at 1 in 1000 dilution in 3% BSA in TBS for 4 h at room temperature. Following washing as described above, blots were developed in 3.4 m*M* 4-chloro-1-naphthol (Sigma), 22% methanol, 0.018% H_2O_2, 15.6 m*M* Tris-HCl pH 7.4, 120.2 m*M* NaCl. Development was terminated by immersion in distilled water followed by drying between sheets of filter paper in the dark.

2.7. Dot blot immunoassay

In order to determine which column fractions from the chromatographic procedure contained metallothioein, dot blots were performed using 100-μl aliquots from each collected fraction. The aliquots were lyophilized and reconstituted in 100 μl of 0.1% TFA containing 2 m*M* $CaCl_2$. A nitrocellulose membrane with 0.45 μm diameter pores (Schleicher and Schuell) was immersed in 0.1% TFA containing 2 m*M* $CaCl_2$ and clamped in a dot blot apparatus (BioRad). The samples were subsequently pipetted into the wells of the dot blot apparatus and a brief vacuum was applied until the solution was carried through the membrane. The wells were washed with 2×100 μl of phosphate-buffered saline under brief vacuum. Immunodetection was performed as aforementioned.

3. Results and discussion

A method of purifying MT-III (and all MT) was undertaken by using differential acetone precipitation [13] and HPLC. As an initial purification step, many brain proteins could be precipitated and eliminated in low to moderate amounts of acetone due to their hydrophobicity. Since metallothioneins are highly soluble, MT isoforms could be differentially precipitated in increasing amounts of acetone. Acetone precipitation of a brain homogenate from normal temporal cortex followed by C_{18} reversed-phase microbore chromatography [14] using a linear gradient from 0% to 60% acetonitrile in 0.094% TFA separated brain metallothioneins from one another (Fig. 1). Western blotting of collected fractions indicated that the protein represented by peak 5 eluted at 37% acetonitrile and was MT-III-positive (Fig. 2). The collected fractions of protein represented by the first two peaks on the same chromatograph eluted at 16% acetonitrile and were shown to be the MT-I and MT-II

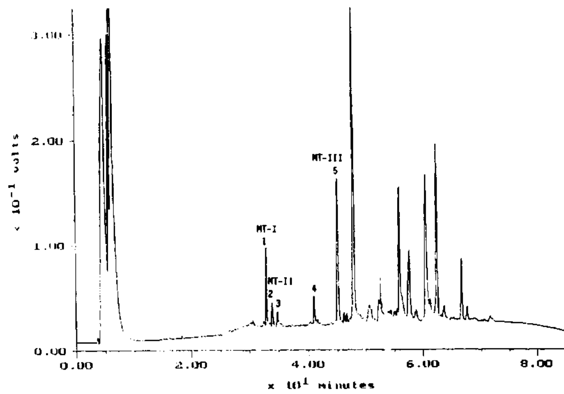

Fig. 1. Microbore reversed-phase HPLC analysis of a differential acetone precipitate of normal temporal cortex. Differential acetone precipitation at 50% (v/v) and 80% (v/v) was employed on a control human brain homogenate. The pellet was reconstituted in 10 mM Tris-HCl and injected into a C_{18} microbore reversed-phase HPLC column (250 × 2 mm I.D., 5 μm/100 nm particle size) which was equilibrated with 0.1% TFA. The column was developed at a flow-rate of 800 μl/min with a linear gradient from 0% to 60% acetonitrile in 0.094% TFA for 60 min. Fractions were collected at 1-min intervals, aliquoted, and dried down for subsequent protein analysis and MT-I,II and MT-III localization. Subsequent dot blots indicated that MT-I,II isoforms are represented by the first two peaks and that MT-III is represented by the fifth peak, as numbered on the chromatograph.

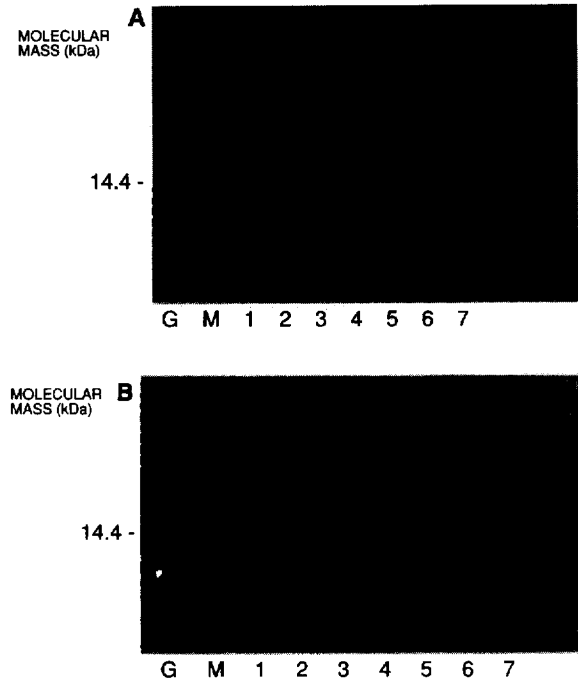

Fig. 2. Western blot of MT-I,II and MT-III from isolated proteins from the microbore reversed-phase HPLC analysis of acetone precipitate from normal temporal cortex homogenate. Gels were transferred to 0.45-μm pore nitrocellulose in CAPS buffer supplemented with 2 mM $CaCl_2$ and then incubated in 2.5% glutaraldehyde prior to immunodetection with a monoclonal antibody to equine renal MT-I,II (A) or the MT-III antibody (B). The MT-I,II antibody detected the protein in lane M and in lane 1 (A) and the MT-III antibody detected protein in lane G and in lanes 6–7 (B). Lane G, 1.5 μg MT-III peptide–BSA. Lane M, 0.5 μg equine renal MT. Lanes 1–3 represent the first three peaks from the chromatograph, lane 4 represents an interpeak aliquot, lane 5 represents the fourth major peak from the chromatograph, and lanes 6 and 7 represent two aliquots of peak 5 from the chromatograph. The proteins represented by the other peaks were not used as they did not immunostain for MT-I,II or MT-III on a dot blot.

isoforms, respectively, by Western blotting and by inference to elution profiles from previously published reversed-phase HPLC of metallothioneins [15]. The peaks corresponding to MT-I,II and MT-III only had absorbance values at 214 nm and not at 280 nm, indicative of proteins that do not have aromatic residues such as metallothioneins. All other collected fractions were not metallothioneins as determined by a dot blot assay (data not shown).

An aliquot of the MT and GIF positive fractions, as determined by a dot blot, was run on a gel and silver strained as shown in Fig. 3. The metallothioneins can be seen to be in an enriched state. As demonstrated from the Western blot, the first two low-molecular-mass bands on the gel were MT-I,II isoforms while the doublet bands immunostained for MT-III. It can be seen in Fig. 2A that the positive control equine renal MT-I,II (lane 2) is also detected along with brain metallothionein and in Fig. 2B that the positive control MT-III peptide-BSA conjugate (lane 1) is also detected along with MT-III.

The chromatographic procedure separated 12 proteins, one of which was MT-III and another series of peaks which were MT-I,II as indicated by Western blotting. A silver-stained gel of the DTT-reduced protein from the MT-III-positive peak showed two bands which may have been due to residual oxidation or represent isoforms

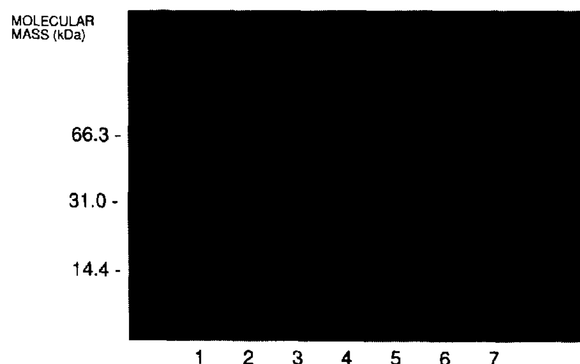

Fig. 3. SDS-PAGE and silver-staining of isolated proteins from the microbore reversed-phase HPLC analysis of acetone precipitate from normal temporal cortex homogenate. Proteins that were separated chromatographically were electrophoresed on a Tricine 10–20% gradient polyacrylamide gel and silver stained. Lanes 1–3 represent the first three peaks from the chromatograph, lane 4 represents an interpeak aliquot, lane 5 represents the fourth major peak from the chromatograph, and lanes 6 and 7 represent two aliquots of peak 5 from the chromatograph. The proteins represented by the other peaks were not used as they did not immunostain for MT-I,II or MT-III on a dot blot.

of MT-III which will be determined in future investigations. The bands are approximately 7 kDa. MT-III eluted at 37% acetonitrile and MT-I,II eluted at 16% acetonitrile. The MT-I,II isoforms may be separated, classically, by anion-exchange [16], if so desired.

Herein is described a very rapid procedure for the purification of brain metallothionein which may have application in addressing toxic-metal effects on levels of metallothionein expression in brain, particularly with regards to aluminum toxicity and Alzheimer's disease.

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