# Actin from Embryonic Chick Brain. Isolation in High Yield and Comparison of Biochemical Properties with Chicken Muscle Actin†

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ABSTRACT: Actin has been isolated from chick embryo brain by a method which results in a 76% recovery of total brain actin with >98% purity as evidenced by densitometry of $NaDodSO_4$-containing acrylamide gels. The isolated brain actin demonstrates the same critical actin concentration for assembly as does skeletal muscle actin when assembly is initiated with 4 mM $MgCl_2$ and 0.1 M KCl. Under these conditions, the rates and extents of brain and muscle actin assembly are identical as followed by viscometry and by absorbancy changes at 232 nm. Similarity in conformation of the brain and muscle G-actins has also been demonstrated by spectropolarimetry in the 260–195-nm region. Biochemical characterization of the isolated chick brain actin and comparison with purified chicken and rabbit skeletal muscle actins indicate that, although the proteins are related, brain contains distinctly unique actin species. Muscle actin generated the $\alpha$ isoelectric focusing species and brain actin the $\beta$ and $\gamma$ species previously observed for muscle and nonmuscle actins, respectively. Amino acid analysis shows chick brain actin contains more Gly and slightly less Asp, Tyr, and Met than does chicken muscle actin. Both actins contain identical composition of the remaining amino acids including one residue of 3-methyl-L-histidine. Both brain and muscle actin also contain 1 mol of $Ca^{2+}$/mol as determined by atomic absorption analysis. Brain actin contained at least one cyanogen bromide peptide not found in chicken muscle actin while the muscle actin contained at least three unique cyanogen bromide peptides.

Brain tissue presents a special problem for actin isolation because of the enormous amounts of tubulin present (Bamburg et al., 1973). Both proteins exhibit similar isoelectric points and assembly properties (Lee et al., 1973; Kasai, 1969; Olmsted & Borisy, 1975). Efforts directed specifically toward brain actin purification began with Puszkin et al. (1968), who obtained a brain fraction containing an actomyosin-like ATPase activity (Berl & Puszkin, 1970) from which an enriched actin fraction could be separated (Puszkin & Berl, 1972). Moring et al. (1975) reported that this actin product also contained very large quantities of tubulin, and subsequently succeeded in isolating a purified actin-like protein from bovine brain (2–5 mg/100 g of whole bovine brain; 90–95% pure). We estimate that bovine brain contains 36 mg of actin per 100 g of whole brain, based upon densitometry of $NaDodSO_4$[1] containing polyacrylamide gels of whole brain extracts, indicating a yield of 6–14% in the purification of Moring et al. (1975).

In addition, many nonmuscle cells of higher eucaryotes contain a low molecular weight protein called profilin which forms a 1:1 complex with G-actin and prevents its polymerization to F-actin (Carlsson et al., 1976). Consequently, many of the highly successful purification methods for nonmuscle actins (Adelstein & Conti, 1972; Yang & Perdue, 1972; Kane, 1975) which rely on enrichment of actin by polymerization directly out of cell extracts could not be utilized (Pardee, 1978). Other methods which resulted in the isolation of 20–30% of the actin from amoeba (Gordon et al., 1976; Spudich, 1974) were also not successful for recovering high yields of homogeneous brain actin (Pardee, 1978).

Another brain actin purification method has been used by Fine & Bray (1971). Approximately 5 mg of actin was isolated from twelve 13 day old brains. This corresponds to a 52% recovery of all actin present based on our analysis of 10 mg of protein per brain, 8% of which is actin. However, in our hands this technique resulted in an actin product of about 80% homogeneity (Pardee, 1978). The more recent purification method of Bray & Thomas (1976) which employs chromatography on DEAE-cellulose resulted in the isolation of homogeneous actin from chick embryo brain. Of the total brain actin content, 23% (44% pure) was recovered after extraction and chromatography by Gordon et al. (1977) by using this method. However, a final product representing but 10% of the total brain actin was obtained (Gordon et al., 1977). Because of the distinct possibility of different types of actins occurring in glial, neuronal, and brain smooth muscle cells, it seems reasonable to suggest that the biochemical properties of brain actin(s) can be confidently determined only on preparations that represent a quantitative recovery of all the actins present in whole brain. Upon this foundation, a meaningful interpretation of the functional differences between brain vs. muscle actin can be constructed.

Initial biochemical comparisons indicated that chick embryo brain actin was biochemically identical with skeletal muscle actin based upon similarities in molecular weight and tryptic peptides (Fine & Bray, 1971). Furthermore, the similarities found in actins from blood platelets (Bettex-Galland & Lüscher, 1965; Adelstein et al., 1971), vertebrate brain (Berl & Puszkin, 1970; Fine & Bray, 1971), and other chicken embryo tissues (Bray, 1972) prompted assertions that all actins were identical and differences in function could be attributed to the presence of ancillary molecules (Bray, 1972).

However, more recent investigations indicate that brain actin exhibits 8 unique tryptic peptides not observed in muscle actin

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; DEAE-cellulose, diethylaminoethylcellulose.

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(Gruenstein & Rich, 1975). Elzinga & Lu (1976) also noted a limited sequence difference between brain and muscle actins which has been confirmed and extended by other workers. Vandekerckhove & Weber (1978) have compared the sequence of bovine brain actin (78% complete) with bovine and rabbit skeletal muscle actin and have found about 25 substitutions between these proteins. Charge differences between brain and muscle actin have been demonstrated on isoelectric focusing gels (Garrels & Gibson, 1976), which can be explained by the amino acid sequence which shows that skeletal muscle actin contains an extra Asp residue near the N terminus accounting for its lower isoelectric point (Vandekerckhove & Weber, 1978).

By using sequence studies which are 70–97% completed, Vandekerckhove & Weber (1978) have identified six different actins in mammalian cells implying a minimum of 6 actin genes. Although the existence of only 6 actin genes remains a possibility, it is necessary to show that the actin used for these sequencing studies is representative of the entire tissue or cell type. The best means of assuring against selective purification of a specific actin species is to isolate the actin in high yield. In this paper, we measure the extent of molecular differences between brain and muscle actins by amino acid analysis, large fragment peptide mapping, isoelectric focusing, and spectropolarimetry by using actin which has been recovered in 70–80% yield from chick embryo brain.

## Experimental Procedure

*Muscle Actin Isolation.* Skeletal muscle actin was isolated from adult chicken breast muscle and rabbit skeletal muscle by the procedure of Spudich & Watt (1971). To eliminate actin oligomers, the addition of a final filtration of the G-actin through a 2 × 80 cm column of Sephadex G-150 resin equilibrated with 2 mM Tris-Cl, pH 7.6, 0.2 mM $Na_2ATP$, and 0.5 mM 2-mercaptoethanol was employed.

*Isolation of Brain Actin in High Yield.* For a typical preparation, twenty 17 day old chick embryo brains were rinsed with water and immediately immersed in 100 mL of 25 °C acetone. Washes were carried out for 15 min each in three successive batches of fresh acetone (Fine & Bray, 1971). Acetone-treated brains were dried in vacuo, ground to a powder, and stored at −20 °C. Actin was extracted by stirring the brain acetone powder for 30 min at 0.5 °C in 50 mL of prechilled extraction buffer consisting of 2 mM Tris-Cl, pH 8.0, 0.2 mM $Na_2ATP$, and 0.5 mM 2-mercaptoethanol. Extracts were centrifuged at 100000*g* (max) for 2 h at 4 °C, and the clear supernatant was decanted. The supernatant was immediately concentrated at 4 °C under nitrogen pressure to approximately 2 mL in an Amicon ultrafiltration cell fitted with a PM-30 membrane which had been preequilibrated with cold extraction buffer. The concentrate (100000*g* concentrate) was loaded onto a 2 × 80 cm Sephadex G-150 column which had been equilibrated with cold extraction buffer, and the eluant was monitored at 280 nm (Figure 1). Fractions eluting in the range of 45 000 daltons (brain actin peak) were pooled and concentrated by ultrafiltration to approximately 6 mL (5 mg/mL) prior to polymerization. The concentrated actin fraction was polymerized as follows: solid $K_2ATP$ and concentrated stock solutions of 4 M KCl and 1 M $MgCl_2$ were sequentially mixed with the brain actin concentrate at 4 °C to achieve final concentrations of 1.0 mM, 0.1 M, and 10 mM, respectively. The polymerization mix was immediately titrated to pH 7.2 with 0.1 N KOH after the addition of ATP and again after $MgCl_2$ addition. After incubation for 2 h at 37 °C, the assembled brain actin was pelleted by centrifugation for 2 h at 37 °C and 100000*g* (max). A gel-like, brain F-actin pellet was obtained after decanting the supernatant. The pellet was suspended in 5 mL of ice-cold extraction buffer and dialyzed for 72 h at 4 °C vs. three 500-mL volumes of extraction buffer. Following dialysis, the depolymerized brain actin was centrifuged at 100000*g* (max) for 2 h at 4 °C, and the supernatant, containing 2–3 mg/mL of >98% homogeneous brain actin, was decanted and stored on ice.

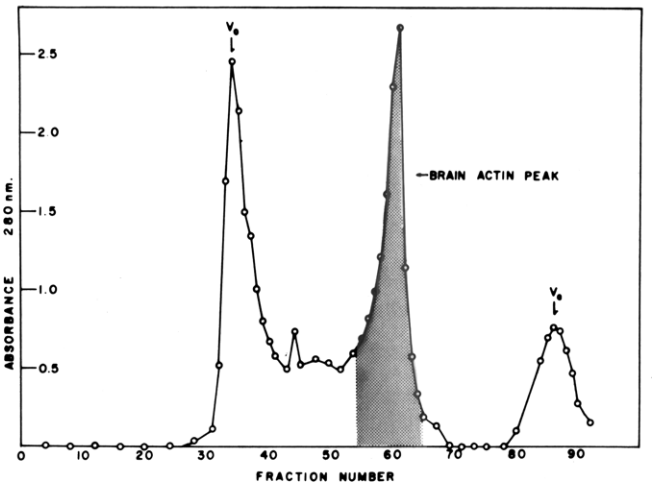


FIGURE 1: Gel filtration of concentrated brain acetone powder extract on Sephadex G-150 resin. Flow rates of 10–12 mL/h were obtained under a constant pressure head of 35 cm, and 3-mL fractions were collected. $V_0$ was determined by elution of Blue Dextran 2000 and $V_e$ calculated from the total column volume (250 mL). Typically, 90–95% of the 280-nm absorbance units loaded were recovered during elution. Actin eluted with a $K_{av}$ of 0.52, corresponding to a molecular weight of approximately 45 000.

*Preparation of Actin Monomers.* For extinction coefficient measurements, spectropolarimetry, and actin assembly studies actin monomers were used. An aliquot (5 mL) containing approximately 2 mg/mL of purified muscle or brain actin which had been dialyzed 3 days at 4 °C against 2 mM Tris-Cl, pH 7.6, 0.2 mM $Na_2ATP$, and 0.5 mM dithiothreitol was loaded onto a 2.5 × 80 cm column of Sephadex G-150 (medium) resin equilibrated in the same buffer except that the Tris concentration was 20 mM. The actin oligomer fraction containing dimers, trimers, and higher oligomers eluted in a broad peak at the column void volume, well resolved from the monomer peak. A column flow rate of 6 mL/h was used. Protein concentration in the monomer peak fractions was approximately 0.3–0.5 mg/mL and this material was used directly for spectroscopic and assembly studies.

*Protein Determinations.* Aliquots from each purification step were dialyzed against water at 4 °C for 24 h and lyophilized. Lyophilized samples were dissolved in 8 M urea, and a portion was analyzed for total protein content by the method of Lowry et al. (1951). The remainder of each sample was reduced and alkylated for polyacrylamide gel electrophoresis. Brain actin purity was estimated by quantitative densitometry of $NaDodSO_4$–polyacrylamide gels. Gels stained with Coomassie blue were scanned at 580 nm using an ISCO gel scanner interfaced with an ISCO Model UA-5 Absorbance Monitor. Total scan area and the brain actin peak area were compared using a polar planimeter, and the percentage of actin to total protein was estimated. The amount of actin present at each stage of purification was then calculated from the total protein present in the fraction.

*$NaDodSO_4$–Polyacrylamide Gel Electrophoresis.* Actins were dialyzed, lyophilized, and solubilized (1–2 mg/mL final concentration) in 1% $NaDodSO_4$ containing 2% 2-mercaptoethanol and immersed in a boiling water bath for 5 min. For

each 1-mL sample, 1 mL of 3 M Tris-Cl, pH 8.5, was added followed by iodoacetamide (150 mg) and the sample incubated at 10–12 °C in the dark for 30 min. 2-Mercaptoethanol (60 μL) was added to the reduced, carboxamidomethylated proteins, and the samples were subsequently dialyzed, lyophilized, and made up to approximately 1 mg/mL in freshly prepared 8 M urea.

Discontinuous, Tris–glycinate gels containing $NaDodSO_4$ were prepared by modification of the method of Davis (1964). Modified resolving gels, pH 8.8, contained 10% acrylamide, 0.05% *N,N'*-methylenebisacrylamide and were poured to 75 mm in 5-mm diameter tubes. Stacking gels contained 3% acrylamide, 0.625% methylenebisacrylamide and were 20 mm in height. The upper electrode buffer (cathode) contained 0.05% $NaDodSO_4$, but $NaDodSO_4$ was not incorporated into the stacking or resolving gels. Reduced and alkylated samples were subjected to electrophoresis at 0.5 mA/tube for 30 min, followed by 1 mA/tube for 1 h, and then 1.5 mA/tube until the Bromophenol blue tracker dye eluted. Gels were soaked in 10 volumes of 20% trichloroacetic acid for 2 h and then overnight in 15% trichloroacetic acid containing 25% isopropyl alcohol (Fairbanks et al., 1971). Staining was with 0.05% Coomassie brilliant blue in 7.5% acetic acid and 17.5% methanol for 8 h. Gels were destained and subsequently stored in 7.5% acetic acid, 17.5% methanol.

*Isoelectric Focusing.* Focusing gels containing urea (9.5 M) and Nonidet P-40 (2%) (Imperial Shell) were run by the method of O'Farrell (1975) using a pH 4–6 gradient in 5-mm diameter gels 90 mm in length. Gel acrylamide concentration was 5% with 0.2% *N,N'*-methylenebisacrylamide employed as the cross-linker. Monoethanolamine (8 mL/L) was employed as the cathode buffer and $H_2SO_4$ (2 mL/L) as the anode buffer. Proteins were applied at the basic end of the gel. Samples were mixed with UltraPure urea (Schwarz/Mann UltraPure), Nonidet P-40, and 2-mercaptoethanol to final concentrations of 9.5 M, 2%, and 5%, respectively, and either applied directly to the gels or stored at −20 °C until electrophoresis could be performed. Gels were prefocused, and samples were applied, overlayered with upper electrode buffer, and focused at 200 V for 7 h. A time-course study showed focusing to be complete on the 5 × 90 mm gels after 6.5 h at 200 V. Ampholines and Nonidet P-40 were extracted from the gels by soaking in 20% trichloroacetic acid for 2 h, then in 10% ethanol, 10% glacial acetic acid for 8 h, with three changes of solution (Rubenstein & Spudich, 1977). Gels were stained in 0.05% Coomassie brilliant blue containing 10% acetic acid and 20% isopropyl alcohol. Destaining was in 10% acetic acid, 20% isopropyl alcohol, or 10% acetic acid only. The pH gradients were determined by slicing a focused blank gel into 3-mm slices, soaking each slice in 1 mL of deionized water for 30 min, and recording the pH of the resulting extract.

*Amino Acid Analysis.* Reduced and carboxamidomethylated brain or muscle actin was dialyzed 48 h against cold, deionized water and lyophilized. Samples containing approximately 0.30 mg of protein were weighed out into acid-cleaned tubes, and 1.0 mL of constant boiling 6 N HCl was added. The hydrolysis solutions were degassed and the tubes sealed under vacuum. Hydrolysis was carried out for 24, 48, and 72 h at 110 °C, and the hydrolysates were dried over NaOH in an evacuated desiccator. The dried amino acids were twice solubilized in 1 mL of deionized water and lyophilized. Samples were redissolved in 6 mL of 0.2 N sodium citrate buffer, pH 3.30, and 1 mL loaded onto each column of a JLC-6AH amino acid analyzer operating in the "micro" mode. A standard mixture (including 3-methylhistidine) containing 25 nmol of each amino acid/mL was used to calibrate the analyzer immediately after each sample analysis.

*Actin-Bound Metal Analysis.* Purified, native brain G-actin in extraction buffer was analyzed for bound $Ca^{2+}$ and $Mg^{2+}$ by atomic absorption. Extraction buffer served as the assay blank control. After aliquots of brain actin monomer (0.5 mg/mL, 11.6 μM) were assayed for bound metal, a known quantity of free $Ca^{2+}$ or $Mg^{2+}$ was added and the sample reanalyzed to internally calibrate the atomic absorption spectrometer.

*Cyanogen Bromide Cleavage.* Samples of reduced, carboxamidomethylated brain or muscle actin containing approximately 2 mg of protein were dialyzed 24 h at 4 °C against deionized water, lyophilized, and dissolved in 1 mL of cold 70% formic acid. A 50× molar excess of BrCN was added (100 μL of 250 mg of BrCN/mL of 70% formic acid), and the reaction was allowed to progress for 12 h at 25 °C under $N_2$. A second 50× molar excess of BrCN was then added for an additional 12-h incubation. The peptide solution was diluted to 100 mL with deionized water, lyophilized, resuspended in 10 mL of water, and lyophilized again. The resulting BrCN peptides were dissolved in 70% formic acid to approximately 5 mg/mL and stored at −20 °C.

*Peptide Mapping.* Approximately 100 μg of either brain or muscle actin cyanogen bromide peptides in 70% formic acid was spotted onto a 160 μm cellulose TLC plate (Eastman Chromogram no. 13255). Electrophoresis was performed in the first direction on a Camag thin-layer electrophoresis unit using Whatman no. 1 filter paper wicks, 3.5 × 3.5 in. Electrophoresis in 63% formic acid was performed for 35 min at 1000 V with an average observed current of 15–20 mA. Wicks which were air-dried and reused gave the best results. Plates were dried for 12 h at 25 °C in a circulating air hood and then chromatographed ascending at 20 °C for 3 h in paper-lined tanks equilibrated with butanol, formic acid (90%), water (14:3:5, v/v). The chromatography solvent was mixed in the strict order formic acid, butanol, water immediately prior to use since phase separation occurred in 3.5–4 h after mixing. Plates were dried overnight at 25 °C, dipped in 0.1% ninhydrin in 95% ethanol, and heated to 75 °C for 35 min. Final development occurred upon storage of plates 24 h in the dark. Spots were marked by lightly outlining in pencil, and spot color was noted. Comparative mapping was performed by spotting a mixture of 100 μg each of brain and muscle peptides onto the plate and directly comparing the maps to individual brain or muscle actin maps.

*Determination of Brain Actin Extinction Coefficient.* An ultraviolet absorption spectrum from 400 to 230 nm on native brain G-actin monomer was obtained, and then duplicate 1-mL aliquots of the sample and buffer were dried to constant weight by lyophilization followed by heating at 110 °C for 48 h. The brain actin absorbance at 280 nm was divided by the net weight of the dried protein to obtain the $\epsilon_{280}^{0.1\%}$ value.

*Spectropolarimetry.* Native brain and rabbit muscle G-actins were analyzed by circular dichroic spectrometry on a Jasco J-41C spectropolarimeter from 260 to 190 nm at 25 °C. Protein concentrations were determined spectrophotometrically at 280 nm using $\epsilon_{cm}^{1\%} = 11.1$. Samples containing 0.3–0.5 mg/mL were scanned in cells of 0.5-mm path length. The spectropolarimeter had been calibrated with *d*-camphor-10-sulfonic acid by the method of Cassim & Yang (1969). Spectra of each protein from two different preparations were determined. Mean residue ellipticity $[\theta]_{MRW}$ was measured at 222 nm and the percent α helix calculated by the method of Chen et al. (1972). Ellipticities were also measured at 213,

Table I: Purification of Chick Embryo Brain Actin

| fraction | total protein[a] (mg) | actin[b] (%) | actin (mg) | yield (%) | purificn |
|---|---|---|---|---|---|
| 17-day chick embryo brain[c] | 338 | 5.6 | 19.0 | 100 | |
| brain acetone extract 100000*g* supernatant | 118 | 15 | 17.7 | 93 | 2.7 |
| 100000*g* concentrate | 118 | 15 | 17.7 | 93 | 2.7 |
| brain actin peak | 33 | 52 | 17.3 | 91 | 9.3 |
| purified brain actin | 14.7 | >98 | 14.4 | 76 | 17.5 |

[a] Determined by the method of Lowry et al. (1951). [b] Determined by densitometry of stained gels. [c] For 20 brains with an average total protein content of 17 mg/brain. The quantitation of actin in chick embryo brain was previously performed (Pardee & Bamburg, 1976).

216, 219, 228, 234, and 237 nm from which an average value for $\beta$ sheet and random coil fractions was calculated (Chen et al., 1972).

*Actin Assembly Measurements.* (*a*) *Ultraviolet Absorption Difference.* The rate and extent of actin assembly, whether from brain or muscle sources, were directly determined from the change in absorbance observed during assembly at 232 nm (Higashi & Oosawa, 1965). Brain actin displays an ultraviolet absorption difference spectrum similar to muscle actin with a maximum in the vicinity of 232 nm (Pardee, 1978). The actin samples to be assembled (0.84 mL at approximately 0.3 mg/mL) were added to cuvettes (1-cm pathlength) and polymerization was followed continuously for 20–30 min at 232 nm with a Gilford 252 spectrophotometer. Assembly curves were continuously plotted on a Gilford 6051 recorder set at 0.1 absorbance unit full scale. Assembly was initiated by adding 30 $\mu$L of 3 M KCl followed by 30 $\mu$L of 120 mM $MgCl_2$ into the sample cuvette with simultaneous additions of water to the reference cuvette containing G-actin. Samples were mixed by inversion and assembly monitored at 30 °C. Samples identical with those used above were monitored for light scattering at 310 nm. No corrections for light scattering were necessary.

(*b*) *Viscometry.* Viscometry of purified actin preparations was performed with a Cannon-Manning 100 semi-micro viscometer by using extraction buffer as the reference solution. Measurements were carried out at 25 °C.

(*c*) *Electron Microscopy.* Samples of purified, polymerized chick brain and chicken breast muscle actins at concentrations of 1 mg/mL in polymerization buffer were diluted 1:5 with buffer and a drop placed onto 400-mesh Formvar-coated grids. Negative staining was with 1% uranyl acetate for 20 s. Blotted grids were examined in a Phillips 200 microscope at 80 kV.

## Results

*Isolation of Chick Embryo Brain Actin in High Yield.* Seventeen day old chick embryo brains contain an average of 17 mg of total protein per brain, 5.6% of which is brain actin (Pardee & Bamburg, 1976). By employing the methodology detailed above, 14 mg of brain actin with a homogeneity greater than 98% was obtained from 20 seventeen day old chick embryo brains, representing a yield of approximately 76% of all actin present in the brain. At each stage of purification, the homogeneity of each fraction was determined by densitometry of stained $NaDodSO_4$–polyacrylamide gels (Figure 2) and a purification table constructed (Table I).

Acetone extractions of less than 45 min did not completely dehydrate the brains. Longer extraction times of up to 3 h did not diminish the amount of actin extracted from the acetone powder, but did result in decreases of polymerization-competent actin in the final purification step. Ninety-one percent of the actin present in chick brain was recovered after acetone treatment, extraction, and gel filtration on Sephadex G-150 resin. $NaDodSO_4$-containing polyacrylamide gel electrophoresis of the extract (Figure 2) showed that tubulin, a major protein constituent of chick brain (Bamburg et al., 1973), was not extracted from the brain acetone powder, while approximately 93% of the total brain actin was solubilized. When $2 \times 10^{-4}$ M $Ca^{2+}$ was included in the extraction buffer, the amount of actin solubilized decreased to 48–50% and an additional 10% actin loss occurred during ultrafiltration.

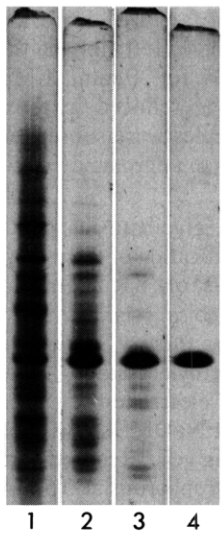


FIGURE 2: $NaDodSO_4$-containing polyacrylamide gels of fractions taken during the purification of brain actin. Gel 1: 30 $\mu$g of 17 day chick embryo brain supernatant containing 7% actin. Gel 2: 30 $\mu$g of brain acetone powder extract containing 15% actin. Gel 3: 30 $\mu$g of brain actin peak (52% actin) eluted from the gel filtration column. Gel 4: 10 $\mu$g of purified brain actin of >98% purity. All fractions were from the same preparation. Purified brain actin comigrated with either rabbit muscle actin or chicken breast muscle actin on $NaDodSO_4$–polyacrylamide gels at all protein loadings tested (1–10 $\mu$g).

Gel filtration of the extract through Sephadex G-150 resin resulted in a well-resolved actin peak (Figure 1). Typically the pooled brain actin fractions contained approximately 1 mg/mL of protein, 50% of which was brain actin (Figure 2, gel 3). The polymerization protocol was derived by determining the maximum obtainable yield under different conditions. $K_2ATP$, $MgCl_2$, and KCl were sequentially added as solids or from concentrated stock solutions, and care was taken not to allow the solution pH to drop below 7.0. The concentrated brain actin fraction was kept at 4 °C and gently mixed during salt addition.

Total actin yields were 25–30% when 0.2 mM $Ca^{2+}$, 10 mM $Mg^{2+}$, and 0.1 M KCl were employed in the polymerization buffer compared with 53% yield when $Ca^{2+}$ was deleted. The addition of 1 mM $K_2ATP$ to the 10 mM $Mg^{2+}$ and 0.1 M KCl at pH 7.2 further increased the final yields to 76%.

Centrifugation of the polymerized brain actin at 37 °C produced a gel-like pellet which slowly dissolved upon mixing with cold (4 °C) extraction buffer. Typically, pellets from a 20-brain preparation were homogenized in 5 mL of cold extraction buffer and dialyzed for 48–72 h which resulted in a clear retentate with no measurable elevated viscosity and which contained little precipitate. After centrifugation of the dialyzed product, a clear supernatant was obtained containing 2.0–2.5 mg/mL of brain actin with a homogeneity greater than 98% on $NaDodSO_4$–acrylamide gels (Figure 2). The final product could be stored on ice for several days with no

Table II: Amino Acid Comparison of Chick Embryo Brain Actin, Chicken Muscle Actin, and Rabbit Muscle Actin[a]

| amino acid | residues/molecule: chick brain actin[c] | chicken muscle actin | rabbit muscle actin[b] |
|---|---|---|---|
| Lys | 19.0 ± 0.3 (19) | 19.0 ± 0.3 (19) | 19 |
| His | 8.1 ± 0.2 (8) | 8.1 ± 0.1 (8) | 8 |
| *N*-Me-His | (1)[d] | (1)[d] | 1 |
| Arg | 18.0 ± 0.1 (18) | 18.1 ± 0.1 (18) | 18 |
| Asn + Asp | 29.9 ± 0.2 (30) | 34.0 ± 0.1 (34) | 34 |
| Thr | 26.6[e] (27) | 27.2[e] (27) | 27 |
| Ser | 21.7[e] (22) | 21.8[e] (22) | 22 |
| Gln + Glu | 40.7 ± 0.6 (41) | 41.1 ± 0.4 (41) | 39 |
| Pro | 19.1 ± 0.4 (19) | 19.4 ± 0.2 (19) | 19 |
| Gly | 35.8 ± 2.3 (36) | 27.0 ± 1.2 (27) | 28 |
| Ala | 29.0 ± 0.4 (29) | 29.0 ± 0.9 (29) | 29 |
| CM-cystine | 5.3 ± 0.1 (5) | 5.1 ± 0.3 (5) | 5 |
| Val | 20.8 ± 0.7 (21) | 20.7 ± 0.1 (21) | 21 |
| Met | 16.4 ± 0.3 (16) | 18.2 ± 0.3 (18) | 16 |
| Ile | 25.7 ± 0.8 (26) | 26.2 ± 0.7 (26) | 30 |
| Leu | 26.8 ± 0.2 (27) | 26.0 ± 0.1 (26) | 26 |
| Tyr | 14.0 ± 0.3 (14) | 15.7 ± 0.1 (16) | 16 |
| Phe | 12.2 ± 0.4 (12) | 11.7 ± 0.1 (12) | 12 |
| Trp | ND | ND | 5 |
| total residues | (371) | (370) | 375 |

[a] Amino acid analysis of purified chick brain actin and chicken muscle actin was compared with the composition of rabbit muscle actin taken from the published sequence. Tryptophan was not determined. Nearest integer values for each amino acid are given in parentheses. [b] Values taken from the sequence determination by Elzinga et al. (1973). [c] Averaged value for five separate hydrolysates including 2-24-, 2-48-, and 1-72-h hydrolysis. Ninety percent confidence interval calculated by the Student's *t* test (Laitinen, 1960). [d] Value estimated from the amino acid analysis of hydrolysates containing 5 nmol of actin/mL. [e] Values obtained by extrapolation to zero hydrolysis time.

degradation as evidenced by a single species when the sample was subjected to electrophoresis on $NaDodSO_4$-containing gels. Preparations kept at −20 °C could withstand several cycles of freeze-thawing without precipitation, but warming to room temperature and subsequent freeze-thawing resulted in a gelatinous actin coagulate which could not be resolubilized.

*Isoelectric Focusing.* When actins purified from chick embryo brain, chicken muscle, and rabbit muscle were subjected to isoelectric focusing on the pH 4–6 polyacrylamide gel system of O'Farrell (1975), rabbit and chicken muscle actins appeared to be composed solely of the $\alpha$ species, while brain actin was composed of $\beta$ and $\gamma$ actin species in a ratio of about 1/1. The banding patterns were in agreement with those observed by others for both muscle and nonmuscle actin (Garrels & Gibson, 1976; Whalen et al., 1976; Rubenstein & Spudich, 1977; Gordon et al., 1977). The measured isoelectric pHs of the $\beta$ and $\gamma$ brain actin species were 5.50 and 5.53, respectively, while the $\alpha$ species from muscle exhibited a p*I* of 5.47.

*Comparative Amino Acid Analysis of Brain and Muscle Actin.* The amino acid compositions of purified actins from chick embryo brain and chicken muscle were compared with rabbit skeletal muscle actin (Elzinga et al., 1973) (Table II). The analyses of skeletal muscle actin from both chicken and rabbit show a very high degree of similarity with differences only in Glu, Met, and Ile detected. The additional 2 Met residues in chicken muscle actin were confirmed by the finding of 19 BrCN peptides (see below). The composition of chick brain actin differs from that of chicken muscle actin in 4 amino acids, namely, Asp, Gly, Met, and Tyr. Again, the differences observed in methionine content seem to be confirmed by the

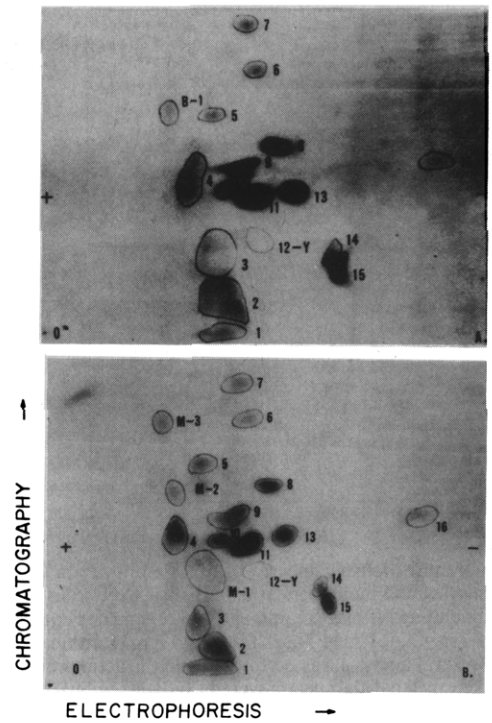


FIGURE 3: Peptide maps of cyanogen bromide cleaved brain and muscle actins. Electrophoresis was performed in the first direction and chromatography in the second. Yellow ninhydrin-stained peptides are designated by "Y" and the origin is marked "O". Peptides 1, 2, and 3 mapped as diffuse spots for both brain and muscle actins. (A) Peptide map of chick embryo brain actin (17 day embryo). (B) Peptide map of chicken breast muscle actin.

finding of only 17 BrCN peptides. The composition of the remaining amino acid residues is identical. *N*-3-Methylhistidine was observed in both chick brain and chicken muscle actin as a very small peak eluting between histidine and ammonia on the short column. Analysis of 5-nmol quantities of brain and muscle actin provided the estimates of one *N*-3-methylhistidine/actin molecule reported in Table II.

*Determination of Actin-Bound $Ca^{2+}$ and $Mg^{2+}$.* Atomic absorption analysis of freshly isolated brain actin monomers indicated the presence of 1.08 mol of $Ca^{2+}$ per mol of brain actin, assuming an actin molecular weight of 42 500 (Elzinga et al., 1973). $Mg^{2+}$ was present in a 0.67/1.0 ratio of $Mg^{2+}$ to actin. These results indicate that $Ca^{2+}$ remains tightly bound to the brain actin during the period of isolation (5 days) in the absence of added $Ca^{2+}$ to the extraction buffer. The presence of 0.67 mol of $Mg^{2+}$/mol of brain actin differed from analysis performed on rabbit skeletal muscle actin isolated in the presence of 0.1 mM $Ca^{2+}$, where only $Ca^{2+}$ was bound to the purified muscle G-actin.

*Cyanogen Bromide Peptide Maps.* Large fragment peptide maps of purified actin from chick brain and chicken muscle are presented in Figure 3. Brain actin (Figure 3A) contained 17 detectable peptides, and muscle actin (Figure 3B) contained 19 peptides in quantitative agreement with the number of methionine residues observed for brain and muscle actins (Table II). Sixteen peptides appeared to be identical (1–16) between the brain and muscle actin. Brain actin contained 1 peptide (B-1) not found in muscle actin, and muscle actin contained 3 unique peptides (M-1, M-2, M-3) not observed in brain actin. Peptides labeled 1, 2, and 3 were always diffuse making exact spot locations difficult in this region. Assignments were based on comapping equal amounts of brain and muscle actin which resulted in the appearance of 20 peptides which coincided with the peptide locations shown in

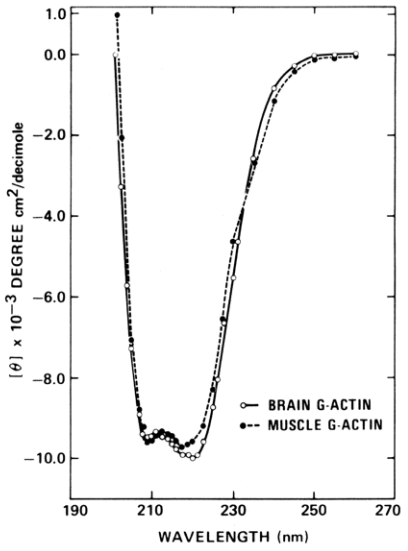


FIGURE 4: Circular dichroic spectra of muscle and brain G-actins. Brain and muscle actin monomers at 0.5 mg/mL were collected from the actin peak eluting off of a column of G-150 Sephadex equilibrated with 20 mM Tris-Cl, 0.1 mM $Na_2ATP$, and 0.5 mM dithioerythritol at pH 7.6. Spectral analysis was performed and the actin immediately assembled to ensure that the spectra were of fully assembly-competent proteins.

Figure 3. Results were obtained for 5–10 maps run on each actin and were consistent for two different preparations of each protein. Electrophoresis had to be performed in high concentrations of formic acid in order to solubilize all the peptides applied to the plates, while chromatography with analytical grade formic acid eliminated the map background coloration encountered when pyridine was employed during chromatography.

*Extinction Coefficient.* The $\epsilon_{280}^{0.1\%}$ for brain actin was calculated to be 1.11 $cm^{-1}$ mL $mg^{-1}$ which, within experimental error, is identical with the reported value for rabbit skeletal muscle actin of 1.108 $cm^{-1}$ mL $mg^{-1}$ (West et al., 1967).

*Circular Dichroic Spectra.* The circular dichroic spectra of brain and muscle G-actins appear nearly identical (Figure 4). Spectra from 260 to 195 nm exhibited maxima at 220 and 208 nm and a minimum at 212 nm. Muscle actin demonstrated a mean residue weight ellipticity at 222 nm of −9372 in agreement with the value of −9960 ± 300 at 222 nm reported by Nagy & Strzelecka-Golaszewska (1972). Brain actin exhibited an ellipticity of −9990 at 222 nm. An α-helix content of 25.3% and 23.2% for brain and muscle actin, respectively, was calculated from the molar ellipticity at 222 nm by the method of Chen et al. (1972). The value for muscle actin is in good agreement with those previously reported by Nagy & Strzelecka-Golaszewska (1972) and Murphy (1971). The contribution to protein secondary structure from β sheet was determined at six different wavelengths by the method of Chen et al. (1972), and the results are summarized in Table III. Within experimental variation, there is no significant difference in α-helix, β-structure, or random coil content between brain and muscle G-actins.

*Actin Assembly Studies.* When equal concentrations of rabbit skeletal muscle actin and chick brain actin were assembled by the addition of KCl and $MgCl_2$ to final concentrations of 0.1 M and 4 mM, respectively, the rates and extent of assembly as measured by viscosity and $\Delta A_{232}$ were very similar (Figure 5). (The $Mg^{2+}$ concentration used here was found to be optimal for the assembly of both actins (Pardee, 1978).) Negative staining electron microscopy of the purified, polymerized, brain actin demonstrated filaments of approximately 4–6 nm in diameter, appearing as tightly coiled double helices (Figure 6). Microscopy fields did not appear to contain aggregates of protein or short actin oligomers, indicating that assembly of the purified brain actin had not been impaired during the purification process. Although other ionic conditions were found where rates and extents of assembly of muscle and brain actin were significantly different (Pardee, 1978), under the conditions used here, both proteins

Table III: Estimated Fraction of α Helix, β Structure, and Random Coil for Actins from Circular Dichroism Spectropolarimetry[a]

| wave length (nm) | brain G-actin | | | muscle G-actin | | |
|---|---|---|---|---|---|---|
| | α helix | β structure | random[b] | α helix | β structure | random[b] |
| 213 | | 0.043 | | | 0.090 | |
| 216 | | 0.128 | | | 0.178 | |
| 219 | | 0.131 | | | 0.154 | |
| 222 | 0.253 | | | 0.232 | | |
| 228 | | 0.251 | | | 0.278 | |
| 234 | | 0.204 | | | 0.164 | |
| 237 | | 0.147 | | | 0.102 | |
| av: | 0.253 | 0.151 | 0.596 | 0.232 | 0.161 | 0.607 |

[a] The α-helix and β-structure contributions were calculated by the method of Chen et al. (1972). [b] Determined by difference.

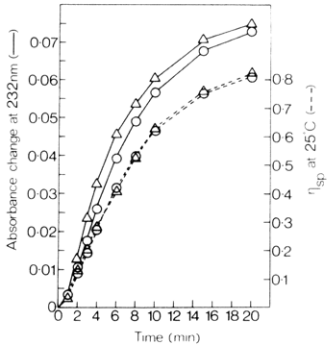


FIGURE 5: Assembly of chick brain and rabbit muscle actins. Solutions of brain (○) and muscle (△) G-actin were induced to assemble into filaments by the addition of KCl and $MgCl_2$ to final concentrations of 0.1 M and 4 mM, respectively. Assembly was followed by measuring either changes in light absorption at 232 nm (—) or increasing sample viscosity (- - -).

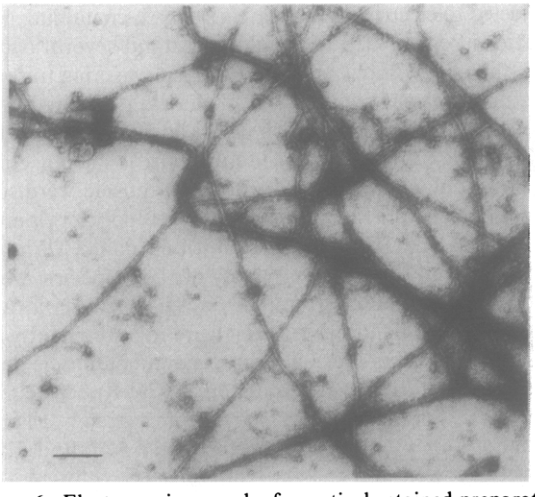

FIGURE 6: Electron micrograph of negatively stained preparation of purified chick embryo brain actin following assembly in 0.1 M KCl, 4 mM $MgCl_2$. Filament diameters are 40–60 Å. Maximum filament lengths of 2–3 μm were obtained. Bar = 0.1 μm. Magnification: ×50 000.

demonstrated the same critical actin concentration for assembly of 20 ± 10 μg/mL as determined from extrapolation of the $\Delta A_{232}$ measurements at equilibrium for different protein concentrations.

## Discussion

Because this isolation procedure results in a 70–80% recovery of actin which is assembly competent, these studies were not limited by possible preferential isolation. Consequently, large quantities of native brain actin(s) are available for biochemical analysis, immunological studies, and in vitro investigations of biological function. There has been a tendency in the literature to underestimate the importance of achieving quantitative yields during protein isolations. The relatively recent discoveries of multiple actin forms should, however, now urge investigators to consider carefully the recovery of the specific protein under investigation to avoid possible purification of a functionally distinct subpopulation of the target protein.

Specifically, mammalian brain contains at least three broad categories of cell species: neuronal cells, glial cells, and smooth muscle cells of the arterial vasculature. Actin is known to reside in neurons, smooth muscle cells, and glial cells. Brain arteries account for 2% of the brain's wet weight (Berl & Puszkin, 1970) with a probable corresponding contribution of 6–8% of smooth muscle actin to the brain actin pool. Purifications which recover 5–15% of the total brain actin are consequently open to criticism concerning the population of actin which has been isolated.

The discovery of profilin in calf brain (Carlsson et al., 1976) suggested that the inability of chick embryo brain actin to assemble in crude extracts (Bray & Thomas, 1976) may be due to the presence of this widely distributed protein. It is not clear at what stage in the purification scheme for brain actin the profilin would be removed. The profilin may be denatured by the acetone dehydration step and thus not extracted in significant quantities, or it may be separated from the actin by gel filtration at low ionic strength. Unfortunately, profilin runs with the dye marker in the $NaDodSO_4$-containing acrylamide gel electrophoresis system used to scan the column fractions and, thus, would have gone undetected. However, higher percentage acrylamide gels showed that no profilin was present in the brain actin containing fractions.

The high degree of similarity between the conformation of brain actin isolated by the method reported here and muscle actin isolated by more classical methods suggests that the isolation of brain actin gives a molecule as native as that from muscle. The finding of one $Ca^{2+}$/molecule of actin from both brain and muscle also suggests that the isolation procedure has not resulted in denaturation of the actin. The rate and degree of assembly of the two actin preparations are also highly similar, indicating that the G-actin from both sources retains the major functional characteristic of the native state, i.e., the ability of the actin to polymerize into filaments.

The role of $Ca^{2+}$ in brain actin assembly appears to differ slightly from its reported effect on the polymerization of muscle actin. Because the omission of $Ca^{2+}$ from the polymerization buffer proved to be a crucial modification, resulting in over a 100% increase in actin assembly (from 25% yield to 53% yield), there is reason to suspect that $Ca^{2+}$ may inhibit or at least not promote brain actin polymerization. Although deviations from muscle actin structure may be small, the effect of lowered $Ca^{2+}$ on the enhancement of brain actin assembly hints at measurable functional differences.

The large cyanogen bromide cleaved peptides of brain and muscle actins reveal the close similarity yet nonidentity of these proteins, confirming the observation by Gruenstein & Rich (1975) and Garrels & Gibson (1976) of nonidentity of $NaDodSO_4$-denatured chick brain and chicken muscle actin tryptic digests. The use of cyanogen bromide proteolytic cleavage allowed complete digestion of actin as evidenced by the absence of protein stain at the map origin and visualization of the theoretical numbers of methionine peptides expected from amino acid analysis. The fewer number of peptides generated by cleavage at methionine residues also makes the method quite useful for determining if differences between similar proteins reside in a confined region of the sequence. Tryptic peptide mapping of actin has been hampered by the inability to achieve complete digestion (Gruenstein & Rich, 1975; Gerday et al., 1968), making a clear assignment of actin differences more difficult.

The demonstration of nonconservative substitutions in amino acid composition, different isoelectric pHs, and nonidentical peptide maps suggest a unique structure for brain actin, a result which concurs with the observations of a separate brain actin gene product reported by Storti & Rich (1976).

The amino acid analysis results reported here for chick embryo brain actin do not closely agree with those taken from the partial sequence of calf brain actin (Elzinga & Lu, 1976; Vandekerckhove and Weber, 1978). With 78% of the calf brain actin sequenced, 14 replacements in 293 residues have been found. Seven substitutions involve sulfur-containing residues, but no substitutions involving acidic residues or glycine have been reported. It is conceivable that the peptides already sequenced correspond closely to muscle actin peptides and as such are easily resolved by the procedures devised to separate rabbit muscle peptides, leaving the unique brain actin peptides for subsequent analysis. However, more detailed comparison of our results must await the completed sequence. Absolute comparison, unfortunately, cannot be made because of the possibility of species-specific actins for calf and embryonic chick brain tissue.

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# Structure of Potato Carboxypeptidase Inhibitor: Disulfide Pairing and Exposure of Aromatic Residues†

T. Richard Leary, Donald T. Grahn, Hans Neurath,* and G. Michael Hass

ABSTRACT: The determination of the covalent structure of a carboxypeptidase inhibitor from potatoes containing 39 amino acid residues has been completed by analysis of the pairing of the six half-cystine residues. Since the native inhibitor is resistant to fragmentation by proteases, the protein was first subjected to cleavage at aspartic acid residues by exposure to 0.03 N HCl at 110 °C for 10 h to yield a fragment containing two chains (residues 6–15 and residues 18–39) held together by three disulfide bonds. Digestion with subtilisin and Pronase, respectively, yielded sets of peptides from which, by diagonal electrophoresis and amino acid analysis, the paired cystinyl residues were identified as Cys-8 to Cys-24, Cys-12 to Cys-27, and Cys-18 to Cys-34. Charge-transfer titration of the native inhibitor with *N*-methylnicotinamide chloride suggests that one of the two tryptophan residues and the single tyrosine residue are exposed to the solvent.

Protein inhibitors of proteases are widely distributed in plants and animals. Many of these have been isolated and characterized by chemical, physicochemical, and enzymatic methods (Fritz et al., 1974; Laskowski & Sealock, 1971).

A protein inhibitor of carboxypeptidases A and B from Russet Burbank potatoes was described by Ryan (1971) and its amino acid sequence was reported by Hass et al. (1975). This inhibitor consists of a single polypeptide chain containing 39 amino acid residues with a molecular weight of 4300 (Hass et al., 1975). The protein contains six half-cystine and no cysteine residues (Figure 1) (Ryan et al., 1974).

Hass et al. (1976) studied the interaction of native and chemically modified potato inhibitor with carboxypeptidase A and found that the inhibitor became inactivated when the terminal α-carboxyl group was blocked by attaching a leucine residue. Reactivation occurred when this leucine residue was removed. No other modification appeared to significantly modify the activity of the inhibitor. However, a complex of carboxypeptidase with acetylated inhibitor protected the acetylated tyrosine-37 from deacylation with hydroxylamine,

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