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Purification and partial amino acid sequence analysis of human erythrocyte acetylcholinesterase

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A single step immunoaffinity purification procedure for human erythrocyte acetylcholinesterase is described which permitted the isolation of milligram quantities of enzyme from 10 U of erythrocytes, with 113 000-fold purification and a yield of about 22%. In SDS-PAGE analysis, the enzyme corresponds to a disulfide linked dimer of 140 kDa which is converted to a 70 kDa monomer upon disulfide reduction. The tryptic peptides generated from purified enzyme were separated by reverse-phase HPLC. Five of these peptides were analysed to determine the amino acid sequences. The obtained sequences showed no homology to the already known amino acid sequences for human serum and brain butyrylcholinesterase and *Torpedo californica* acetylcholinesterase.

Acetylcholinesterase; Immunoaffinity chromatography; Amino acid sequence

1. INTRODUCTION

Acetylcholinesterase (AChE), an essential enzyme of the nervous system, rapidly terminates the action of acetylcholine released into the synapse. The enzyme exists in multiple molecular forms [1] with similar kinetic properties. The asymmetric and globular forms differ in their cellular localization [1], in number of catalytic subunits [2], in level of hydrophobicity [3] and mode of glycosylation [4]. In blood, amphipathic acetylcholinesterase dimers of globular form are bound to the erythrocyte membrane [5,6]. The present study was undertaken to determine the partial amino acid sequence for human erythrocyte acetylcholinesterase, which can further be used for isolating the gene of the enzyme, as no such information is available to date. Attempts to identify the gene for human acetylcholinesterase using the probes derived from the *Torpedo* enzyme cDNA sequence [7] have resulted in the isolation of genes for pseudocholinesterase [8].

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2. MATERIALS AND METHODS

2.1. *Extraction of acetylcholinesterase from human erythrocytes*

Packed human erythrocytes from 10- to 20-day-old collection of blood obtained from Baltimore Red Cross blood program (Baltimore, MD, USA) were washed three times with a cold, isotonic solution of 5 mM sodium phosphate, pH 7.5, containing 0.15 M sodium chloride. The cells were then lysed by slowly pouring and stirring into 20 vols of cold lysis buffer (5 mM sodium phosphate, pH 7.5, containing 0.02% sodium azide). The erythrocyte membranes were separated from soluble proteins and simultaneously concentrated to approx. 2 l using Millipore pellicon high volume molecular filtration set-up as described [9]. The membranes were then washed with 20 l of cold lysis buffer using the above mentioned set-up and collected by centrifugation at 4°C for 30 min at 10000 × *g*. The proteins of the washed membranes were solubilized by adding Triton X-100 to a final concentration of 1% and stirring the mixture for 15 h at 4°C. The supernatant fluid was collected by centrifugation at 100000 × *g* for 1 h at 4°C and then diluted 5-fold by adding 4 vols of lysis buffer before loading onto the monoclonal antibody affinity column.

2.2. *Monoclonal antibody affinity column preparation*

Hybridoma cells secreting monoclonal antibody AE-4, specific for human acetylcholinesterase, were obtained from Dr D. Fambrough (Carnegie Institute, Baltimore, MD, USA) [10]. Large amounts of immunoglobulin were obtained by growing cells as ascites tumors in mice. Ascites fluid was inactivated at

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56°C for 30 min and clarified by centrifugation at 100000 × *g* for 1 h. The antibody was purified from ascites fluid by chromatography on a protein A-Sepharose column (data not shown). The purified IgG was coupled to Sepharose CL-4B according to the procedure described [11]. A 1.5 × 10 cm column containing 30 mg of AE-4 antibody coupled to 10 ml of Sepharose CL-4B beads was washed with several volumes of 20 mM sodium phosphate buffer, pH 7.5, before applying the erythrocyte membrane extract.

2.3. *Purification of acetylcholinesterase*

The diluted extract was loaded onto the AE-4 antibody affinity column at a flow rate of 1 ml/min, at 4°C. After the sample was loaded, column was washed successively with 30 ml each of (i) 20 mM sodium phosphate, pH 7.5, containing 0.2% Triton X-100, (ii) 20 mM sodium phosphate, pH 7.5, containing 0.5% β-D-octylglucoside, (iii) 0.1 M borate buffer, pH 8.0, containing 1 M sodium chloride and 0.5% β-D-octylglucoside, (iv) 50 mM diethylamine, pH 10.0, with 0.5% β-D-octylglucoside. The protein was then eluted with 50 mM diethylamine, pH 10.5, containing 0.5% β-D-octylglucoside. Fractions of 3 ml were collected in tubes containing 0.5 ml of 0.5 M monobasic sodium phosphate with 0.5% β-D-octylglucoside. Individual fractions were assayed for enzyme activity according to Ellman et al. [12]. The active fractions were pooled and concentrated to 1 ml by vacuum dialysis against 100 mM sodium phosphate, pH 8.0, containing 0.5% β-D-octylglucoside. Protein concentration was determined as described in [13].

2.4. *Enzyme assay*

Acetylcholinesterase activity was estimated according to Ellman et al. [12]. The assay mixture contained 5 μl of 50 mM dithiobisnitrobenzoic acid, 5 μl enzyme sample, 300 μl of 100 mM sodium phosphate buffer, pH 7.5, and 2 μl of 75 mM acetylthiocholine substrate. Appearance of the nitrothiobenzoic acid adduct of thiocholine product was measured spectrophotometrically at several time points as the increase in absorbance at 405 nm.

2.5. *SDS-PAGE analysis*

Vertical, 10% polyacrylamide slab gel was prepared and run according to the system described by Laemmli [14]. The gels were stained with silver nitrate for protein visualization [15]. The molecular mass for the purified AChE was estimated from standard curve of log kDa versus relative migration of polypeptide standards.

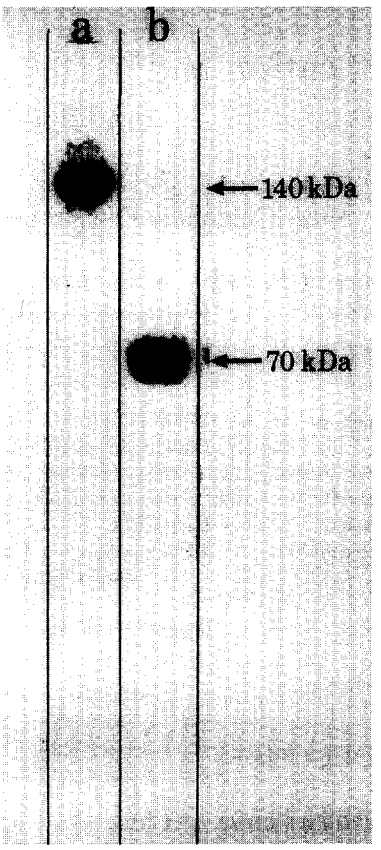

Fig.1. SDS-PAGE analysis for purified AChE (20 μg) before (a) and after disulfide reduction (b). The molecular mass was calculated as described in the text.

Table 1

Purification of human erythrocyte acetylcholinesterase[a]

Step	Total volume (ml)	Total protein[b] (mg)	Activity[c] (ΔA/min)[d]	Recovery (percent)	Specific activity (ΔA/min per mg)	Fold purification
Hemolysate	21217	488286	292971		0.6	1
Red cell ghosts	1132	5040	262080	89	52	486
100000 × *g* supernatant	1027	994	176932	60	178	296
Antibody affinity	0.9	0.95	64771	22	68180	113633

[a] Results are the mean value of four purifications
[b] Protein was measured as described in [13]
[c] Enzyme activity was measured as described in [12]
[d] Change in absorbance at 405 nm

2.6. *Trypsin digestion of acetylcholinesterase and separation of peptides*

Purified enzyme (70 μg in 2 ml of 20 mM sodium phosphate buffer, pH 7.6, containing 0.2% β-D-octylglucoside) was incubated twice with 1.5 μg trypsin at room temperature for 4 h each. The peptide mixture was applied to a Vydac reverse-phase C4 column (4.6 mm × 25 cm) equipped with a Beckman 421 gradient controller. The solvents were (A) 0.1% trifluoroacetic acid (TFA) and (B) acetonitrile propanol (2:1) containing 0.1% TFA. The column temperature was maintained at 45°C. The peptides were eluted at a flow rate of 1 ml/min with the gradient program shown in fig.2.

2.7. *Amino acid sequence analysis*

Individual peptide peaks from reverse-phase HPLC were dried under vacuum, redissolved in 100 μl of 50% acetic acid (aldehyde free) and analysed with an Applied Biosystems model 470 A gas-phase sequencer with an on-line PTH-amino acid analyser.

3. RESULTS AND DISCUSSION

3.1. *Purification of erythrocyte acetylcholinesterase*

The results of a typical purification experiment are summarized in table 1. 1 mg of pure enzyme could be obtained from 10 U of packed erythrocytes. This yield is similar to that reported by Rosenberry et al. [5], using the acridinium ligand procedure. The recovery of the enzyme activity was found to be 22% with a fold purification of over 113000. The Triton X-100 used to solubilize the enzyme was displaced during affinity chromatography by another detergent β-D-octylglucoside, which could be easily dialysed and did not interfere with the separation of peptides on reverse-phase HPLC. The elution of enzyme from the antibody column at pH 10.5 was found to be important as the enzyme eluted at higher pH values was partially denatured and less active (data not shown).

The preparation was found to be homogeneous by SDS-PAGE analysis (fig.1). The enzyme was present as a dimer (140 kDa) before and as a monomer (70 kDa) after disulfide reduction.

3.2. *Fractionation of tryptic peptides and amino acid sequence analysis*

Fig.2 shows the elution profile obtained by reverse-phase HPLC of the tryptic peptides of acetylcholinesterase. Identical profiles were obtained for the peptide mixtures in three separate experiments. Approx. 19 peptides were resolved. Five of these peptides have yielded satisfactory sequence data (table 2), totaling for 105 residues. The sequences obtained show no homology with the human butyrylcholinesterase [8] or *Torpedo* acetylcholinesterase [7]. The erythrocyte acetylcholinesterase being globular in nature is expected to be different than the asymmetric form of the en-

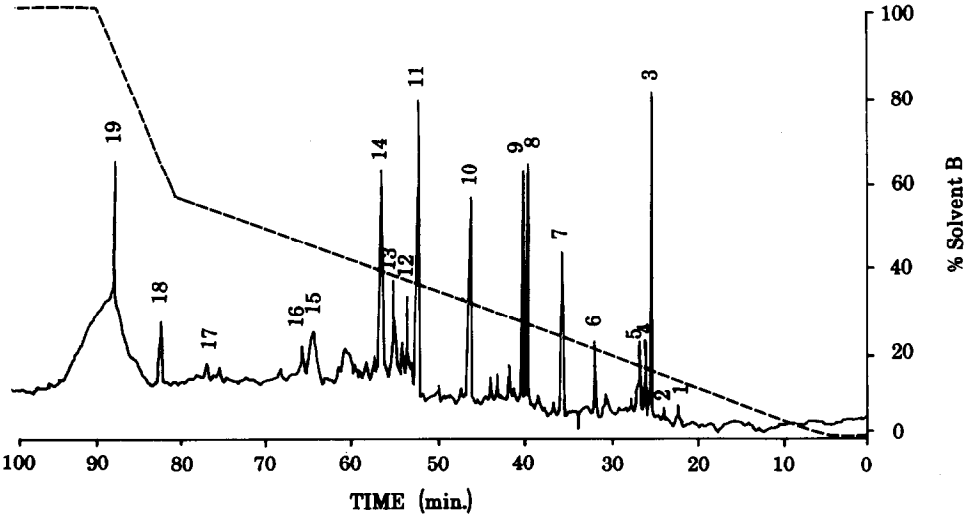

Fig.2. Preparation of tryptic peptides of AChE. AChE (70 μg) in 2 ml of 20 mM sodium phosphate, pH 7.6, containing 0.2% octylglucoside was treated at room temperature for 8 h with a total of 3 μg of trypsin. The digest was applied to a Vydac C4 column (4.6 mm × 25 cm), maintained at 45°C. The solvents were (A) 0.1% TFA and (B) acetonitrile/propanol (2:1) containing 0.1% TFA. The peptides were eluted at a flow rate of 1 ml/min with the following gradient program: 0% B for 5 min, 0% B to 55% B in 75 min, 55% B to 100% B in 10 min and 100% B for 10 min. The peptides are numbered in their order of elution from the column.

Table 2

Sequence analysis of AChE tryptic peptides

Cycle number	Peptide 7		Peptide 9		Peptide 10		Peptide 11		Peptide 16	
	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)
1	Ala	X	Thr	99	X	X	Val	216	X	X
2	Val	224	Arg	50	Pro	276	Gly	142	X	X
3	Leu	265	Pro	210	Gln	116	Val	202	Thr	28
4	Gln	188	X	X	Try	156	Pro	296	Leu	75
5	Ser	56	Gln	73	Pro	218	Gln	119	Asp	32
6	Gly	129	Val	118	Pro	225	Val	182	Val	19
7	Ala	176	Leu	113	Tyr	144	Ser	40	Pro	49
8	Pro	190	Val	118	Thr	71	Asp	56	Leu	50
9	Asn	85	X	X	Ala	158	Leu	160	X	X
10	Gly	124	His	25	Gly	141	Ala	117	Met	36
11	Pro	131	Glu	71	Ala	101	Ala	126	Gly	27
12	Try	46	Asp	64	Gln	132	Glu	92	Val	33
13	Ala	103	His	25	Gln	122	Ala	106	Pro	37
14	Thr	35	Val	85	Tyr	116	Val	110	X	X
15	Val	82	Leu	94	Val	125	Val	92	Gly	25
16	Gly	101	Pro	98	Ser	26	Leu	115	Tyr	15
17	Met	74	Gln	48	Leu	93	Asp	22		
18	Gly	44	Glu	50	Asp	36	Tyr	73		
19			Asp	59	Leu	75	Thr	29		
20			Val	20	Arg	34	Asp	29		
21			Phe	45	Pro	85	Try	36		
22					Leu	63	Leu	64		
23					Glu	48	X	X		
24					Val	49	Pro	60		
25							X	X		
26							Asp	16		
27							Pro	46		
Average repetitive yield		88.35		88		89.7		95.2		86

X, unidentified residue

zyme, the gene for which is cloned from *Torpedo californica* [7]. We are now using the obtained amino acid sequences to generate the oligonucleotide probes and identify the gene for human erythrocyte acetylcholinesterase.

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