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Purification of Soluble Acetylcholinesterase from Sheep Liver by Affinity Chromatography

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Abstract The purpose of this study was to develop a protocol for the purification of acetylcholinesterase (AChE, acetylcholine acetylhydrolase, E.C.3.1.1.7) enzyme and to extend a purification method for further enzyme characterization. A further aim was to study whether the edrophonium's pharmacologic action is due primarily to the inhibition or inactivation of AChE at sites of cholinergic transmission. The purification of a soluble AChE from sheep liver using affinity chromatography on Concanavalin A–Sepharose 4B and edrophonium–Sepharose 6B is studied. The affinity matrix was synthesized by coupling an inhibitor edrophonium to epoxy-activated Sepharose at flow rate of 0.5 ml/min. AChE is a pivotal enzyme in the cholinergic nervous system. Its primary function is to catalyze hydrolysis of released acetylcholine (ACh) and thus maintain homeostasis of this neurotransmitter in the central and peripheral nervous systems. Hence, AChE is important in both pharmacological and toxicological mechanisms. It was purified 842-fold with a specific activity of 21 U/mg protein. Sodium dodecyl sulfate (SDS) electrophoresis resulted in a monomeric molecular weight of 67.04 kDa, while on gel chromatography using Sephacryl S-200 under nondenaturing conditions to be 201.5 kDa. Based on the molecular weight obtained by gel filtration, the purified AChE was assumed to be a tetrameric form.

Keywords Purification · Soluble acetylcholinesterase · Affinity chromatography

Introduction

Acetylcholinesterase (AChE, E.C.3.1.1.7) is an specialized carboxylic ester hydrolases that catalyze the hydrolysis of choline esters, which is systematically called acetylcholine acetylhydrolase. Other names include true cholinesterase, specific cholinesterase, red blood cell cholinesterase, erythrocyte cholinesterase, and cholinesterase I. The preferred substrate for AChE is acetylcholine (ACh) [1–3]. AChE serve a pivotal role in regulating nerve

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impulse transmission by rapid hydrolysis of the neurotransmitter ACh [4, 5]. AChE is present in liver, muscle, kidney, spleen, and nervous tissues [2, 6]. Most of the information about the properties of purified AChE has been obtained from studies of the AChE from the electric tissue of the eel *Electrophorus electricus* [7]. Lord in [8] partially purified AChE from the German cockroach *Blatella germanica* by grinding insects in sodium taurochlorate, dialyzing against di-sodium hydrogen phosphate, precipitating with ammonium sulfate, dialyzing against sodium citrate, incubating in protamine sulfate, dialyzing against water, and precipitating in acetone with the final sample being dissolved in sodium citrate buffer. Leuzinger and Baker in [9] purified AChE as a crystalline and electrophoretically homogenous form by chromatographic procedures. Efficient purification of AChE has since become possible using affinity chromatography, which has been described by Cuatrecasas et al. [10] as permitting a given enzyme to be readily separated from a mixture of proteins by its selective and reversible adsorption on a resin to which a specific competitive inhibitor of that enzyme has been covalently attached. Early affinity techniques for AChE purification were investigated by Reavill and Plummer [11] who compared the efficiency of three affinity columns. Recently, a variety of affinity resins such as tacrine [12], procaimide [13], edrophonium [14], and *m*-tri-methylaminophenylamine [15] have been developed for the purification of AChE from various organisms. Most of these ligands are specific inhibitors of AChE. We attempted to purify the soluble AChE from sheep liver using the two-step affinity chromatography such as Concanavalin A–Sepharose 4B column and edrophonium–Sepharose 6B column that turned out to be very rapid and sensitive.

Chatonnet and Lockridge [16] observed that AChE possesses two forms. In globular forms, the quaternary structure is defined in a negative fashion, by the lack of collagen like tails, and generally identify these forms as monomer (G_1), dimmer (G_2), and tetramers (G_4), attached together by disulfide bond, and each unit contains esteratic active sites of serine hydroxyl group and anionic sites of quaternary ammonium group, located in vertebrate and invertebrate. Other forms are asymmetric or collagen-like tailed forms; the quaternary structure are characterized by the presence of a collagen-like tail, which is formed by the triple helical association of three collagenic subunits (Q subunits), and each subunit may be attached to one catalytic tetramer (G_4). The asymmetric forms consist of either one, two, or three catalytic tetramers, and these forms are noted as A_4, A_8, and A_{12} forms respectively [17]. Localized at liver, skeletal neuromuscular junction, synapses, brain, heart muscle, and peripheral ganglia, they have glycosylated head which are joined together by sulfhydryl groups containing the active sites and collagen tails that attach the enzyme to the cell surface [18–22]. Furthermore, the heterogeneity of the enzyme has been characterized by its solubilization and hydrodynamic properties, reflecting discrepancies in both its mode of attachment to cellular structures and in its quaternary structure, and they can exist as soluble or membrane-associated species [14].

Materials and Methods

Sample Collection

Meat from healthy food animals (sheep) from local abattoirs in Cornwall (Callington and Launceston), UK, was used in this study. The sample (liver) was transported on ice to the laboratory at the University of Plymouth for immediate processing. During sample collection from the animal, it was ensured that there was no possibility of the introduction

of anti-AChE compounds from the skin of the animals. As noted by Fairbrother et al. [23], this can be a source of contamination by anti-AChE.

Sample Preparation

Ten grams of liver tissue was removed using a scalpel, cut into small pieces (3–4 mm^3), and rinsed until the blood was fully removed. The tissue was then placed on ice in 50-ml tubes (10 mm internal diameter) and homogenized using a mechanically driven homogenizer (Model X520-D, T6 probe, Bennett and Company, Weston-super-Mare, UK) with sodium phosphate buffer (0.1 M, pH 8) containing 0.5 M NaCl at a ratio of 1 part of tissue to 9 parts of buffer, and a speed of 10,000 rpm. Homogenization required between 2 and 5 min depending on the tissue; after every 30 s or so of homogenization, the mixture was rested for 10 s to allow cooling. The homogenate was then centrifuged in 50-ml tubes by using (MES, T8 probe, Europa 284) at 30,000 *g* for 1 h, at 4 °C [14].

Enzyme Activity Measurement

Acetylcholinesterase activity was determined by the Ellman method [24], adapted for use with microtiter plates as described by Haigh et al. [25], and using acetylthiocholine iodide as substrate (1 mM final concentration) for measuring AChE activity. In preparing solutions during measuring, it is essential that the substances have been stored properly and have not exceeded their shelf life; however, substrates and reagents in solution have a much shorter duration of stability and should not to be used on experiment extending over 1 day, and kept on ice during use. Briefly, 0.02 ml of sample and 0.24 ml of assay mixture [9.75 ml of 0.1 M sodium phosphate buffer, pH 8.0, containing 1 mM EDTA, and 0.25 ml of 0.2 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)] were mixed and allowed to stand for 5 min, and then, 0.04 ml of substrate solution was added. The absorbance increase was monitored for 5 min at 410 nm, at 25 °C in a plate reader (OptiMax, Molecular Devices, Sunnyvale, CA) [25, 26]. There may be some non-enzymic (endogenous) reaction between the sample and the DTNB which may interfere with the analysis. To control for this, a pre-incubation of DTNB and sample is performed prior to the addition of substrate. In each case, the rate of absorbance increase was corrected by subtracting the rate observed for a reagent blank (i.e., without sample). All measurements were carried out in triplicate. The use of a multi-pipette for the addition of substrate is recommended as the reaction proceeds quickly.

Determination of Protein

The protein content was quantified either by measuring the absorbance at 280 nm [27] or by Bradford Method (colorimetric protein assay at 595 nm) [28] based on the binding of Coomassie brilliant blue dye to proteins. Bovine serum albumin (BSA) is unique, which plays an important role in stabilizing protein structure. The BSA standards were made at concentrations of 0, 0.2, 0.4, 0.6, 0.8, 1, and 1.2 mg/ml BSA in DW.

Samples (50 μl) were placed in dry clean tubes and the volume was made up to 2.55 ml by the addition of the Bradford buffer (100 mg coomassie Brilliant Blue in 50 ml 95% ethanol, add 100 ml 85% phosphoric acid), volume was made up to 1 litter by DW and when the dye has completely dissolved, and filter through Whatman filter paper. Then sample with Bradford buffer was mixed briefly by vortex machine (MS1 Minishaker, IKA Works, Inc.) and incubated for 5 min at room temperature 20 °C, and analyzed spectrophotometrically using a Helious Betra UV–Vis spectrophotometer (UK).

Synthesis of Edrophonium–Sepharose Affinity Gel

Preparation of affinity gel was followed by the process of Anthony and Ian [29] with minor modification of Son et al. [14]. Epoxy-activated Sepharose was hydrated and washed with deionised DW on a sintered glass filter as recommended by the producer by the Sigma Chemical Company (Poole, UK). Before use, the gel could be washed in sequence with 10 volumes each of 0.1 M sodium acetate buffer (pH 4.5) 0.012 M sodium borate buffer (pH 10) and deionised DW. The gel slurry was dried on a Buchner funnel and transferred into a solution (12 mM borate buffer, pH 11) containing 20 mM edrophonium chloride (1 part gel/2 parts mixture solution). The pH of aliquots was then adjusted to 12 by the addition of 1 M NaOH. The mixture was shaken for 48 h at 50 °C on a incubator (LEEC, UK). The efficiency of edrophonium coupled to Sepharose 6B was measured based on the variation at 280 nm. All purification steps were performed at 4 °C, according to Anthony and Ian [29].

Isolation and Purification of Acetylcholinesterase by Affinity Chromatography

Essentially, this followed the method of Son et al. [14] by which AChE retained on an affinity Concanavalin A–Sepharose 4B column (GE Healthcare, UK Ltd.) was equilibrated with 50 mM sodium phosphate buffer (pH 7.4) and was followed by 0.5 M NaCl at 4 °C previous to packing liver extracts onto the column. The column was washed with the sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl until the protein content of the eluate was under the detection limit at 280 nm. AChE was then eluted with the sodium phosphate buffer (pH 7.4) containing 0.5 M α-methyl-D-mannoside at a flow rate of 30 ml/h. Active fractions monitored as a sole peak were collected and pooled, and then concentrated by using Amicon Ultra Centrifugal Filter Devises (Millipore, Carrigtwohill, Ireland). The eluant was then applied onto a column of edrophonium–Sepharose 6B previously equilibrated with 50 mM sodium phosphate buffer, pH 7.4 containing 0.5 M NaCl. Then, the column was washed with the 50 mM sodium phosphate buffer, pH 7.4, containing 0.5 M NaCl until the protein content was below 0.01 at 280 nm. The enzyme was then specifically eluted with 50 mM sodium phosphate buffer (pH 7.4) containing 20 mM edrophonium chloride. Each fraction of 0.5 ml was collected by a peristaltic pump (Miniplus 3, Gilson, UK) connected to a fraction collector (FC 2,112 Fraction Collector, Redirac, UK), and the active fractions were pooled and dialyzed overnight against 50 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl, with three changes of the buffer [14, 29].

Sephacryl S-200 HR

Molecular weight was estimated under non-denaturing conditions by gel filtration technique (molecular exclusion) using Sephacryl S-200. High molecular weight proteins will go down through a column quickly, while lower molecular weight proteins will go down through a column slowly. This is because the structure of the gel beads within the column excludes molecules that are too big to pass through the bead pores. A standard protein markers mixture (1 ml) containing (in milligram) carbonic anhydrase 1.5, β-amylase 2, alcohol dehydrogenase 2.5, cytochrome C 4, BSA 5, and apoferritin 5 were applied to a Sephacryl S-200 column, followed by 100 ml of 0.15 M sodium phosphate buffer (pH 7.2), flow rate (0.5 ml/min), and volume of each fraction collected, a sample (about 1.81) detecting at 595 nm after loading to the column. The column (2.4×24 cm) was calibrated with standard molecular weight: carbonic anhydrase (29,000), BSA (66,000), alcohol dehydrogenase (150,000), β-amylase (200,000), and apoferritin (443,000).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate (SDS) gels were prepared according to the method of Laemmli [30]. Polymerization was achieved by the addition of ammonium persulfate (APS) and *N*, *N*, *N'*, *N'*-Tetramethylethylenediamine (TEMED). Protein samples were dissolved with three times concentrated sample buffer at a ratio 3:1. Of fraction samples, 15 μl was mixed with 5 μl of sample buffer containing (0.2 M Tris buffer (pH 6.8), 10% (*w*/*v*) SDS, 20% (*w*/*v*) glycerol, 0.05% (*w*/*v*) bromphenol blue). Next, the prepared samples were completely loaded on the gel, and electrophoresis was carried out in a Mini Protean 3 system (Bio-Rad, UK) at a constant current of about 60 V until the dye front reached the resolving gel and then at 120 V until the dye front had reached the bottom of the gel.

After removal of the staking gel, the resolving gel was placed in stainer [0.125% Coomassie blue G-250 in 10% (*v*/*v*) phosphoric acid, 10% (*w*/*v*) ammonium sulfate, 20% (*v*/*v*) methanol for 2 h at room temperature 25 °C] and left overnight at room temperature with constants shaking. Gels were then destained by placing in destainer. The destainer [10% (*v*/*v*) methanol and 10% (*v*/*v*) acetic acid] was changed regularly until the gel was fully destained. All chemicals and protein markers used in this study were of analytical grade.

Estimation of the Relative Mobility

The relative mobility (*Rf*) refers to the mobility of the protein of interest measured with reference to a tracking dye. Standard molecular weight markers were run on every SDS gel conducted. After destaining, the *Rf* of each marker was determined according to Garfin [31] as follows:

$$Rf = \frac{\text{Distance of protein migration from top of resolving gel}}{\text{Distance of tracking dye migration from top of gel}}$$

The *Rf* values were then plotted vs. the log of known molecular weights. This allowed the construction of a calibration curve from which the *Rf* values of the unknown protein could be read off to give the molecular weight of the protein (Fig. 5).

Results and Discussion

Purification of Acetylcholinesterase

The present approach to purification of sheep liver AChE entailing affinity chromatography was based on the approach of Anthony and Ian [29] and Son et al. [14]. AChE is primarily involved in cholinergic synaptic transmission and found in a variety of neuronal and non-neuronal tissues [5]. The purification of soluble AChE from sheep liver is summarized in Table 1. Total protein activity was detected to be 183 mg in 50 ml of homogenate, with a specific AChE activity of 0.025 U/mg of protein. After affinity 1, the purified AChE contained 3.9 mg of protein in 41 ml of supernatant, with a specific activity of 0.289 U/mg of protein. The supernatant obtained by ultracentrifuge has about 66% of the total AChE activity and about 46% of the total protein recovery. When the supernatant was applied onto Concanavalin A–Sepharose 4B column, AChE was quantitatively adsorbed by Concanavalin A–Sepharose 4B column (Fig. 1). About 25% of the enzyme and 2% of protein were eluted from the column with 0.5 M α-methyl-D-mannoside, and purification fold was nearly 12. The edrophonium–Sepharose 6B affinity chromatography resulted in an AChE activity of 0.382 (U) and a purification fold of 842.

Table 1 Purification of AChE from sheep liver

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Protein recovery (%)	Activity recovery (%)	Purification factor
Homogenate	50	183.0	4.6	0.025	100	100	1
Supernatant	41	83.3	2.9	0.036	45.5	65.7	1.5
Affinity 1: Concanavalin A–Sepharose 4B column	22	3.9	1.1	0.289	2.1	24.7	11.6
Affinity 2: Edrophonium–Sepharose 6B column	10	0.018	0.382	20.9	0.009	8.4	841.9

The specific activity of AChE, expressed as micromole hydrolyzed per minute per milligram of protein. The recovery (%) of protein and activity was based on the total protein and AChE activity, respectively

Although there were some tailings at the end of a peak shoulder in the adsorption of enzyme from the column, a reasonable amount of enzyme was recovered. It has been known that globular form of AChE, predominant in the mammalian liver and muscle, is a glycoprotein [19]. The chromatographic behavior of sheep liver AChE on Concanavalin A–Sepharose 6B column resin indicates that soluble AChE from sheep liver may be of glycoprotein nature [32]. The enzyme eluted with 0.5 M α-methyl-D-mannoside showed a high protein with very sharp peak with AChE activity (Fig. 1).

Then, the pooled sample concentrated and loaded on the edrophonium–Sepharose 6B column. After the column was washed with 50 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl, AChE was eluted with edrophonium chloride. They demonstrated a very sharp peak with high AChE activity but with very low protein. However, this enzyme did not show any enzyme activity because of the inhibitory action of edrophonium bound to the active site of AChE. Most of the non-specifically bound proteins were removed in 50 mM sodium phosphate buffer and 0.5 M NaCl washing (Fig. 2).

The purity of the purified AChE is quite compatible with that of AChE from other sources such as 600-fold for rat liver [33], cattle erythrocyte (930-fold) [34], cattle serum (44,000-fold) [35], and housefly (400-fold) [36]. The specific activity of the AChE we

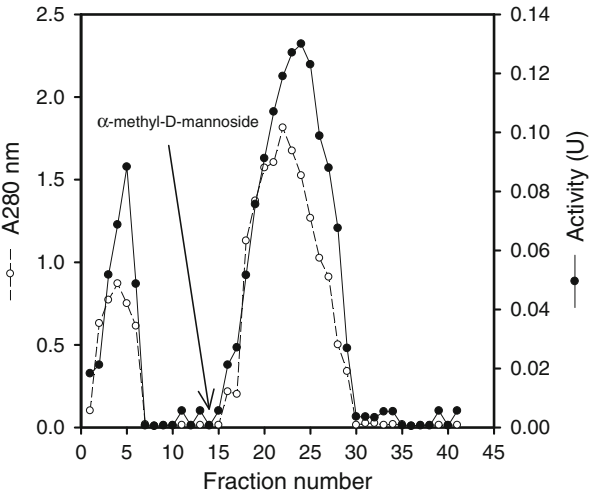

Fig. 1 A typical elution profile for the chromatography of sheep liver AChE on Concanavalin A–Sepharose 4B column (1.5× 5 cm) previously equilibrated with 0.05 M sodium phosphate buffer containing 0.5 M α-methyl-D-mannoside at a flow rate of 0.5 ml/min

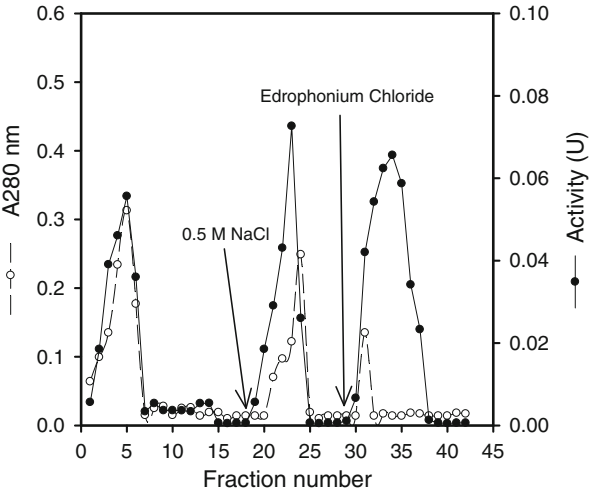

Fig. 2 A typical elution profile for the chromatography of sheep liver AChE on edrophonium–Sepharose 6B column (1×15 cm) previously equilibrated with 50 mM sodium phosphate buffer containing 20 mM edrophonium chloride at a flow rate of 0.5 ml/min

obtained from liver of sheep (Table 1) is much lower with those found by Eileen [37], Son et al. [14], and Im et al. [36]. However, these authors use brain tissues instead of liver which we used. In addition, a very high AChE activity found in the brain compared to other tissues. This factor may explain the apparent difference in specific activity.

Molecular Weight Determination

The molecular weight by gel filtration analysis of sheep liver AChE was also calculated from the calibration curve and estimated to be 201.5 kDa as tetrameric using Sephacryl S-200 (Fig. 3). Our results (Fig. 3) are lower when compared with other food animals, e.g., cattle

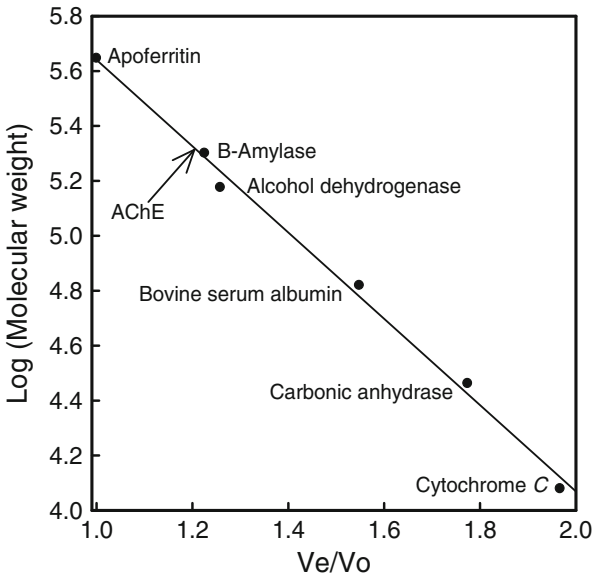

Fig. 3 Calibration curve for gel permeation determination of the molecular weight of purified AChE by Sephacryl S-200 HR chromatography. The protein markers used in order of increasing molecular weight: Cytochrome *C* (12,000), carbonic anhydrase (29,000), BSA (66,000), alcohol dehydrogenase (150,000), *b*-amylase (200,000), and apoferritin (443,000). Dextran blue (2,000,000) was used to determine the void volume (*Vo*), while *Ve* is the elution volume

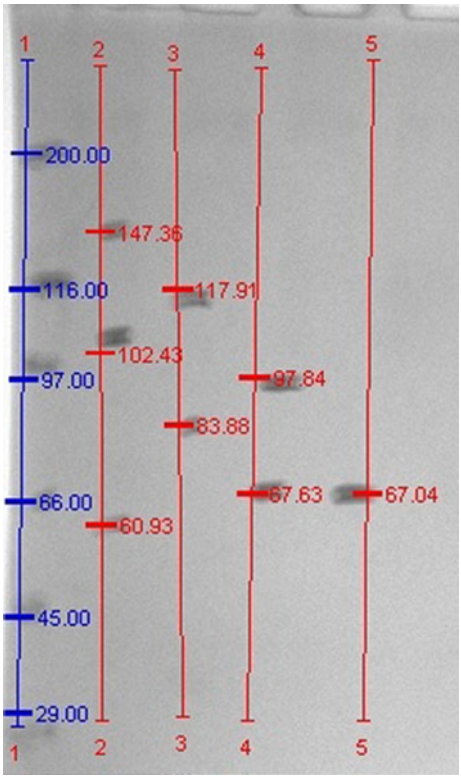

Fig. 4 SDS–polyacrylamide gel electrophoresis pattern of AChE from sheep liver. SDS–PAGE was conducted in gel and the protein stained with Coomassie blue. *Lane 1* standard proteins (molecular weights insert the figure) are carbonic anhydrase (29,000), egg albumin (45,000), BSA (66,000), phosphorylase *b* (97,000), β-galactosidase (116,000), and myosin (200,000). *Lane 2* supernatant AChE. *Lane 3* 50 mM sodium phosphate buffer fraction on Concanavalin A–Sepharose. *Lane 4* 50 mM sodium phosphate buffer fraction on Concanavalin A–Sepharose. *Lane 5* 20 mM edrophonium fraction on edrophonium–Sepharose

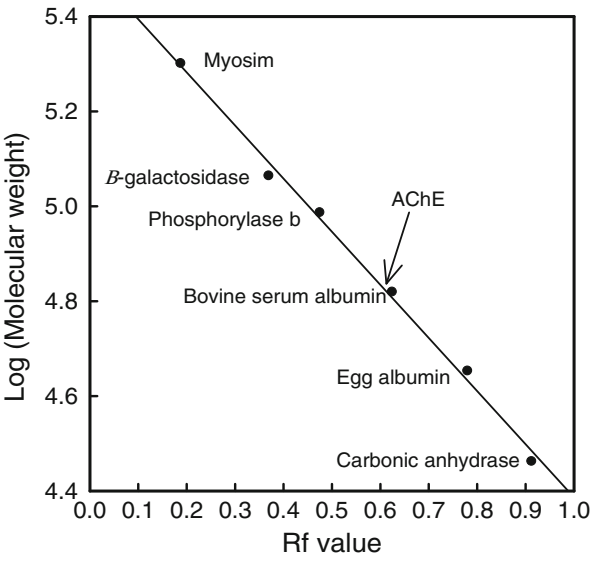

Fig. 5 A typical standard calibration curve for an SDS gel. The protein markers used in order of increasing molecular weight: Carbonic anhydrase (29,000), egg albumin (45,000), BSA (66,000), phosphorylase *b* (97,000), β-galactosidase (116,000), and myosin (200,000)

[35]. However, this author used serum instead of liver tissues. This factor may explain the apparent difference in tetrameric molecular weight.

Monomeric subunit molecular weight of AChE was calculated from the calibration curve and estimated to be 67.04 kDa as single monomeric subunits using 10% SDS-polyacrylamide gel electrophoresis (Coomassie blue stained) (Figs. 4 and 5). It was higher than observed in quail brain (62.5 kDa) [14], human brain (66 kDa) [38], human serum (65 kDa) [39], and electric organ of the electric eel (25–59 kDa) [40], whereas lower than in studied *Torpedo californica* (71–82 kDa) [41, 42], cattle serum (83 kDa) [35], and cattle superior cervical ganglia (75 kDa) [43]. The above results differ with our results due to these authors using different tissues instead of liver tissue. Finally, we found that in this case, purification was most successful on a column containing edrophonium chloride covalently linked to epoxy-activated Sepharose 6B and eluted with α-methyl-D-mannoside.

Conclusions

In conclusion, we succeeded in establishing a gentle solubilization technique that provided a favorable detergent during further purification procedure by stabilizing the native form of this fragile protein. Secondly, we could purify AChE by a two-step separation on Concanavalin A–Sepharose 4B column followed by edrophonium–Sepharose 6B column. This protocol, in our opinion, (combined use of Concanavalin A–Sepharose 4B and edrophonium affinity 6B chromatography) could be a useful resource for purifying soluble AChE from sheep liver, which is readily applicable to the purification of soluble AChE from other sources.

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