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Acetylcholinesterase from *Bungarus* venom: a monomeric species

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Abstract The venom of *Bungarus fasciatus*, an *Elapidae* snake, contains a high level of AChE activity. Partial peptide sequences show that it is closely homologous to other AChEs. *Bungarus* venom AChE is a non-amphiphilic monomeric species, a molecular form of AChE which has not been previously found in significant levels in other tissues. The composition of carbohydrates suggests the presence of *N*-glycans of the 'complex' and 'hybrid' types. Ion exchange chromatography, isoelectric focusing and electrophoresis in non-denaturing and denaturing conditions reveal a complex microheterogeneity of this enzyme, which is partly related to its glycosylation.

Key words: Acetylcholinesterase; Snake venom; *Bungarus fasciatus*

1. Introduction

Acetylcholinesterase (AChE, E.C. 3.1.1.7) plays a vital role in cholinergic transmission, ensuring an extremely rapid hydrolysis of the neurotransmitter acetylcholine. AChE exists in oligomeric forms, which are integrated in various ways in cholinergic synapses, in the nervous system and muscles [1]. AChE also occurs in non-cholinergic tissues, e.g. in blood cells [2].

The venoms of some Elapid snakes constitute a remarkable source of 'non-cholinergic' AChE [3]. In particular, AChE may be purified in significant quantities from the venom of *Bungarus fasciatus*. In this report, we show that this enzyme presents a strong sequence homology with AChEs from various invertebrate and vertebrate species, in agreement with previous studies on AChE from the venom of *Naja naja oxiana* [4,5]. It differs, however, from AChEs from other vertebrate tissues by the fact that it consists entirely of soluble, non-amphiphilic monomers. In an attempt to understand the significance of AChE in venoms, we analyzed whether it was toxic by itself, or acted synergistically with other venom components.

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Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; AcSCh, acetylthiocholine; BuSCh, butyrylthiocholine; IEF, isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; PI-PLC, phosphatidylinositol specific phospholipase C; PrSCh, propionylthiocholine

2. Materials and methods

2.1. Materials

Bungarus fasciatus venom was from the stock of Institut Pasteur. Reagents for polyacrylamide gel electrophoresis (PAGE), electrofocusing and Mono Q column were from Pharmacia (Uppsala, Sweden). All salts and other reagents were from Merck (Darmstadt, Germany) or from Prolabo (Paris, France). Phosphatidylinositol specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* was from Immunotech (Marseilles, France) and neuraminidase from *Arthrobacter ureafaciens* was from Boehringer (Mannheim, Germany). *N*-Glycanase from *Flavobacterium meningosepticum* was from Genzyme (Boston, MA, USA).

2.2. AChE assay

AChE activity was determined by the colorimetric method of [6], using acetylthiocholine, propionylthiocholine or butyrylthiocholine as substrate. Unless otherwise specified, the assay medium contained 0.75 mM acetylthiocholine iodide, 0.25 mM dithio-bis-dinitrobenzoic acid, 0.01 M sodium phosphate, pH 7.4, and the reaction was monitored at 412 nm.

2.3. Purification of AChE; sedimentation and electrophoretic analyses

Lyophilized *Bungarus* venom was solubilized in 0.1 M sodium phosphate buffer, pH 7.4 (100 mg/ml), under gentle stirring. An affinity gel for AChE was prepared by coupling Sepharose 4B to an *m*-carboxyphenyldimethylethyl ligand, as previously described [7]. The reconstituted venom (2 ml) was applied to 2 ml of affinity gel, equilibrated in the same buffer (fixation buffer). The column was washed with 10 ml of the fixation buffer, 10 ml of buffer complemented with 0.25 M NaCl, and finally eluted with 0.1 M sodium phosphate, 0.4 M NaCl, 0.02 M decamethonium bromide. Active fractions were pooled (affinity purified AChE) and extensively dialyzed, for 72 h, at 4°C, against several changes of 0.1 M sodium phosphate pH 7.4.

Sedimentation in sucrose gradients, without detergent or in the presence of 1% Triton X-100 or Brij-96, was performed as described previously (Bon et al., 1988). Isoelectric focusing (IEF) in polyacrylamide gels between pH 3 and pH 9 was performed in a Phastsystem apparatus (LKB Pharmacia, Uppsala, Sweden). Gels were fixed with 20% trichloroacetic acid, stained in methanol/acetic acid/water (3/1/6) containing 0.02% Coomassie brilliant blue R and 0.1% $CaSO_4$, and destained in the same solution. Polyacrylamide gel electrophoresis (PAGE) in denaturing and in non-denaturing conditions was performed as previously described [8]. AChE activity was revealed by the method of [9].

2.4. Peptide sequencing

Tryptic peptides were obtained after trypsin digestion of affinity purified AChE from *Bungarus* venom. They were purified by on-line reverse phase HPLC in a C_8 column and sequenced by Edman degradation with an Applied Biosystem 102-A analyzer.

2.5. Determination of carbohydrate composition

For the determination of neutral monosaccharides, 4–20 μg of affinity purified *Bungarus* venom AChE was mixed with 400 μl of 2 M trifluoroacetic acid and incubated at 100°C for 5 h. For basic monosaccharides, hydrolysis of a similar sample was performed with 6 N HCl, under the same conditions. For *N*-acetylneuraminic acid, 0.15 mg of AChE was digested with neuraminidase from *Arthrobacter ureafaciens* for 15 h at 37°C, in 50 mM sodium acetate buffer pH

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5.5, in the presence of 0.5 mg/ml bovine serum albumin. In all cases, the reaction medium was evaporated to dryness. After addition of H_2O and filtration through a membrane of 0.45 μm pore size, an aliquot was analyzed by HPLC with a Bio LC Dionex chromatograph in a basic Carbopac PA1 column (Ø4 mm, length 250 mm) using gradient elution with sodium hydroxide for monosaccharides, and isocratic elution with sodium acetate for neuraminic acid. Three independent assays were performed for neutral and basic monosaccharides, and two for sialic acid. Although glucose was found in some assays of neutral sugars, this was erratic and most likely resulted from contamination, as frequently observed. Otherwise, the results showed high reproducibility, with less than 5% variation for glucose, mannose, *N*-acetylglucosamine and sialic acid, and fair reproducibility for galactose, with about 12% variation.

2.5. Fractionation of affinity purified Bungarus venom AChE by ion exchange chromatography on a Mono-Q column

A sample (1 ml) of affinity purified AChE was dialyzed against 25 mM Tris-HCl pH 7.5 containing 25 mM $MgCl_2$, then applied to a Mono Q column (HR 5/5, about 0.4 ml), previously equilibrated with the same buffer. Elution was carried out at a flow rate of 1 ml/min, first with a small volume of buffer (isocratic elution), then with a linear NaCl gradient from 0 to 0.5 M. Fractions (1 ml) were collected in assay tubes containing 0.1 ml of 1 mg/ml bacitracin, to prevent adsorption of the enzyme to the tube walls.

2.7. Treatment with neuraminidase and PI-PLC

Neuraminidase-treated AChE, obtained as described in Section 2.6, was subjected to chromatography on a Mono Q column. For IEF, 20 μl samples of 1.5 mg/ml of AChE were incubated with 0.2 mg/ml of neuraminidase from *Clostridium perfringens* (Boehringer, Mannheim) in 50 mM sodium acetate buffer, pH 6.5, for 2 h at 37°C; for non-denaturing PAGE, fractions from the Mono Q column were treated with 50 μg/ml neuraminidase, in the same manner. Most of the AChE activity was retained after such incubation. Treatment with PI-PLC from *Bacillus thuringiensis* was performed as previously described [8]. Deglycosylation of denatured AChE was performed by *N*-glycanase (Genzyme), as specified by the manufacturer, prior to sodium dodecyl sulfate PAGE.

2.8. Toxicity in vivo

In lethality assays, we used 3 week old male Swiss mice weighing 18–20 g, from Charles River (St Aubin-lès-Elbeuf, France). Samples of 0.2 ml of *Bungarus fasciatus* venom or of affinity purified AChE were injected intravenously. Crude *Bungarus fasciatus* venom (1 g) was dissolved in 20 ml of water. The material which did not adsorb to a 2 ml affinity column (see above) was the AChE-depleted venom. The purified enzyme, eluted 20 mM decamethonium bromide, was extensively dialyzed against 20 mM sodium phosphate pH 7.0 containing 20 mM NaCl, concentrated by lyophilization, then gel-filtered in the same buffer through a Sephadex G-25 column.

The lethal potency (LD_{50}) and the confidence limits of LD_{50} were calculated by the statistical method of Spearman-Kärber, as recommended by the World Health Organization (World Health Organization Bulletin, offset publication No. 58, pp. 25–27, 1981). Three mice were injected with each dose, and the doses differed by a factor of 1.414 ($\sqrt{2}$).

3. Results

3.1. Bungarus fasciatus venom contains a true AChE

Bungarus venom contains an enzyme which hydrolyzes acetylthiocholine faster than propionylthiocholine, and has essentially no activity on butyrylthiocholine. It also presents an excess substrate inhibition, above 3 mM acetylthiocholine. It is inhibited by eserine (physostigmine), with an IC_{50} of about 2×10^{-9} M. This enzyme thus possesses all the characteristics of a typical AChE.

Bungarus venom AChE could be successfully purified by affinity chromatography on *m*-carboxyphenyldimethylethyl-EAH-Sepharose [7], with a yield of 85%, producing 2–5 mg AChE/g of dry venom. The N-terminal sequence of affinity purified *Bungarus* venom AChE was blocked, but we determined peptide sequences from several tryptic fragments. These partial sequences could be aligned with the primary sequence of other AChEs (Fig. 1).

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Torpedo   DDDSELLVNTKSGKVMRTRIPVLSSHISAFLGIPFAEPPVGNMRFRRPEPKKPWSGVWNA  60
Torpedo   STYPNNCQQYVDEQFPGFPGSEMWNPNREMSEDCLYLNIWVPSPRPKSATVMLWIYGGGF 120
Bungarus                                                      LALQWI
                                                              .:::::.
Torpedo   YSGSSTLDVYNGKYLAYTEEVVLVSLSYRVGAFGFLALHGSQEAPGNMGLLDQRMALQWV 180
Bungarus  QNNIHPFGG                                  AILQSGGPNAPWATVSPAE
            .::  :::                                 :::::::.:: :::.:: ::
Torpedo   HDNIQFFGGDPKTVTLFGESAGRASVGMHILSPGSRDLFRRAILQSGSPNCPWASVSVAE 240
Bungarus  S                                                     FPFVSVIDGDF
          .                                                     : :: :::::.:
Torpedo   GRRRAVELRRNLNCNLNSDEDLIQCLREKKPQELIDVEWNVLPFDSIFRFSFVPVIDGEF 300
Bungarus  FPDTPEAML      ETQLLLGVVKDEGSYFLIYGLPGFSK
          :: . : ::       ::.::::: :::::.:::.:: :::::
Torpedo   FPTSLESMLNAGNFKKTQILLGVNKDEGSFFLLYGAPGFSKDSESKISREDFMSGVKLSV 360
Torpedo   PHANDLGLDAVTLQYTDWMDDNNGIKNRDGLDDIVGDHNVICPLMHFVNKYTKFGNGTYL 420
Bungarus                                                      YWANFARTG
                                                              ::: ::.::
Torpedo   YFFNHRASNLVWPEWMGVIHGYEIEFVFGLPLVKELNYTAEEEALSRRIMHYWATFAKTG 480
Torpedo   NPNEPHSQESKWPLFTTKEQKFIDLNTEPIKVHQRLFWNQFLPKLLNATETIDEAERQWV 540
Torpedo   KTEFHRWSSYMMHWKNQFDQYQRHENCAEL                               570
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Fig. 1. Alignment of partial peptide sequences from *Bungarus* venom AChE with the primary sequence of *Torpedo marmorata* AChE. Single and double dots indicate similar and identical residues between the two enzymes.

3.2. Macromolecular structure: a non-amphiphilic monomer

In sucrose gradients, *Bungarus* AChE sedimented as a monodisperse peak of 4.5 S (Fig. 2). The same sedimentation was observed with crude venom and with purified AChE and was not affected by addition of the non-denaturing detergents Triton X-100 or Brij-96. In gel filtration chromatography through a Sephadex column, this enzyme eluted at approximately the same position as bovine serum albumin, indicating that its Stokes radius is close to 4.5 nm. Together, these hydrodynamic analyses indicate that *Bungarus* venom AChE is a non-amphiphilic monomeric species. In addition, the affinity purified enzyme showed the same pattern in PAGE, under denaturing conditions, with or without reduction.

3.3. Carbohydrate composition of Bungarus venom AChE

Affinity purified *Bungarus* venom AChE contains about 7% of its weight of carbohydrates: 37% mannose, 32% *N*-acetylglucosamine, 11% galactose, 9% fucose, 9% *N*-acetylneuraminic acid, indicating that the protein carries mostly *N*-glycans

Table 1
AChE does not contribute to the lethal potency of the venom

Fraction	Protein (mg)	LD_{50} (mg/kg)	AChE activity (U/mg)
Crude venom	1000	2.4 ± 0.5	750 ± 30
Venom depleted of AChE	995 ± 5	2.8 ± 0.5	2 ± 1
Affinity purified AChE	9 ± 1	>80	72 000 ± 4000

Values are means and standard errors of two experiments.

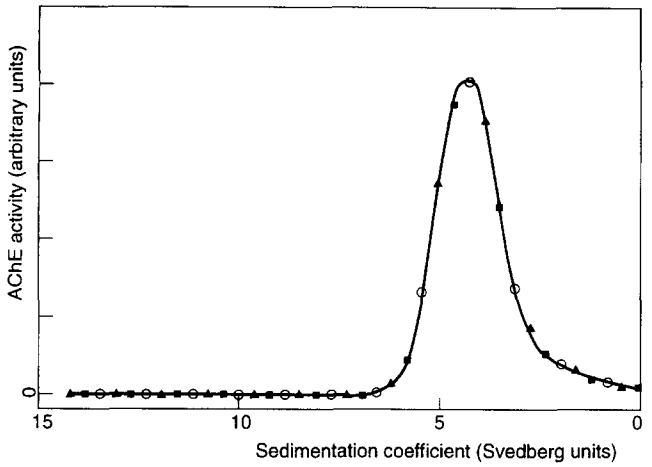

Fig. 2. Sedimentation profile of *Bungarus* venom AChE. The sedimentation coefficients were deduced from the positions of the peaks of the internal sedimentation standards, catalase (11.3 S) and alkaline phosphatase (6.1 S). The sedimentation of AChE was identical in the presence of 1% Triton X-100 (▲), 1% Brij-96 (■), or in the absence of detergent in the gradient medium (○), indicating that the *Bungarus* venom enzyme is a non-amphiphilic species (4.5 S).

of the 'complex' type; the high proportion of mannose may correspond to small amounts of *N*-glycans of the 'hybrid' type [10]. In addition, the presence of a minor component of *N*-acetylgalactosamine (2%) suggests the possible presence of *O*-linked glycans. The amount of neuraminic acid corresponds to approximately two residues per AChE molecule, on average.

3.4. Electric charge and microheterogeneity of Bungarus venom AChE

When subjected to exchange chromatography on a Mono Q cationic column, affinity purified *Bungarus* AChE was eluted as a small peak (isocratic elution) followed by a broad peak (salt gradient elution), suggesting charge heterogeneity (Fig. 3).

In PAGE under denaturing conditions, each of the peaks eluted from the Mono Q column was found to contain a major AChE subunit, around 66–68 kDa (this band migrated slightly faster in the case of the more acidic fraction 10 than in the case of fraction 4), and a minor one at 63 kDa (Fig. 5A). In the affinity purified enzyme, the superposition of the two doublets produced a more diffuse pattern. After digestion with *N*-glycanase, all these enzyme fractions yielded the same thin band, at 57 kDa, indicating that the heterogeneity of their apparent mass was due to differences of their *N*-glycans.

Non-denaturing PAGE gave rise to 4–5 bands, and IEF also resolved affinity purified AChE into 8–9 distinct bands, with pIs ranging from 5.2 to 5.8. Analysis of a series of fractions eluted from the Mono Q column showed that they contained variable proportions of these bands, according to their electric charge (Fig. 4). Clearly, these components were not resolved in the chromatographic conditions used in Fig. 3.

Digestion by neuraminidase reduced the heterogeneity observed in non-denaturing PAGE and in IEF (Fig. 5B,C). In addition, neuraminidase treatment significantly modified the elution profile from the Mono Q column, increasing the proportion of enzyme eluted in the first peak, and shifting the second peak towards lower salt concentration (not shown). The fact that a large fraction was still eluted at higher ionic strength may result from an incomplete removal of sialic acids, under our experimental conditions.

3.5. Lack of toxicity of Bungarus venom AChE

We examined whether AChE possesses an intrinsic toxicity, or reinforces the effect of other venom components. Injection of affinity purified AChE, up to 80 mg/kg, induced no lethality, whereas the crude venom killed the mice with an LD_{50} of about 2.5 mg/kg (Table 1). Moreover, the lethal potency of a venom which had been depleted from its AChE was the same as that of complete venom. Addition of a large excess of purified AChE to depleted venom (4 mg of AChE added to 1 mg of venom, i.e. 1000 times its original content in AChE) was found to slightly increase its lethal potency (about 10%), but this effect could be entirely accounted for by the presence of decamethonium, which is used to elute AChE from the affinity column, and is not completely eliminated even after prolonged dialysis and gel filtration. We did not, therefore, observe any synergistic effect of AChE with other venom components.

4. Discussion

In this report, we show that the venom of *Bungarus fasciatus* contains a true AChE, which presents characteristic catalytic properties, as first reported by Kumar and Elliott [3]. Its molecular mass is similar to that of AChE catalytic subunits from *Torpedo* and mammals, and partial peptide sequences establish that its catalytic domain is homologous to that of other AChEs. This enzyme is a monomeric, non-amphiphilic species, and thus differs from all AChE forms that had previously been identified in other tissues. The cloning of *Bungarus* AChE showed that the venom enzyme corresponds in fact to a novel type of subunit [11], possessing a hydrophobic C-terminal sequence that differs from those encoded by the alternative exons H and T [1]. We found that this enzyme remained monomeric, in sucrose gradients, up to 0.2 mg/ml at the peak [12]. It is possible that dimers may form at higher concentrations, since Raba et al. [13] showed that AChE from cobra (*Naja naja oxiana*) venom was monomeric up to 0.2 mg/ml but reversibly aggregated into dimers around 2 mg/ml, in the analytical centrifuge.

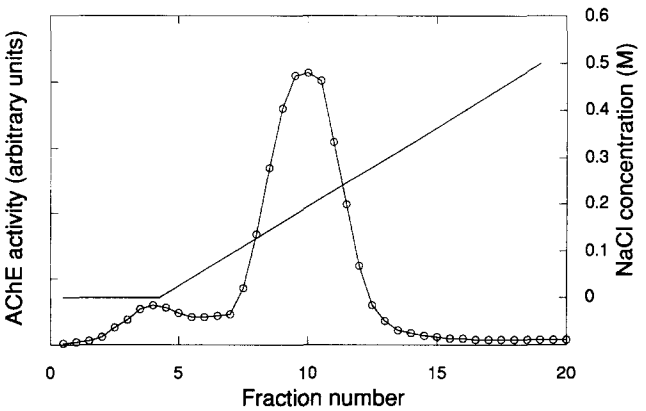

Fig. 3. Chromatography on a Mono Q column: elution profile of AChE activity. The scale shown on the right axis corresponds to the NaCl concentration in the elution buffer.

Ion exchange chromatography, non-denaturing PAGE and IEF revealed a charge heterogeneity of *Bungarus* venom AChE, with multiple components ranging from pI 5.2 to pI 5.8. The effect of neuraminidase showed that this is partly due to its content in sialic acids, in agreement with the fact that *Bungarus* AChE contains 7% carbohydrates, with a characteristic composition of *N*-glycans of the 'complex' and 'hybrid' types. A similar heterogeneity was observed for the cobra venom AChE [13]. In non-denaturing PAGE, the polydispersity of this enzyme is remarkably more pronounced than for AChEs of other tissues, which have been studied previously (see for example [14]). We observed a similar polydispersity in the case of AChEs from the venoms of other *Elapidae* snakes, *Naja naja*, *Ophiophagus hanna*, *Haemachatus haemachates* (not shown), and also in the case of recombinant *Bungarus* venom AChE, expressed in COS cells [11], ruling out the hypothesis that it would result from differences between individual snakes or modifications during storage of the venom. Raba et al. [13] reported that, in the case of cobra venom AChE, neuraminidase digestion modified the isoelectric focusing pattern, but did not abolish the multiplicity of bands, in agreement with the present observations. These authors suggested, therefore, that, in addition to glycosylation heterogeneity, charge isoforms may reflect variations in free glutamic or aspartic acids, produced by post-translational deamidation of glutamines and asparagines [15]. Such a modification may also contribute to the heterogeneity of *Bungarus* venom AChE, and would explain the fact that neuraminidase treatment did not produce a single electrophoretic band.

The physiological significance, if any, of AChE in snake venoms raises an extremely intriguing question. AChE exists at high levels in the venom of snakes from four genera of the *Elapidae* family (*Bungarus*, *Haemachatus*, *Naja*, *Ophiophagus*), but not in the venom of the *Dendroaspis* genus (mambas), which contains the anti-AChE toxin fasciculin (Y. Frobert,

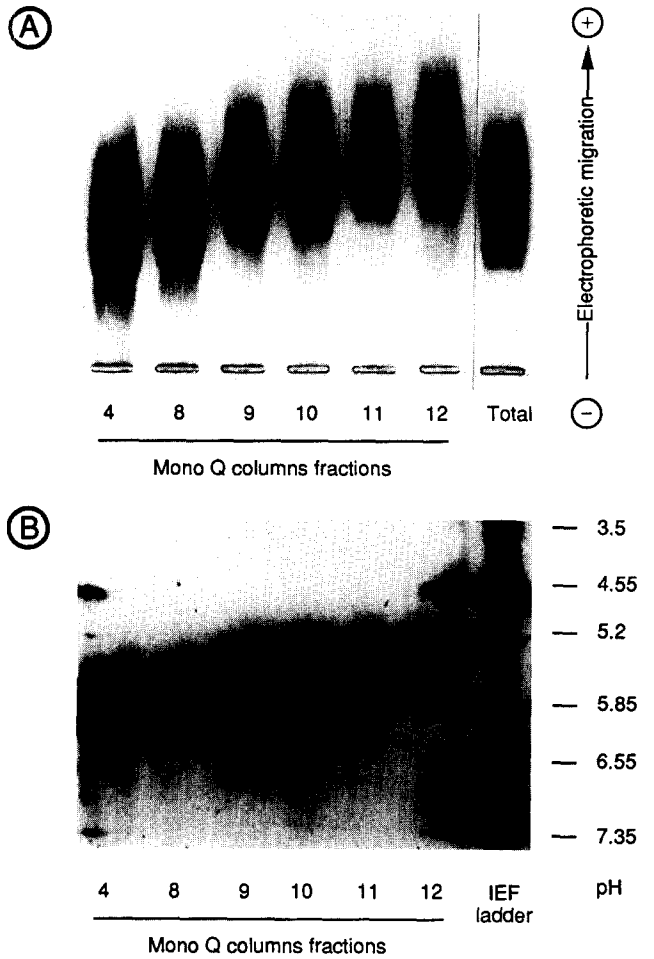

Fig. 4. Microheterogeneity of *Bungarus* venom AChE, in non-denaturing PAGE and IEF. Affinity purified AChE, as well as fractions from the Mono Q column (Fig. 3) were analyzed (A) by non-denaturing PAGE in a 10% polyacrylamide gels and staining of the AChE activity and (B) by IEF, followed by staining of the protein. The column fractions which were eluted at higher salt concentration were enriched in more negatively charged components.

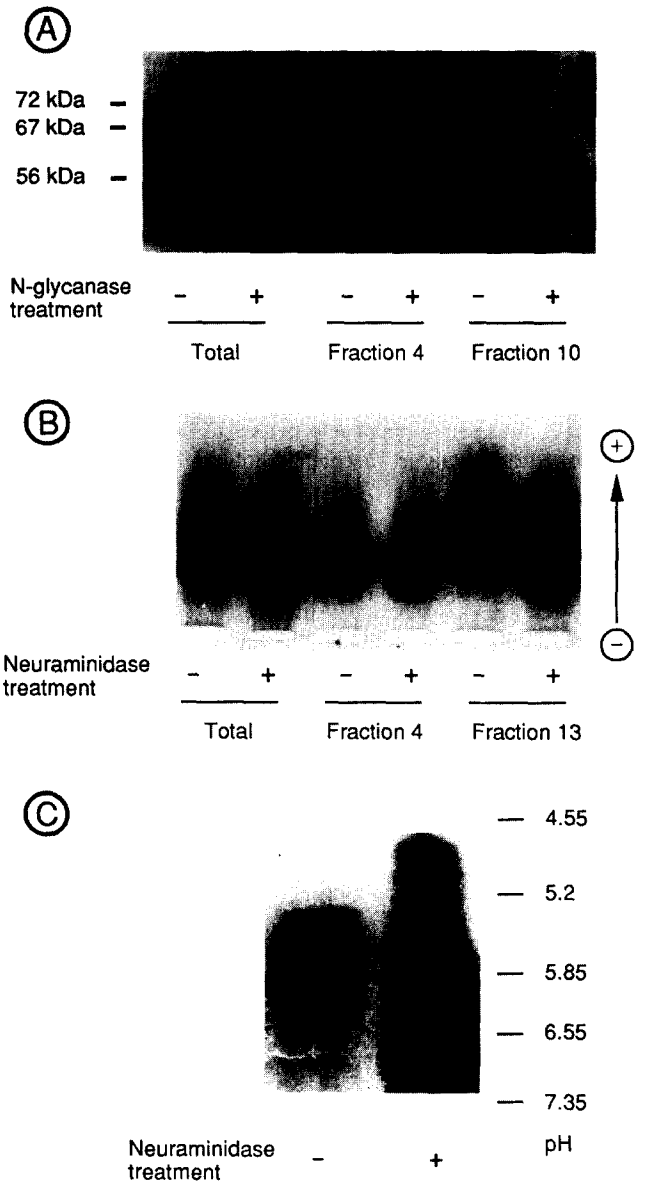

Fig. 5. Effect of *N*-glycanase and neuraminidase on *Bungarus* venom AChE. Affinity purified AChE and fractions from the minor and major peaks, eluted at low and high ionic strength from the Mono Q column (Fig. 3) were denatured and analyzed by PAGE in the presence of sodium dodecyl sulfate and dithiothreitol, with or without treatment by *N*-glycanase, as indicated (A); the effect of neuraminidase on active AChE was analyzed in non-denaturing conditions, by PAGE (B) and by IEF (C).

X. Cousin, C. Créminon, M.-H. Rémy, J.-M. Chatel, S. Bon, C. Bon and J. Grassi, in preparation). Since the venom of *Elapidae* snakes contains α-toxins which block the nicotinic receptor at neuromuscular junctions, it might be thought that AChE synergistically participates in a concerted blockade of cholinergic synapses by eliminating acetylcholine. On the other hand, this particular enzyme might exert a toxic effect, related to its molecular structure rather than to its catalytic activity. We found, however, that this enzyme is not toxic to mice, even at very high doses, in agreement with previous studies [16,17]. Moreover, it did not reinforce the toxicity of other venom components. Its presence in snake venoms therefore remains a mystery.

In any case, because of its relative abundance and original properties, this venom enzyme provides an excellent model for analyzing the catalytic mechanism of AChE, in a soluble monomeric form.

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