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Epitopes recognized by anti-reduced and alkylated acetylcholinesterase antibodies[1]

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ABSTRACT Peptides of the reduced and alkylated acetylcholinesterase (RA-AChE) from the electric organ of Torpediniformes *Torpedo torpedo* subjected to bromo-cynogen (CNBr) cleavage or/and peptic digestion conserved well the antigen-antibody reactivity with anti-RA-AChE monoclonal antibodies E9, F6, and F12, whereas peptides produced by CNBr and tryptic treatments lost all the reactivity. Periodate oxidation of the RA-AChE or glycopeptidase digestion of the CNBr cleaved RA-AChE did not change the antigen-antibody reactivity. It implied that the epitopes recognized by the 3 anti-RA-AChE monoclonal antibodies are all peptide determinants rather than carbohydrate determinants.

KEY WORDS acetylcholinesterase; monoclonal antibodies; antigenic determinants

Acetylcholinesterase (acetylcholine acetylhydrolase, EC 3, 1, 1, 7, AChE) is one of the essential enzymes in the nervous system. Immunological properties of the enzyme protein have been investigated[1-4]. However, little is known about its antigenic determinants (epitopes) which is reckoned as another important domain of the enzyme next to the catalytic center. It becomes a major issue in the study of the structure of enzyme protein. In this paper, AChE purified from the electric organ of Torpediniformes *Torpedo torpedo* was reduced and alkylated to unfold the enzyme molecule for thorough exposure of the continuous antigenic determinants buried in the protein molecule. Monoclonal antibodies directed to the reduced and alkylated acetylcholinesterase (RA-AChE) were made use of in exploration of the epitope property.

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MATERIALS AND METHODS

o-Phenylenediamine, purchased from Merck; trypsin, pepsin and glycopeptidase F, from Sigma; CNBr-activated Sepharose 4B, from Pharmacia LKB; horse-radish peroxidase labeled goat-anti-mouse IgG, product of Institute of Microbiology and Epidemiology; other reagents were all chemically pure. ELISA microplates, produced by Tianjin Plexiglass Factory; Titertek Multiskan Type Mcc/340 MK II, Flow product.

RA-AChE and anti-RA-AChE monoclonal antibody ascites was prepared[5].

ELISA of small molecular peptide was carried out[5]. The microplates were pretreated with 200 μl/well of the coating buffer (phosphate buffer 50 mmol · L^{-1}, pH 8.0) containing 0.1 % 1,5-penta dial for 3 h, washed once with the coating buffer (200 μl/well), and then went on for ELISA.

Cleavage of RA-AChE by CNBr[7] Aliquots (5 mg) of lyophilized RA-AChE were dissolved in 0.2 ml CNBr solution (10 mg · ml^{-1}, in 70 % formic acid), incubated at 25 C in nitrogen for 4, 12, or 24 h, and mixed with 1.8 ml deionized water and lyophilized again.

Tryptic digestion of CNBr-Cleaved peptide CNBr-Cleaved peptides (5 mg) of RA-AChE were dissolved in 2 ml of NH_4HCO_3 50 mmol · L^{-1} (pH 8.0), and mixed with 0.2 ml of packed Sepharose 4B immobilized with trypsin. After shaking at 37 ℃ for 16 h, the filtrate was lyophilized.

Peptic digestion of CNBr-Cleaved peptide[8] CNBr-Cleaved peptides (5 mg) of RA-AChE were dissolved in 2.5 ml HAc 50 mmol · L^{-1}, and incubated with 50 μg pepsin (in 50 μl deionized water) at 37 ℃ for 18 h. The hydrolysate was boiled for 5 min and then lyophilized.

Glycopeptidase F treatment[9] CNBr-Cleaved RA-AChE peptides (1 mg) were incubated with 0.2 U glycopeptidase F in 0.5 ml phosphate buffer 50 mmol · L^{-1} (pH 7.0) at 37 ℃ for 18 h and heated at 100 ℃ for 3 min to terminate the reaction.

Periodate oxidation[10] To the antigen (RA-AChE)-coated microplate, 200 μl/well of washing buffer (phosphate buffer 10 mmol · L^{-1}, 0.05 % Tween 20, ph 7.2) was added. The microplate was washed thrice 30 min later. After washing with acetate buffer 50 mmol · L^{-1} (pH 4.5), the periodate (20 mmol · L^{-1} in acetate buffer, 200 μl/well) was added to each well. The plate was left in dark for 1 h, and then washed with acetate buffer 3 times. Glycine (1 %, in 50 mmol · L^{-1} phosphate buffer, pH 8.0, freshly prepared) was used for blockage (30 min). ELISA was carried out after 5 cycles of washing. Acetate buffer was used in the non-oxidized control instead of the periodate.

RESULTS AND DISCUSSION

Cleavage and cutting of RA-AChE RA-AChE was chemically cleft by CNBr at 25 ℃ for 24 h and then cut with trypsin or pepsin. As shown in SDS-PAGE, the cleavage of RA-AChE was nearly completed after CNBr treatment for 24 h (Fig 1 b, c) and the enzymatic cutting rendered the cleavage even more thoroughly (Fig 1 d, e).

Immunoreactivity of antibodies with RA-AChE and its cleaved peptides Immunoreactivity of monoclonal antibodies E9, F6, and F12 in mouse ascitic fluid ($1:10^2 \sim 1:10^5$ dilution) was tested with RA-AChE, CNBr-RA-AChE, trypsin-CNBr-RA-AChE or pepsin-CNBr-RA-AChE (0.5 μg/well). The pepsin-CNBr-RA-AChE pepetides reacted well with E9, F6, or F12. However, the trypsin-CNBr-RA-AChE peptides lost all their antigenicity (Tab 1). The results implied that epitopes directed by E9, F6, and F12 might not contain methionine, phenylalanine, tryptophan, and leucine residues. But lysine and/or arginine residue probably constituted the most important constituents of the epitopes.

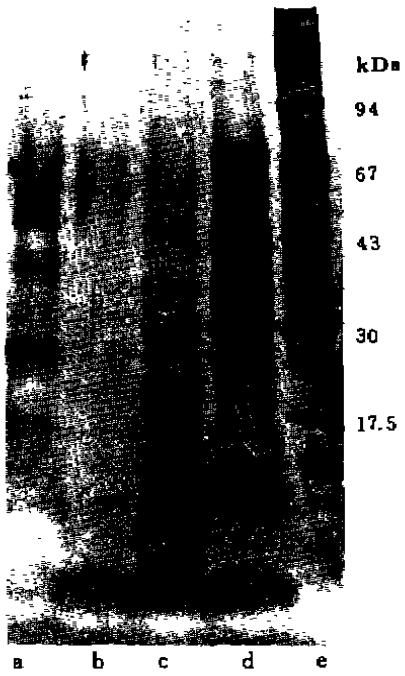

Fig 1. SDS-PAGE of cleaved peptides of RA-AChE. Gel gradient, 4 — 30 %; silver staining. a) RA-AChE cleaved by CNBr and digested by pepsin. b) RA-AChE clove by CNBr and digested by trypsin. c) RA-AChE clove by CNBr for 24 h. d) RA-AChE cleaved by CNBr for 4 h. e) Marker: phosphorylase B, 94 kDa; bovine serum albumin, 67 kDa; actin, 43 kDa; carbonic anhydrase, 30 kDa; TMV coat protein, 17.5 kDa.

Property of epitopes recognized by anti-RA-AChE monoclonal antibodies AChE is a glycoprotein of known molecular structure. The specific sequences of the polypeptides and/or its carbohydrate side chains might constitute the epitopes. Changes in the antigen-antibody reactions between anti-RA-AChE

Tab 1. Influence of enzymatic and chemical cleavages on antigenicity of reduced and alkylated-AChE. $n=3$, $\bar{x}\pm s$.

Monoclonal antibody	Dilution	Absorbance at λ_{492} (ELISA) RA-AChE	RA-AChE-CNBr	RA-AChE-CNBr-trypsin	RA-AChE-CNBr-pepsin
E9	1 : 10^4	2.37±0.11	1.27±0.05	0.03±0.00	1.62±0.10
	1 : 10^5	0.78±0.02	0.51±0.10	0.03±0.00	0.91±0.09
F6	1 : 10^4	2.28±0.10	1.86±0.10	0.06±0.01	1.17±0.07
	1 : 10^5	0.54±0.01	0.51±0.05	0.06±0.02	0.77±0.04
F12	1 : 10^4	2.09±0.15	1.83±0.04	0.10±0.00	1.81±0.11
	1 : 10^5	0.95±0.08	0.97±0.05	0.07±0.01	1.28±0.02
SP2/0	1 : 10^4		0.11±0.02	0.08±0.01	0.10±0.01
	1 : 10^5		0.08±0.01	0.07±0.01	0.07±0.00

Tab 2. Influence of periodate oxidation or glycopeptidase F treatment on antigenicity of reduced and alkylated-AChE and its cleaved peptides. $n=3$, $\bar{x}\pm s$.

Antigen	Dilution of monoclonal antibody	Absorbance at λ_{492} (ELISA) E9	F6	F12
RA-AChE	1 : 10^5	2.58±0.05	3.24±0.21	3.02±0.17
	1 : 10^4	1.72±0.07	1.97±0.09	1.40±0.04
RA-AChE-periodate	1 : 10^3	2.34±0.09	3.28±0.29	3.14±0.09
	1 : 10^4	1.64±0.11	2.12±0.08	1.46±0.09
RA-AChE-CNBr	1 : 10^4	1.27±0.05	1.86±0.10	1.83±0.04
	1 : 10^5	0.51±0.10	0.51±0.05	0.97±0.05
RA-AChE-CNBr-glycopeptidase	1 : 10^4	1.42±0.07	2.26±0.20	1.79±0.14
	1 : 10^5	0.49±0.05	0.54±0.04	0.86±0.06

monoclonal antibodies and AChE or its cleaved peptides before and/or after the modification of carbohydrate side chains by glycopeptidase hydrolysis or periodate oxidation would reflect somehow the features of the epitopes. The results showed that the modifications did not influence the reactivity between antigens and monoclonal antibodies (Tab 2). The 3 epitopes directed by E9, F6, and F12 all belong to the peptide-type rather than the carbohydrate-type. They are buried inside the native enzyme molecule, and the glycosylation might occur only at the glutamine residues exposed to the surface of the protein.

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抗还原并烷化的 AChE 单克隆抗体识别的抗原决定基

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A **摘要** 电器官还原并烷化的乙酰胆碱酯酶(RA-AChE)的溴化氰裂解肽或又经胃蛋白酶切割的肽与抗 RA-AChE 单克隆抗体 E9、F6 及 F12 仍有抗原抗体反应. 经溴化氰及胰蛋白酶割的肽与 3 者反应消失. 氧化破坏 RA-AChE 糖侧链、溴化氰裂解的 RA-AChE 又经酶切除糖侧链后, 与 3 者反应无变化. E9、F6 及 F12 所识别的抗原决定簇是多肽型, 不是多糖型.

关键词 乙酰胆碱酯酶; 单克隆抗体; 抗原决定基 单克体