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Localization of the peptidase activity of human serum butyrylcholinesterase in a – 50-kDa fragment obtained by limited α-chymotrypsin digestion

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Purified human serum butyrylcholinesterase (≈ 90-kDa subunit) is known to exhibit aryl acylamidase and peptidase activity. Limited α-chymotrypsin digestion of the purified butyrylcholinesterase gave three major protein fragments of ≈ 50 kDa, ≈ 21 kDa and ≈ 20 kDa. In our earlier studies [Rao and Balasubramanian (1989) *Eur. J. Biochem. 179*, 639 – 644] we characterized the ≈ 20-kDa fragment and showed that it exhibited both butyrylcholinesterase and aryl acylamidase activities. In the present studies the ≈ 50-kDa fragment is characterized. This fragment, after isolation by Sephadex G-75 chromatography from a chymotryptic digest of purified butyrylcholinesterase, exhibited only peptidase activity and was devoid of cholinesterase and aryl acylamidase activities. It could bind to a column of *Ricinus communis* agglutinin bound to Sepharose, indicating its glycosylated nature and the presence of galactose. The peptidase activity in the ≈ 50-kDa fragment could be immunoprecipitated by a polyclonal antibody raised against purified butyrylcholinesterase. SDS-gel electrophoresis of this fragment isolated by *R. communis* agglutinin – Sepharose and Sephadex G-75 chromatography showed a protein band of ≈ 50 kDa by silver staining. Amino-terminal sequence analysis of the ≈ 50-kDa fragment gave the sequence of Gly-Pro-Thr-Val-Asp which corresponded to amino acid residues 291 – 295 in the butyrylcholinesterase sequence [Lockridge et al. (1987) *J. Biol. Chem. 262*, 549 – 557]. The combined results suggested that α-chymotrypsin digestion of human serum butyrylcholinesterase resulted in the formation of a ≈ 20-kDa fragment exhibiting both cholinesterase and aryl acylamidase activities and a ≈ 50-kDa fragment exhibiting only peptidase activity.

Human serum butyrylcholinesterase (BtChE) is a tetramer with a subunit of ≈ 90 kDa [1]. The enzyme is a glycoprotein with galactose and mannose as the major neutral sugar residues [2]. Purified human serum BtChE is known to exhibit an aryl acylamidase activity [3, 4] and a peptidase activity [5]. The presence of both these activities on purified human serum BtChE was demonstrated by their identical behaviour on column chromatographic and affinity chromatographic procedures, gel filtration, gel electrophoresis and their co-precipitation at different dilutions of antibody raised against purified BtChE. In our earlier report [6] we showed that limited α-chymotrypsin digestion of purified human serum BtChE results in the formation of three major fragments, of ≈ 20 kDa, ≈ 21 kDa and ≈ 50 kDa, as observed by SDS-gel electrophoresis. Of the two low-molecular-mass fragments, the ≈ 20-kDa one is enzymatically active and exhibits both BtChE and aryl acylamidase activities. Sequencing of amino acids from the amino terminal showed that the ≈ 20-kDa fragment had the sequence Arg-Val-Gly-Ala-Leu that fits in with amino acid residues 147 – 151 in the BtChE sequence given by Lockridge [2]. In this report we demonstrate that the ≈ 50-kDa fragment resulting from α-chymotrypsin digestion of purified human serum BtChE exhibits the peptidase activity. N-terminal sequence analysis of this ≈ 50-kDa protein fragment showed the sequence Gly-Pro-Thr-Val-Asp corresponding to the amino acid residues 291 – 295 in the BtChE sequence. Further, the comparative characteristics of the intact BtChE, ≈ 20-kDa and ≈ 50-kDa protein fragments and active-site labelling are also described.

MATERIALS AND METHODS

Materials

α-Chymotrypsin (treated with N^{α}-tosyl-L-lysine chloromethane), chicken egg white trypsin/chymotrypsin inhibitor and Bolton-Hunter reagent [*N*-succinimidyl-3-(4-hydroxyphenyl) propionate] were purchased from Sigma Chemical Co. USA. *Ricinus communis* agglutinin 120 (RCA_1) was purified from *R. communis* beans and coupled to Sepharose 4B as described earlier [6, 7]. [^{3}H]Diisopropyl fluorophosphate, from Amersham International, was a kind gift from Dr K.S. Krishnan (Tata Institute of Fundamental Research, Bombay). Sodium [^{125}I]iodide in dilute NaOH was obtained from Bhabha Atomic Research Centre, Bombay. Other chemicals were procured as described earlier [3].

Purification of human serum BtChE

BtChE was purified by the procedure described earlier [8] involving DEAE-cellulose and procainamide – Sepharose affinity chromatography.

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Abbreviations. BtChE, butyrylcholinesterase; RCA_1, *Ricinus communis* agglutinin 120; iPr_2P-F, diisopropyl fluorophosphate.

Enzymes. Butyrylcholinesterase (EC 3.1.1.8); aryl acylamidase (EC 3.5.1.13).

α-Chymotrypsin digestion

Proteolysis was performed essentially as described earlier [6]. In brief, the purified human serum BtChE was digested with α-chymotrypsin (BtChE/chymotrypsin = 1:0.01, by mass) in a 0.2-ml reaction mixture containing 100 mM Tris/HCl, 1 mM EDTA, pH 7.5. After incubation at 37°C for 6 h, the reaction was terminated by the addition of the chymotrypsin inhibitor.

Digestion with immobilized chymotrypsin

Chymotrypsin was immobilized on Sepharose 4B by coupling 5 mg chymotrypsin to 5 ml cyanogen-bromide-activated Sepharose 4B [9]. The washed gel was packed in a column (1.6 × 2 cm) and equilibrated with 20 mM phosphate pH 7.2. Purified BtChE (0.5 ml) was introduced into the gel, incubated for the desired period at 37°C and eluted with the same buffer.

Labelling with [^{3}H]iPr$_2$P-F

Purified serum BtChE (50–100 μg at a concentration of 4 μg/0.1 ml) was allowed to react with a slight molar excess of [^{3}H]iPr$_2$*P*-F (specific activity 3.5 Ci/mmol) and kept at 4°C for 5 days to achieve aging. The free [^{3}H]iPr$_2$*P*-F was separated from the bound by passing the sample through a Sephadex G-75 column followed by dialysis at 4°C of the pooled active fractions against distilled water. Preferential labelling of esteratic and non-esteratic sites was done according to Small and Chubb [10]. In order to label the esteratic site of BtChE preferentially, purified BtChE was incubated with [^{3}H]iPr$_2$*P*-F for only 20 min. The non-esteratic site was labelled preferentially by pre-incubating BtChE for 1 h with 200 μM eserine to block the esteratic site. [^{3}H]iPr$_2$*P*-F was then added and the incubation continued for 3 h. After incubation, the reaction mixture was applied onto the Sephadex G-75 column to separate bound radioactivity from free [^{3}H]iPr$_2$*P*-F.

Iodination of purified BtChE

Iodination was carried out as described by Bolton and Hunter [11]. Crystalline *N*-succinimidyl 3-(4-hydroxyphenyl)propionate (0.25 μg) was treated with 10 μl (1.4 mCi) of ^{125}I and 50 μl (1 mg/ml) chloramine T in 20 mM potassium phosphate pH 7.5. The reaction was terminated by the addition of 120 μl (1 mg/ml) sodium metabisulphite in the same buffer, followed by the addition of 5 μl dimethyl formamide. The hydroxysuccinimide ester was extracted into benzene and recovered by evaporation of the solvent under vacuum. Purified human serum BtChE (10 μg in 10 μl 0.1 M borate pH 8.5) was added to the dried iodinated ester and ^{125}I-labelled BtChE was separated from the other labelled products of the conjugation reaction by applying the sample on a Sephadex G-75 column.

SDS/polyacrylamide gel electrophoresis

Samples were boiled for 3 min in the protein dissociation buffer (0.186 M Tris/HCl pH 7.6, 6% 2-mercaptoethanol, 20% glycerol, 6% SDS and 0.001% bromophenol blue) and analyzed on 10% sodium dodecyl sulphate/polyacrylamide slab gels [6] according to Laemmli [12]. The gels were fixed and stained with Coomassie brilliant blue R or silver nitrate [13]. Alternatively, after destaining the Coomassie-blue-stained gels in acetic acid/water/methanol (10:40:50, by vol.), the gel was dried on a gel drier and autoradiography was carried out by keeping the dried gel in contact with a Curix X-ray film at −20°C for 5–8 days. The [^{3}H]iPr$_2$*P*-labelled BtChE on the gel was detected fluorographically according to Skinner and Griswold [14]. The gels were soaked in acetic acid for 5 min, transferred to 20% (mass/vol.) PPO in acetic acid for 2 h, again soaked in water for 30 min, dried and kept in contact with X-ray film at −70°C for 12 days.

Sephadex G-75 gel filtration

A 0.5-ml sample was applied on a Sephadex G-75 column (36 × 1.1 cm) pre-equilibrated with 20 mM potassium phosphate pH 7.2 containing 0.05 M NaCl. Elution was carried out by the same buffer at a flow rate of 4 ml/h and 1-ml fractions were collected. Each fraction, after dialysis against 20 mM phosphate pH 7.2, was assayed for the enzyme activities.

RCA$_1$-Sepharose chromatography

Samples (0.5 ml) were applied onto an RCA$_1$-Sepharose column (3.5 × 1.1 cm) pre-equilibrated with 20 mM potassium phosphate pH 7.2, washed with the same buffer and eluted with 0.5 M lactose, 1 M NaCl, 20 mM potassium phosphate pH 7.2. Fractions of 1 ml were collected.

Immunological studies

Antibody against purified BtChE was raised in a rabbit and purified by DEAE-cellulose chromatography as described earlier [3]. Immunoprecipitation was performed by cross-reacting different concentrations of antibody with the enzyme in 0.4 ml 20 mM potassium phosphate pH 7.2. After incubation at 4°C for 24 h, the samples were centrifuged at 10000 × *g* for 30 min and the supernatant was assayed for the enzyme activity.

Amino-terminal sequence analysis

Manual micro-sequence analysis was carried out according to Chang [15] using the dimethylaminoazobenzene isothiocyanate/phenylisothiocyanate (DABITC/PITC) double-coupling technique.

Enzyme assays and other procedures

Assay procedures of BtChE and aryl acylamidase, as well as enzyme units, were as described earlier [6].

The peptidase activity was assayed fluorometrically as described earlier [5] using the dipeptide Phe-Leu as substrate. The amount of the substrate hydrolyzed was determined from a standard curve obtained with known concentrations of an equimolar mixture of phenylalanine and leucine. One unit of peptidase activity is defined as the amount catalysing the liberation of 1 nmol each of phenylalanine and leucine/h. In some experiments [Leu^5]enkephalin was also used as a substrate for measuring peptidase activity [5].

Protein was determined according to Lowry et al. [16] with crystalline bovine serum albumin as standard or by absorbance at 280 nm for column effluents.

Radioactivity due to [^{3}H]iPr$_2$*P*-labelling was determined by counting 0.1-ml samples in 1 ml Bray's scintillation fluid in dioxan [17] in an LKB liquid scintillation counter.

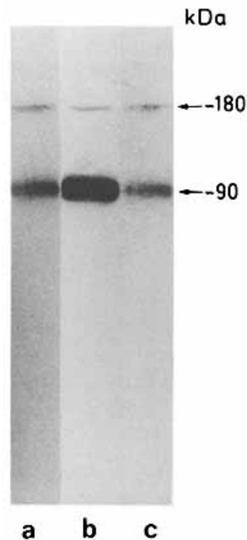

Fig. 1. *SDS-gel electrophoresis of purified human serum BtChE.* (a) Purified enzyme stained with Coomassie brilliant blue. (b) Autoradiogram of purified ^{125}I-labelled enzyme subjected to electrophoresis. (c) Autoradiogram of purified [^{3}H]iPr$_2$*P*-labelled enzyme subjected to electrophoresis

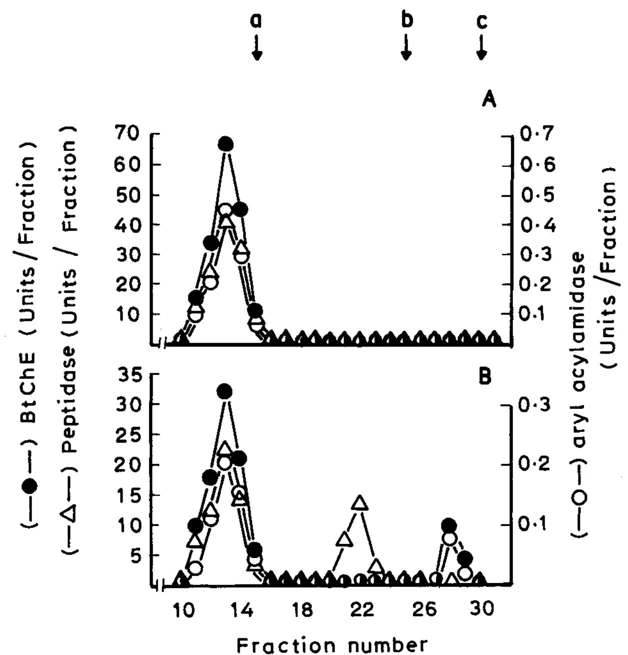

Fig. 2. *Sephadex G-75 chromatography and elution profile of (A) purified BtChE and (B) BtChE digested with α-chymotrypsin for 6 h.* Each fraction was assayed for BtChE (●), aryl acylamidase (○) and peptidase (△) activity. Arrows (↓) indicate the molecular mass standards used; (a) bovine serum albumin, 68 kDa; (b) carbonic anhydrase, 29 kDa; (c) cytochrome *c*, 12.4 kDa. Details of chromatography and chymotryptic digestion are given under Materials and Methods

Inhibition studies

Inhibition studies were performed by pre-incubating the enzyme with the inhibitor for 10 min at 37°C followed by assay. The inhibitors were added to the assay blanks also to avoid any interference by them.

RESULTS

SDS/polyacrylamide gel electrophoresis of the unlabelled and labelled BtChE

The SDS-gel electrophoresis pattern of the purified BtChE under reducing conditions is shown in Fig. 1. The monomeric form of the enzyme as a major protein band of ≈ 90 kDa and the dimeric form of the enzyme as a minor band of ≈ 180 kDa were seen, as observed by Lockridge et al. [18]. A similar kind of pattern was seen when purified BtChE was labelled with [^{3}H]iPr$_2$*P*-F or ^{125}I followed by SDS-gel electrophoresis and autoradiography (Fig. 1). Both the monomer and dimer incorporated radioactivity, the former to a much greater extent in comparison to the latter. The identical electrophoretic patterns of the unlabelled and labelled protein showed that it is essentially homogeneous.

α-Chymotrypsin digestion and Sephadex G-75 gel filtration

The Sephadex G-75 gel filtration pattern of the purified enzyme is shown in Fig. 2A. The elution profile showed the BtChE, aryl acylamidase and peptidase activities co-eluting as a single peak at the void volume. The ratios of BtChE/aryl acylamidase activity and BtChE/peptidase activity remained constant in all the eluted fractions. After digestion of the purified enzyme with chymotrypsin for 6 h and chromatography of the digested sample on Sephadex G-75, two peaks of co-eluting BtChE and aryl acylamidase activity were seen, one corresponding to the undigested enzyme and another to a smaller molecule of ≈ 20 kDa (Fig. 2B). In our earlier study [6], we reported this ≈ 20-kDa fragment exhibiting both aryl acylamidase and BtChE activity. In the present study we determined the peptidase activity exhibited by BtChE in the Sephadex G-75 fractions. Two peaks of peptidase activity were seen, one coinciding with the undigested enzyme and another at a molecular mass of ≈ 50 kDa (Fig. 2B). The peptidase activity peaks were measurable using not only Phe-Leu as substrate but also with another known substrate [Leu^{5}]enkephalin (results not shown). Whereas the ≈ 20-kDa peak showed both BtChE and aryl acylamidase activities, the ≈ 50-kDa peak exhibited only peptidase activity. These results suggested that, apart from a ≈ 20-kDa fragment exhibiting both cholinesterase and aryl acylamidase activity [6], α-chymotrypsin digestion of purified BtChE also gave rise to a ≈ 50-kDa fragment exhibiting only the peptidase activity.

Similar results as given above were obtained when BtChE was digested for 6 h with chymotrypsin coupled to Sepharose 4B and the digest passed through the Sephadex G-75 column, thereby excluding the possibility of any peptidase activity arising from the α-chymotrypsin. Keeping the purified BtChE alone at pH 7.5 at 37°C for 6–12 h followed by passage through Sephadex G-75 or subjection to SDS-gel electrophoresis did not provide any evidence for cleavage of BtChE, ruling out the possibility of any endogenous chymotrypsin-like activity in the purified BtChE. Moreover, α-chymotrypsin at the concentration used for digestion did not exhibit any peptidase activity towards Phe-Leu under the usual assay conditions.

RCA$_1$-Sepharose chromatography

Purified BtChE and the associated aryl acylamidase and peptidase activities could fully bind to a *Ricinus communis*

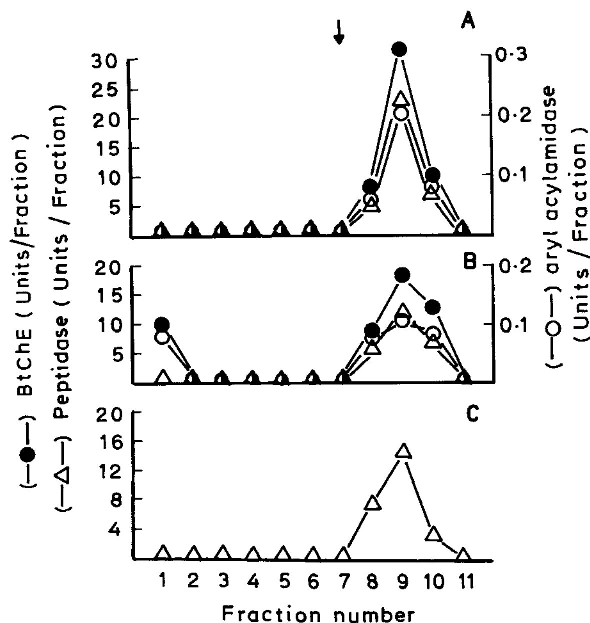

Fig. 3. *RCA_1-Sepharose chromatography of (A) purified BtChE, (B) BtChE digested with α-chymotrypsin for 6 h and (C) the ≈ 50-kDa fragment exhibiting peptidase activity recovered from Sephadex G-75 column.* Each fraction was assayed for BtChE (●), aryl acylamidase (○) and peptidase (△) activity. Arrow (↓) indicates the start of elution with lactose. Details of chromatography and chymotryptic digestion are given under Materials and Methods

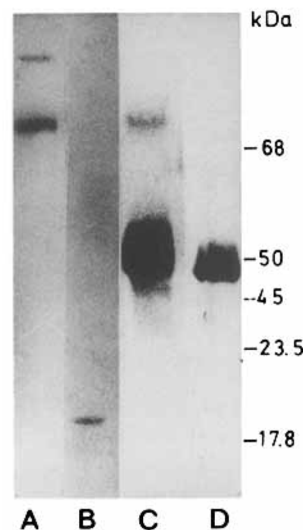

Fig. 4. *SDS/polyacrylamide gel electrophoresis pattern of (A) purified BtChE, (B) the ≈ 20-kDa chymotryptic fragment of purified BtChE isolated from the flow-through fraction of RCA_1-Sepharose chromatography followed by Sephadex G-75 chromatography, (C) the pooled eluted fraction from RCA_1-Sepharose of the chymotryptic digest of purified BtChE, (D) the ≈ 50-kDa chymotryptic fragment of purified BtChE exhibiting peptidase activity isolated by Sephadex G-75 chromatography followed by RCA_1-Sepharose chromatography and elution.* Lanes (A) and (B) were stained by Coomassie blue and lanes (C) and (D) by silver nitrate

agglutinin – Sepharose column and could be eluted to the extent of 97% (Fig. 3 A). In an earlier study [6], we showed that chymotryptic digestion of BtChE resulted in the formation of a ≈ 20-kDa fragment that exhibited both aryl acylamidase and BtChE activities and that could not bind to RCA_1-Sepharose. When we measured peptidase activity we found that all the peptidase activity in the chymotrypsin digest of BtChE could bind to RCA_1-Sepharose and could be fully eluted (Fig. 3 B). The enzyme eluted from RCA_1-Sepharose was subjected to Sephadex G-75 chromatography. Two peaks of peptidase activity, one at the void volume corresponding to the undigested BtChE and another at a molecular mass of ≈ 50 kDa, were observed (result not shown). In further experiments, it was observed that the ≈ 50-kDa fragment which exhibited peptidase activity when obtained from the Sephadex G-75 column could completely bind to RCA_1-Sepharose and be eluted to the extent of 94% (Fig. 3 C). These results suggested that, in addition to a ≈ 20-kDa active protein fragment exhibiting both BtChE and aryl acylamidase activity that could not bind to RCA_1-Sepharose, a ≈ 50-kDa fragment exhibiting only peptidase activity that could fully bind to a RCA_1-Sepharose column was formed by chymotryptic digestion of BtChE.

SDS-gel electrophoresis of the low-molecular-mass fragments of BtChE obtained after chymotryptic digestion

Purified BtChE showed a single major protein band of ≈ 90 kDa as shown in Fig. 4 A. After digestion of BtChE with α-chymotrypsin and passage through a RCA_1-Sepharose column, the flow-through fraction, exhibiting aryl acylamidase and BtChE activities, and the eluted fractions, exhibiting BtChE, aryl acylamidase and peptidase activities, were subjected to SDS/PAGE. The BtChE fragment that did not bind to RCA_1-Sepharose after isolation by Sephadex G-75 chromatography showed a single protein band of ≈ 20 kDa as reported earlier [6] (Fig. 4 B). The eluate from RCA_1-Sepharose that showed BtChE, aryl acylamidase and peptidase activities gave two protein bands of ≈ 90 kDa and ≈ 50 kDa (Fig. 4 C). The fragment that exhibited only peptidase activity isolated from the chymotryptic digest of BtChE by Sephadex G-75 chromatography gave a protein band of ≈ 50 kDa on SDS/PAGE (Fig. 4 D). These results confirmed that chymotryptic digestion of BtChE resulted in the formation of a ≈ 50-kDa protein that exhibited peptidase activity exclusively and a ≈ 20-kDa protein that exhibited both BtChE and aryl acylamidase activity. The results also indicated that the ≈ 20-kDa fragment was devoid of galactose as reported earlier [6] but the ≈ 50-kDa fragment contained galactose.

Immunoprecipitation studies

Polyclonal antibody raised against the purified BtChE could immunoprecipitate both the purified intact BtChE and the ≈ 50-kDa fragment exhibiting peptidase activity (Fig. 5).

Comparative characteristics of the ≈ 50-kDa and ≈ 20-kDa fragments with the parent enzyme

As shown in Table 1, potent cholinesterase inhibitors like eserine, diisopropyl fluorophosphate and tetra-isopropyl-pyrophosphoramide had no inhibitory effect on the peptidase activity exhibited by the parent enzyme and the ≈ 50-kDa fragment. There was a slight activation of the peptidase activity in the presence of eserine, tetra-isopropylpyrophosphoramide and diisopropyl fluorophosphate as observed earlier [5] (Table 1). On the other hand, both cholinesterase and aryl acylamidase activities of the ≈ 20-kDa fragment

Table 1. *Effect of various cholinesterase inhibitors on the enzyme activities exhibited by intact BtChE, ≈50-kDa fragment and ≈20-kDa fragment*
Control enzyme activity in the absence of inhibitor is taken as 100%

Inhibitor	Concn	Cholinesterase		Peptidase		Aryl acylamidase	
		parent BtChE	≈20-kDa fragment	parent BtChE	≈50-kDa fragment	parent BtChE	≈20-kDa fragment
	mM	% control					
Eserine	0.05	32	12	125	107	18	15
	0.10	16	0	132	128	7	6
Procainamide	50	36	22	52	46	20	14
	100	6	0	52	50	7	5
Tetra-isopropyl-pyrophosphoramide	0.005	0	0	140	142	0	0
	0.025	0	0	148	152	0	0
Diisopropyl fluorophosphate	0.02	19	0	130	128	0	0
	0.10	0	0	138	136	0	0

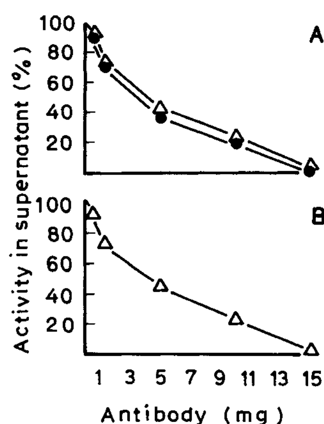

Fig. 5. *Immunoprecipitation of (A) purified BtChE and (B) the ≈ 50-kDa fragment exhibiting peptidase activity by different concentrations of the antibody raised against purified BtChE.* Both BtChE (●) and peptidase (△) activities were measured. Details of the experiment are given under Materials and Methods

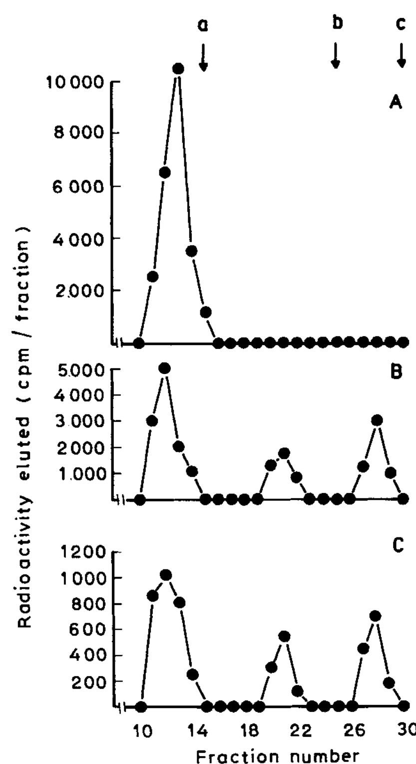

Fig. 6. *Sephadex G-75 chromatography of [^{3}H]iPr$_2$P-labelled BtChE.* The [^{3}H]iPr$_2$*P*-labelled BtChE or its chymotryptic digest (0.5-ml sample) was passed through a Sephadex G-75 column (36 × 1.1 cm) and eluted with 20 mM phosphate pH 7.2, 0.05 M NaCl. Fractions of 1 ml were collected. Elution profile of (A) [^{3}H]iPr$_2$*P*-labelled purified BtChE, (B) α-chymotrypsin digest of [^{3}H]iPr$_2$*P*-labelled BtChE and (C) [^{3}H]iPr$_2$*P*-labelled BtChE in the presence of eserine followed by α-chymotrypsin digestion are shown. The radioactivity of samples applied on Sephadex G-75 were different in each experiment, A, B and C. The molecular mass standards a, b and c are as mentioned in Fig. 2

and the parent enzyme were significantly inhibited by these inhibitors. These results showed that the response towards the inhibitors by both the proteolytic fragments (≈ 50 kDa and ≈ 20 kDa) arising from the parent enzyme upon α-chymotrypsin digestion is similar to that of the parent enzyme.

We have shown earlier that the aryl acylamidase activity of BtChE is inhibited by serotonin and activated by tyramine [3]. We examined the effect of these two biogenic amines on the aryl acylamidase activity of the ≈ 20-kDa fragment and found that serotonin (1 – 10 mM) caused an inhibition of 10 – 80% and tyramine (1 – 10 mM) stimulated the activity 2 – 12-fold, similar to findings made with the parent BtChE (data not shown).

Amino-terminal sequence analysis of the ≈ 50-kDa protein fragment

Purified BtChE was digested with α-chymotrypsin for 6 h, loaded onto the RCA$_1$-Sepharose column and the eluted fractions from the RCA$_1$-Sepharose were pooled and subjected to Sephadex G-75 gel filtration. The ≈ 50-kDa fragment exhibiting peptidase activity was collected, dialyzed against water and used for amino-terminal sequence analysis. The following N-terminal sequence of five amino acids was obtained: Gly-Pro-Thr-Val-Asp. This sequence was identical to the amino acid residues 291 – 295 in the BtChE sequence given by Lockridge et al. [2].

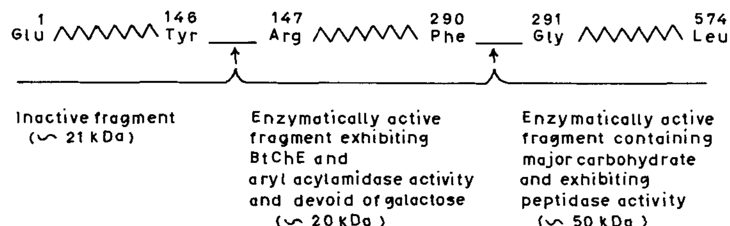

Fig. 7. *Schematic diagram showing the major protein fragments arising by limited α-chymotrypsin digestion of human serum BtChE and the nature of the fragments*

α-Chymotrypsin digestion and Sephadex G-75 chromatography of [^{3}H]iPr$_2$P-labelled BtChE

The [^{3}H]iPr$_2$*P*-labelled BtChE showed chromatographic behaviour on Sephadex G-75 identical to that of the unlabelled protein and emerged as a sharp peak at the void volume (Fig. 6A). The labelled BtChE was then digested with chymotrypsin for 6 h and the sample was loaded on the above column. Three peaks of radioactivity were seen on elution, one corresponding to the protein in the void volume, a second at a molecular mass corresponding to ≈ 50 kDa and a third at a lower molecular mass corresponding to ≈ 20 kDa (Fig. 6B). Since the ≈ 50-kDa fragment that exhibited peptidase activity did not show either cholinesterase or aryl acylamidase activity and was not sensitive to cholinesterase inhibitors, we were interested in seeing if the active site of the peptidase was distinct from the esteratic site of BtChE. We carried out [^{3}H]iPr$_2$*P*-labelling of BtChE in the presence and absence of eserine according to Small and Chubb as described under Methods [10]. The labelled BtChE was then digested with α-chymotrypsin and subjected to Sephadex G-75 chromatography when three peaks of radioactivity were seen similar to the peaks shown in Fig. 6B. The ratio of radioactivity of ≈ 50-kDa/≈ 20-kDa fragments was 0.75 when the labelling was done in the absence of eserine and 0.71 when labelling was done in the presence of eserine (Fig. 6C).

DISCUSSION

Our previous work [3, 5] has shown that purified human serum BtChE exhibited an aryl acylamidase and a peptidase activity. In subsequent studies [6] we showed that limited α-chymotrypsin digestion of the purified human serum BtChE resulted in three major protein fragments of ≈ 21 kDa, ≈ 20 kDa and ≈ 50 kDa on SDS-gel electrophoresis. The two low-molecular-mass fragments were stained well by Coomassie blue, while the ≈ 50-kDa fragment was poorly stained and needed silver staining for detection. We characterized [6] the ≈ 20-kDa fragment and showed that it exhibited both BtChE and aryl acylamidase activities and that it had the N-terminal sequence Arg-Val-Gly-Ala-Leu which agreed with amino acid residues 147 – 151 in the BtChE sequence given by Lockridge et al. [2]. We also showed that the ≈ 20-kDa fragment was devoid of galactose and could not bind to RCA$_1$-Sepharose, unlike the parent enzyme [6]. In the present study, we have characterized the ≈ 50-kDa fragment that did not have cholinesterase or aryl acylamidase activity but exhibited a peptidase activity exclusively. This ≈ 50-kDa fragment, which could be separated by Sephadex G-75 column chromatography, bound completely to a RCA$_1$-Sepharose column indicating its glycosylated nature and the presence of galactose residues. The peptidase activity exhibited by the ≈ 50-kDa protein could be immunoprecipitated by a polyclonal antibody raised against the purified parent BtChE. The response of both the ≈ 20-kDa and ≈ 50-kDa fragments towards cholinesterase inhibitors was similar to the parent enzyme. The ≈ 50-kDa fragment did not involve the active-site Ser^{198} residue, as evidenced by the N-terminal amino acid sequencing; this could explain the insensitivity of the peptidase activity in the parent BtChE [5], as well as the ≈ 50-kDa fragment, to many classical cholinesterase inhibitors. However the slight stimulatory effect of peptidase activity by these inhibitors [5] was retained by the ≈ 50-kDa fragment. The overall results suggested that the ≈ 20-kDa and ≈ 50-kDa fragments obtained by chymotryptic cleavage of the parent BtChE still retained their binding sites for the inhibitors and behaved in a manner similar to the parent BtChE.

When purified [^{3}H]iPr$_2$*P*-labelled BtChE was digested with chymotrypsin and subjected to Sephadex G-75 chromatography three peaks of radioactivity corresponding to the parent enzyme, ≈ 50-kDa fragment and ≈ 20-kDa fragment were seen. Since the ≈ 20-kDa fragment has an active-site serine residue, as reported earlier [6], the appearance of a radioactive peak for this fragment was understandable. The amino acid residue involved in the [^{3}H]iPr$_2$*P*-labelling of the ≈ 50-kDa fragment is however not clear. Recently Small and Chubb [10] reported that a trypsin-like endopeptidase activity was present in acetylcholinesterase and that it was associated with a serine residue at a site distinct from the esteratic site. We carried out preferential labelling with [^{3}H]iPr$_2$*P*-F as described by Small and Chubb [10] in the presence and absence of eserine, an esteratic site inhibitor. If two distinct serine residues were responsible for the cholinesterase and peptidase activities then labelling with [^{3}H]iPr$_2$*P*-F for a short period in the absence of eserine is expected to occur only on the esteratic site of BtChE and the addition of eserine is expected to block the esteratic site labelling of BtChE with the resulting incorporation of radioactivity into the peptidase fragment only [10]. In our experiment, both in the presence and absence of eserine, radioactivity from [^{3}H]iPr$_2$*P*-F was incorporated into the ≈ 50-kDa and ≈ 20-kDa protein fragments. It has been proposed that the active-site center of cholinesterase, in addition to a reactive serine residue, may also include a histidyl residue involved in the catalytic triad [19, 20] which, however, has not yet been located. Ozols [21] has proposed that the iPr$_2$*P*-binding histidine residue of a ≈ 60-kDa glycoprotein esterase from liver microsomal membrane may correspond to His^{438} of serum cholinesterase, based on the sequence similarity between the two proteins. It is possible that the ≈ 50-kDa fragment incorporates radioactivity from [^{3}H]iPr$_2$*P*-F at this histidyl residue.

We reported earlier [6] that the partial manual sequencing of the N-terminal amino acids of the ≈ 20-kDa fragment showed the sequence Arg-Val-Gly-Ala-Leu which corresponds to the amino acid residues 147 – 151 of BtChE [2]. Sequencing of the N-terminal amino acids of the ≈ 50-kDa fragment

showed the sequence Gly-Pro-Thr-Val-Asp, which agreed with amino acid residues 291–295 in the BtChE sequence [2], suggesting that the chymotryptic cleavage generating the ≈ 50-kDa fragment has taken place between Phe^{290} and Gly^{291} in the BtChE molecule. The scheme given in Fig. 7 summarizes the conclusions and indicates the amino acid residues encompassed by the three protein fragments arising from purified BtChE by α-chymotrypsin digestion.

Finally, the identity of the five-amino-acid sequence at the N-terminus of the ≈ 50-kDa fragment (exhibiting peptidase activity) with amino acid residues 291–295 in the BtChE sequence [2] provides convincing evidence that the fragment exhibiting peptidase activity is part of the BtChE molecule.

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