Article

Comparison of Hupresin® to procainamide-Sepharose for purification of butyrylcholinesterase and acetylcholinesterase

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Abstract: Affinity chromatography on procainamide-Sepharose has been an important step in the purification of butyrylcholinesterase (BChE) and acetylcholinesterase (AChE) since its introduction in 1978. The procainamide affinity gel has limitations. In the present report a new affinity gel called Hupresin® was evaluated for its ability to purify truncated, partly-deglycosylated human butyrylcholinesterase (rHuBChE) expressed in a stably transfected CHO cell line. We present a detailed example of the purification of rHuBChE secreted into 3940 mL of serum-free culture medium. The starting material contained 13,163 units of BChE activity (20.9 mg). rHuBChE was purified to homogeneity in a single step by passage over 82 mL of Hupresin® and elution with 0.1 M tetramethylammonium bromide in 20 mM TrisCl pH 7.5. The fraction with the highest specific activity of 630 units/mg contained 11 mg of BChE. Hupresin® is superior to procainamide-Sepharose for purification of BChE, but is not suitable for purifying native AChE because Hupresin® binds AChE so tightly that AChE is desorbed with denaturing solvents such as 50% acetonitrile or 1% trifluoroacetic acid. Procainamide-Sepharose will continue to be useful for purification of AChE.

Keywords: Hupresin®, affinity chromatography, procainamide-Sepharose, BChE, AChE

1. Introduction

Affinity chromatography on procainamide Sepharose 4B beads has been successfully used as part of purification protocols for BChE since 1978 [17; 19; 27-29]. Though chromatography on procainamide beads greatly enriches samples for BChE, the resultant partially-purified BChE requires additional manipulations to make the BChE homogeneous. A new affinity matrix, introduced by Brazzolotto et al, yielded pure truncated rHuBChE from the culture medium of transfected insect cells [5] in a single step. The new affinity matrix, called Hupresin®, is commercially available from CHEMFORASE. All 9 native N-linked glycosylation sites were present on the rHuBChE secreted by insect cells, but the glycans were not capped with sialic acid. The present work demonstrates that affinity chromatography on Hupresin® can yield pure rHuBChE in a single step for BChE expressed in CHO cells, where at least 72% of the glycans are capped with sialic acid [43].

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2. Results

2.1. Purification of rHuBChE on 82 mL Hupresin®

The particle-free, red culture medium containing 13,163 units of BChE in 3940 mL was loaded onto 82 mL Hupresin®. The red color of the phenol red indicator eluted during loading along with 1251 units of BChE activity. See Table 1. Washing with 1 L of 20 mM TrisCl pH 7.5 eluted more of the red color and left the Hupresin® colored beige. Contaminating proteins were washed off with 1 L of 0.1 M NaCl in 20 mM TrisCl pH 7.5 followed by 0.5 L of 0.3 M NaCl in 20 mM TrisCl pH 7.5. Fractions of 20 mL were collected during elution with 0.1 M tetramethylammonium bromide (TMA) in 0.1 M TrisCl pH 7.5. The first 80 mL of eluate containing 86 units of BChE activity was discarded. The next 60 mL of eluate containing 6922 units of BChE activity with a specific activity of 630 units/mg were saved. Less pure BChE was recovered in later fractions with specific activities of 596 and 531 units/mg. BChE recovered in 0.1 M TMA represented 77% of the starting activity.

Table 1. Purification of rHuBChE on 82 mL Hupresin®

	Volume, mL	Units	Units/mL	A_{280nm}	Units/mg	BChE mg	% pure
Culture medium	3940	13,163	3.34	Red		20.9	
Flow thru	3700	1251	0.33	Red		2.0	
Buffer wash	1000	19	0.02	Pink			
0.1 M NaCl	1000	79	0.08	0.025		0.1	
0.3 M NaCl	500	230	0.46	0.311		0.4	
0.1 M TMA	80	86	1.07	0.077	25	0.1	
"	60	6922	15.4	0.330	630	11.0	100
"	100	2286	22.86	0.069	596	3.6	95
"	160	897	5.60	0.019	531	1.4	84

We have been using Hupresin since the year 2012 for purification of rHuBChE [5]. The scale of the starting material has ranged from several hundred mL to 5 L of culture medium. Over the years we have eluted BChE from Hupresin with 0.5 M TMA, 0.1 M procaine, 0.1 M procainamide, and finally settled on 0.1 M TMA in either 20 mM TrisCl pH 7.5 or 20 mM sodium phosphate pH 8.0 as the elution buffer of choice. The conditions that yield pure HuBChE at the lowest cost of reagents are described in detail for the example we present in this report.

2.2. Cleaning Hupresin®

After BChE had been eluted with 0.1 M TMA, the Hupresin® had a faint yellow color in the top 20% of the column. The Hupresin® was returned to a pristine white color by washing with 200 mL of 0.1 M NaOH [32]. The pH was neutralized by washing with 200 mL of 0.1 M citric acid pH 4.5, 500 mL of water, and 150 mL of 20 mM TrisCl pH 7.5, 0.05% azide. The washed Hupresin® was stored in 20 mM TrisCl pH 7.5, 0.05% azide at 4˚C.

2.3. Dialysis and concentration

The 3 grades of rHuBChE were dialyzed in separate cellulose membrane dialysis tubing against 4 x 5 Liters of 20 mM TrisCl pH 8 at 4˚C to remove tetramethylammonium bromide. The dialyzed BChE was concentrated in an Amicon stirred cell fitted with a PM10 membrane (10,000 molecular weight cut-off). A total of 8.4 mg of the purest rHuBChE was recovered (Table 2), representing a loss of 24% during the dialysis and concentration steps.

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Table 2. Yield of concentrated rHuBChE

Specific activity, u/mg	mg/mL	Volume, mL	Total mg
630	2.1	4	8.4
594	1.2	2.6	3.1
531	0.4	2.8	1.1

2.4. SDS gel electrophoresis

The purity of the dialyzed, concentrated rHuBChE samples was visualized on the Coomassie blue stained SDS gel in Figure 1. The 630 and 594 u/mg samples in lanes 1-6 have a single band at about 72 kDa, consistent with a BChE monomer of 530 amino acids (59,711 Da) decorated with 6 glycans. The plasma-derived HuBChE tetramer in lane 8 has a nonreducible dimer band at 170 kDa and a monomer band at 85 kDa for 574 amino acids (68,419 Da) plus 9 glycans per subunit. The 531 u/mg BChE in lanes 9-11 has faint contaminating bands at 85 and 70 kDa.

The rHuBChE L530stop 4sugars-off protein, purified by Hupresin® affinity chromatography, has been successfully used for crystal structure analysis of HuBChE bound to the reversible inhibitors decamethonium, thioflavin T, propidium, huprine 19, and ethopropazine [42].

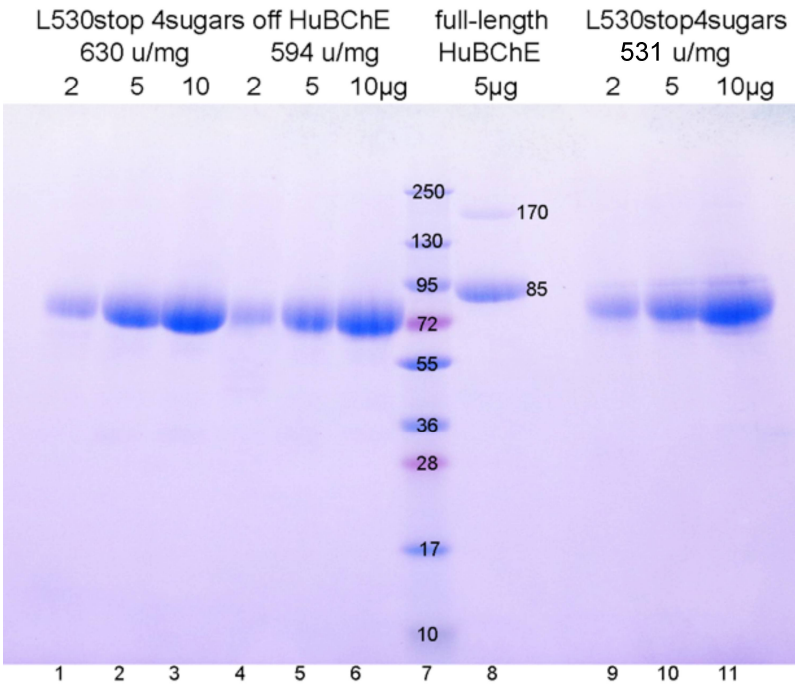

Figure 1. Coomassie blue stained SDS gel for samples purified on Hupresin. Samples had been boiled in the presence of dithiothreitol to reduce disulfide bonds.

3. Discussion

3.1. Hupresin® for purifying BChE

Hupresin® is a good affinity gel for purifying BChE. 1) The huprine ligand inhibits HuBChE with an IC_{50} of 0.99 µM [38], a value compatible with release of bound BChE by competitive inhibitors or ions. 2) The huprine ligand is coupled to Sepharose 4B via a 10-atom spacer between the Sepharose beads and the ligand. The spacer extends the ligand out of the Sepharose backbone, making room for the BChE protein to interact with the ligand as illustrated in the crystal structure of huprine bound to HuBChE [42]. 3) The maximum density of ligand bound per mL Sepharose is determined by the concentration of spacer (6-aminohexanoic acid) on the Sepharose gel. ECH-Sepharose 4B (GE

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Healthcare 17-0571-01) yields the Hupresin® affinity gel that successfully purified rHuBChE in this report. Table 3 cites use of Hupresin® to purify BChE from human plasma, porcine milk, an insect expression system, and a plant expression system.

3.2. Hupresin® compared to procainamide affinity gel for purifying BChE

The procainamide affinity gel introduced by Lockridge and La Du in 1978 [28] for purification of BChE from human plasma has been used worldwide with success (references in Table 3). The procainamide ligand is inexpensive, readily available from commercial sources, and is easily conjugated to ECH-Sepharose. An important advantage of the procainamide affinity gel over Hupresin® is that the GE-Healthcare Co. produces the procainamide affinity gel under GMP conditions, as mandated by the Food and Drug Administration. Pure HuBChE enzyme intended for injection into humans must be purified using GMP quality affinity gel [53]. In the year 2018 Hupresin® is not yet available as a GMP-certified affinity gel.

Research laboratories find Hupresin® superior to the procainamide affinity gel for purifying BChE from human plasma and other sources [3; 5; 32]. Table 3 lists uses of the procainamide and Hupresin® affinity gels for purifying BChE from a variety of animal plasma, porcine milk and expression systems. Hupresin® is new, so the list of applications for Hupresin® is short.

During the 40 years that the procainamide affinity gel has been used by researchers it has become clear that BChE in complex media such as plasma, milk, or plant extracts requires several additional steps to yield pure product. Less complex starting materials like serum-free culture medium can yield pure BChE in a single chromatography step on procainamide Sepharose. Over the years we have searched for better affinity ligands for purifying HuBChE. We tested proflavine, propidium, tacrine, Nile blue, polyproline, cibacron blue Sepharose (Sigma-Aldrich), and phenyl Sepharose (GE Healthcare), but none were as good as procainamide.

Hupresin® is the only affinity gel that works better than procainamide Sepharose for purifying BChE. Hupresin® binds more HuBChE from plasma per mL gel and is more selective compared to procainamide Sepharose. Hupresin® does not yield pure BChE from plasma in one step, but passage of plasma through Hupresin® yields a highly enriched HuBChE that is suitable for mass spectrometry analysis of nerve agent exposure [3]. Pure HuBChE can be obtained from plasma in 2 chromatography steps: anion exchange chromatography at pH 4.5 followed by Hupresin® affinity chromatography at pH 8.

Table 3. BChE purified on procainamide and Hupresin® affinity gels

Procainamide purifies	Eluant	Reference
HuBChE in plasma or serum	NaCl gradient or 0.2 M procainamide or 1 M NaCl	17; 29; 30
Plasma-derived HuBChE in Cohn fraction IV-4	1 M NaCl	45
Macaque BChE in plasma	0.2 M procainamide	39
Macaca radiata BChE in serum	0.05 M procainamide	14
Equine BChE in plasma	0.1 M procainamide gradient	35
Porcine BChE in milk	0.1 M procainamide	44
Felis Catus BChE in plasma	0.2 M procainamide	54
Rat BChE in serum	0.05 M procainamide	14
Mouse BChE in serum	1 M NaCl	50
Chicken BChE in serum	0.05 M or 0.2 M procainamide	14; 54
HuBChE covalently modified on the active site serine with soman, sarin, VX, tabun, cyclosarin,	NaCl gradient or 20 mM procainamide in 0.1 M NaCl or 1 M	13; 20; 25; 51; 52

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chlorpyrifos oxon, O-methoate,	NaCl or 2 M NaCl	
Marmoset BChE in plasma modified on the active site serine with soman, tabun	0.6 M NaCl	36
rHuBChE expressed in CHO cells	0.2 M procainamide or 0.1 M tetramethylammonium Br or 1 M NaCl or NaCl gradient	19; 27; 31; 40
rHuBChE from milk of transgenic goats	0.5 M NaCl	18
rHuBChE expressed in silkworm	0.2 M procainamide	24
rHuBChE expressed in tobacco *N. benthamiana*	NaCl and procainamide	15
Cocaine hydrolase mutants of rHuBChE	0.2 M procainamide	49; 54
Cocaine hydrolase mutants of rMouse BChE	0.1 M procainamide, 0.3 M NaCl	8
rRat BChE expressed in CHO cells	0.2 M procainamide	2
rMouse BChE expressed by adenovirus in HEK293 cells	1 M NaCl, 0.2 M choline	37
Procainamide does not bind		
Bovine BChE in plasma or serum		10
rBovine BChE expressed in CHO cells		10
Hupresin® purifies		
HuBChE in plasma	0.1 M tetramethylammonium Br or 0.1 M procaine	32; 46
rHuBChE truncated monomer expressed in CHO cells	0.1 M tetramethylammonium Br	Present report
rHuBChE expressed in rice *Oryza sativa*	0.5 M tetramethylammonium Cl	9
rHuBChE expressed in insect cells (no sialic acid on glycans)	0.5 M tetramethylammonium Cl	5
porcine BChE in porcine milk	0.1 M tetramethylammonium Br	44
Sarin-inhibited HuBChE in plasma	0.5 M tetramethylammonium Cl	3
Hupresin® does not bind		
rHuBChE with 47 mutations expressed in bacteria		4
rHuBChE acyl loop mutants		Brazzolotto (unpublished)
Bovine BChE in plasma or serum		10
rBovine BChE expressed in CHO cells		10

3.3. Hupresin® compared to procainamide Sepharose for purifying AChE

Though the procainamide affinity gel was developed for purification of HuBChE, we and others have found that it successfully purifies human acetylcholinesterase (HuAChE). Table 4 lists the applications for which procainamide Sepharose has been used to purify AChE.

In contrast, Hupresin® cannot be used to purify active HuAChE because it binds HuAChE too tightly. HuAChE is not released from Hupresin® by nondenaturing buffers. It can only be released with denaturing agents such as 1% trifluoroacetic acid or 50% acetonitrile [33]. This limits the application of Hupresin® for purification of HuAChE to projects that can make use of denatured enzyme, such as

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detection of nerve agent exposure by mass spectrometry. CHEMFORASE is synthesizing and testing new affinity ligands that will be useful for purifying AChE.

Table 4. Active AChE purified on procainamide affinity gel.

Procainamide purifies	Eluant	Reference
Fetal bovine serum AChE tetramers	10 mM decamethonium	11
AChE from brain of *Channa micropeltes* fish	1 M NaCl	47
AChE from Cotton aphid (*Aphis gossypii Glover)*	0.1 M procainamide	23
AChE from oyster *Crassostrea gigas*	1 M NaCl, 1 mM procainamide	1
rHuAChE monomers expressed in CHO cells	0.5 M tetramethylammonium I, 1 M NaCl, 1 mM decamethonium Cl	6
rHuAChE expressed in HEK293 cells	0.15 M decamethonium	22
rHuAChE expressed in tobacco *N. benthamiana*	0.2 M procainamide	16; 41
rMouse AChE monomers expressed in HEK293 cells	0.1 M decamethonium Br	21
rDrosophila *melanogaster* AChE expressed in insect cells	1 M NaCl, 10 mM procainamide	12
Hupresin® binds native HuAChE but releases denatured AChE		
Human erythrocyte AChE	1% Trifluoroacetic acid or 50% acetonitrile	33

3.4. Mass spectrometry for analysis of nerve agent exposure

Hupresin® has been used to isolate sarin-modified BChE tetramers from human plasma [3] and soman-modified AChE dimers from human red blood cells [33]. The yield of sarin-modified BChE was sufficiently high that the modified active site peptide could be detected by mass spectrometry. Use of the same enrichment protocol on procainamide-Sepharose yielded no detectable BChE active site peptide because contaminating proteins suppressed ionization of the peptide of interest.

The crystal structure of rHuBChE with huprine 19 shows the ligand is located deep within the active site gorge near the active site Ser198 [42]. This suggests that Hupresin® binding to BChE should be limited when Ser198 is modified with bulky organophosphates; recovery of sarin-modified peptides may depend on binding of Hupresin® to uninhibited subunits in the BChE tetramer.

Some protocols have successfully used affinity chromatography on procainamide-Sepharose to extract nerve agent modified BChE from human and marmoset plasma [13; 20; 25; 36; 51; 52].

The most successful methods to date for extracting nerve agent modified HuBChE and HuAChE from biological fluids use immobilized monoclonal antibodies to purify the proteins in preparation for mass spectrometry [7; 34; 48]. Binding to the antibodies is highly specific yielding samples with fewer contaminating proteins than samples enriched by affinity chromatography on either procainamide or Hupresin®. The immunopurified BChE and AChE proteins are released with denaturing agents.

4. Materials and Methods

Hupresin® was synthesized by Emilie David at CHEMFORASE, Mont-Saint-Aignan, France, emilie.david@chemforase.com . The ligand is a custom-synthesized hybrid of tacrine and huperzine [5; 38]. Materials are from the following sources: Ultraculture (Lonza 12-725F), methionine sulfoximine (Sigma 76078), tetramethylammonium bromide (Sigma 195758), Stericup Durapore (Millipore Merck, 1000 ml receiver bottle Cat No SCGPU11RE), Nalgene sterile filter unit with a 0.45 micron PES membrane (Thermo Scientific 167-0045).

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4.1. BChE activity assay.

Activity was measured at 25˚C in 2 mL of 0.1 M potassium phosphate pH 7.0 in 1 cm quartz cuvettes containing 0.5 mM dithiobisnitrobenzoic acid and 1 mM butyrylthiocholine iodide, on a Gilford spectrophotometer interfaced to a MacLab computer. Increase in absorbance at 412 nm was recorded for 1 min and converted to µmoles per min using E_{412nm} = 13,600 M^{-1} cm^{-1}. Units of activity are expressed as µmoles butyrylthiocholine hydrolyzed per min.

4.2. Expression of rHuBChE in serum-free culture medium

A stable cell line of CHO-K1 cells expressing truncated L530stop, 4 sugars off HuBChE [31] was grown in T75 flasks. The deleted glycosylation sites N17/455/481/486 unmasked N485 for glycosylation, so that the rHuBChE contained 6 glycans. N485 is not glycated in plasma-derived HuBChE [26].

The adherent cells were fed every 5 days with 35 mL of serum free, glutamine-free Ultraculture containing 25 µM methionine sulfoximine. The glutamine synthetase inhibitor, methionine sulfoximine, suppresses endogenous glutamine synthesis, while driving expression of recombinant glutamine synthetase encoded in the pGS plasmid vector. Culture medium was collected into sterile bottles over a period of 4 months and stored at 4˚C before the rHuBChE was purified. The culture medium contained 13,164 units of BChE activity (20.9 mg) in a volume of 3940 mL (5.3 mg/L).

4.3. Filtration of culture medium containing rHuBChE

Cells were allowed to settle to the bottom of the storage bottles before the clear portion was filtered through a 0.45 or 0.22 micron Nalgene sterile filter unit. The turbid portion was clarified by centrifugation before it was filtered. It was essential to remove cells and cell debris to avoid clogging the chromatography gel.

4.4. Loading filtered culture medium on Hupresin® column

A Pharmacia C26/40 glass column was cleaned with 500 mL of 0.1 M sodium hydroxide and rinsed with several liters of water before the column was packed with 82 mL of Hupresin®. The Hupresin® column was equilibrated with 20 mM TrisCl pH 7.5 at room temperature. The filtered culture medium was loaded on the Hupresin® column by gravity flow at room temperature.

4.5. Calculation of specific activity and mg of rHuBChE

Specific activity in units/mg was calculated from absorbance at 280 nm using the relationship that a 1 mg/ml solution of HuBChE has an absorbance of 1.8 at 280 nm. The yield of rBChE in mg was calculated using 630 units/mg as the specific activity for pure rHuBChE.

5. Conclusion

Procainamide Sepharose has been used since 1978 to purify BChE from a variety of sources. A new affinity gel, Hupresin®, is now available. Hupresin® is a better affinity gel for purifying BChE and is recommended over procainamide Sepharose for that purpose. Hupresin® is stable and can be reused many times. Between runs Hupresin® can be sanitized and cleaned with 0.1 M sodium hydroxide. Procainamide Sepharose will continue to be useful for purifying AChE because Hupresin® binds, but does not release native AChE.

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