Isolation of a galactose-free 20-kDa fragment exhibiting butyrylcholine esterase and aryl acylamidase activity from human serum butyrylcholine esterase by limited α-chymotrypsin digestion

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Purified human serum butyrylcholine esterase (≈ 90-kDa subunit), which also exhibits aryl acylamidase activity, was subjected to limited α-chymotrypsin digestion. Three major protein fragments of ≈ 50 kDa, ≈ 21 kDa and ≈ 20 kDa were found to be produced, as observed by SDS-gel electrophoresis of the chymotryptic digest. The purified butyrylcholine esterase could fully bind to a *Ricinus-communis*-agglutinin-Sepharose column but after chymotryptic digestion about 15–20% of the enzyme activity remained unbound and was recovered in the run-through fractions. Sephadex G-75 chromatography of the chymotryptic digest showed an enzymatically active fragment eluted at an approximate molecular mass of 20 kDa, apart from the undigested butyrylcholine esterase eluted at the void volume. The butyrylcholine esterase fragment that did not bind to *Ricinus communis* agglutinin also was eluted at an approximate molecular mass of 20 kDa from a Sephadex G-75 column. This enzymatically active low-molecular-mass fragment from Sephadex G-75 chromatography showed a single protein band of ≈ 20 kDa on SDS-gel electrophoresis. Neutral sugar analysis of the ≈ 20 kDa fragment showed the presence of mannose only, whereas the undigested butyrylcholine esterase showed the presence of both mannose and galactose. Amino-terminal-sequence analysis of the ≈ 20 kDa fragment showed the sequence Arg-Val-Gly-Ala-Leu, which agrees with amino acid residues 147–151 reported for human serum butyrylcholine esterase [Lockridge et al. (1987) *J. Biol. Chem. 262*, 549–557]. Both cholinesterase and aryl acylamidase activities were co-eluted in all chromatographic procedures. The results suggested that limited α-chymotrypsin digestion of human serum butyrylcholine esterase resulted in the formation of a ≈ 20-kDa enzymatically active fragment with Arg^{147} as its N-terminal residue and which was devoid of galactose.

Human serum butyrylcholine esterase (BuChE) is a globular tetrameric molecule with a subunit of ≈ 90 kDa [1]. It has four identical subunits, two monomers covalently linked through a single disulfide bond into a dimer and two such dimers hydrophobically linked into a tetramer [2]. It is a glycoprotein with 23.9% of the mass due to carbohydrate, with galactose and mannose as neutral sugar residues [3]. Previous work from this laboratory [4] as well as others [5] have shown that an aryl acylamidase (AAA) activity is exhibited by human serum butyrylcholine esterase. Both activities have been identified with the same protein on the basis of their identical behaviour on column chromatographic and affinity chromatographic procedures, gel filtration, gel electrophoresis, their co-precipitation by anti-BuChE antibody and identical response to potential cholinesterase inhibitors [4]. An interesting feature of this AAA activity was its specific inhibition by serotonin and activation by tyramine [4, 5]. Studies on chemical modification of different amino acids of the human serum BuChE have suggested that AAA and cholinesterase activities reside at non-identical but overlapping active sites on the BuChE protein [6].

The existence of multifunctional proteins has been recognized in several mammalian and non-mammalian systems. Work in different laboratories [7–10] has indicated that multifunctional proteins can exist as separate independent domains connected by polypeptide bridges that are sensitive to proteolytic attack. Cleavage of such sensitive regions by various proteases has allowed the isolation of functionally active domains [7–10]. Crystallographic studies for a number of proteins reveal that any large protein with a molecular mass exceeding 30 kDa can have multiple domains [11]. It was therefore considered to be of interest to subject human serum BuChE to limited protease digestion and examine the possibility of separating the AAA and BuChE activities. In the present work we have isolated and characterized a protein fragment of ≈ 20 kDa from BuChE, by limited chymotryptic digestion, which exhibits both AAA and BuChE activities. This protein fragment contains only mannose residues in contrast to the parent BuChE, which contains both galactose and mannose residues.

MATERIALS AND METHODS

Materials

α-Chymotrypsin (treated with N^{α}-tosyl-L-lysine chloromethane) and chicken egg-white trypsin/chymotrypsin inhibi-

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Abbreviations. BuChE, butyrylcholine esterase; AAA, aryl acylamidase; RCA_1, *Ricinus communis* agglutinin 120.

Enzymes. Butyrylcholine esterase (EC 3.1.1.8); aryl acylamidase (EC 3.5.1.13).

tor were purchased from Sigma Chemical Co., USA. Sephadex G-75 was from Pharmacia, Sweden. *Ricinus communis* agglutinin 120 (RCA_1) was purified from *Ricinus communis* beans by guar-gum affinity chromatography and Sephadex G-200 gel filtration [12]. It was then coupled to Sepharose 4B [13], resulting in 6.6 mg lectin bound/ml packed gel. Outdated human plasma was obtained from the hospital blood bank. Other chemicals were procured as described earlier [4].

Purification of human serum BuChE

BuChE was purified to homogeneity from human plasma by DEAE-cellulose and procainamide-Sepharose affinity chromatography as described earlier [6].

Chymotryptic digestion

The purified BuChE was digested with α-chymotrypsin in an 0.2-ml reaction mixture containing 100 mM Tris/HCl, 1 mM EDTA, pH 7.5 [7]. The ratio of BuChE to chymotrypsin varied from 1:0.02 to 1:0.01 (by mass) in the various experiments. After incubation at 37°C for different periods the reaction was terminated by the addition of the chymotrypsin inhibitor [twice the amount (by mass) of chymotrypsin used for digestion].

SDS/polyacrylamide gel electrophoresis

Samples (50 µl) were boiled for 3 min after the addition of 25 µl protein-dissociating buffer (0.186 M Tris/HCl, pH 7.6, 6% 2-mercaptoethanol, 20% glycerol, 6% SDS and 0.001% bromophenol blue) and subjected to SDS-gel electrophoresis on 10% slab gels, according to Laemmli [14]. The gels were fixed and stained with Coomassie brilliant blue R or silver nitrate [15].

Polyacrylamide gel electrophoresis

Gel electrophoresis under non-denaturing conditions was carried out according to Davis [16] in glycine/Tris buffer, pH 8.3, on 7% polyacrylamide gel. After electrophoresis, the gels were cut into 0.5-mm thick slices, the enzyme extracted into 0.2 ml 20 mM potassium phosphate, pH 7.2, dialyzed against the same buffer to remove glycine/Tris and assayed for activity.

Chromatography on RCA_1-Sepharose

RCA_1-Sepharose chromatography was performed on a 3.5 × 1.1 cm column in 20 mM potassium phosphate, pH 7.2. After loading on 0.5-ml sample, the column was washed with 5 ml of the above buffer and eluted with 0.5 M lactose/1 M NaCl/20 mM potassium phosphate, pH 7.2. Fractions of 1 ml were collected.

Sephadex G-75 gel filtration

A Sephadex G-75 column (36 × 1.1 cm) was pre-equilibrated with 20 mM potassium phosphate, pH 7.2, containing 0.5 M NaCl. The sample (0.6 ml) was applied and elution was carried out using the above buffer at a flow rate of 4 ml/h. Fractions of 1 ml were collected. Each fraction, after dialysis against 20 mM potassium phosphate, pH 7.2, was assayed for enzyme activities.

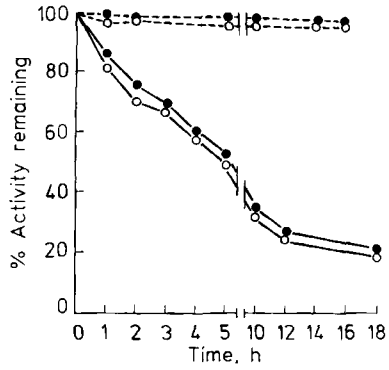

Fig. 1. *Effect of α-chymotrypsin digestion on BuChE and AAA activities.* Purified enzyme (5 µg) was incubated at 37°C with 0.05 µg α-chymotrypsin in a total vol. of 0.2 ml 100 mM Tris/HCl buffer, 1 mM EDTA, pH 7.5. At different time periods the reaction was terminated by the addition of 0.1 µg chymotrypsin inhibitor. The BuChE (●) and AAA (○) activities were determined using butyryl thiocholine and *o*-nitroacetanilide as substrates, as given in Materials and Methods. (——) Activities in the presence of chymotrypsin; (- - -) activities in the absence of chymotrypsin

Analysis of neutral sugars

A 60-µg protein sample was hydrolyzed in 2 M HCl at 100°C for 4 h, in a sealed tube under nitrogen, and the neutral sugar fraction was isolated by Dowex column chromatography [17]. The neutral sugars were identified by paper chromatography using the solvent system ethyl acetate/pyridine/*n*-butanol/butyric acid/water (10:10:5:1:5) [18] and by staining with alkaline silver nitrate. Total neutral sugars were estimated by the phenol/sulphuric acid method [19] using D-mannose as the standard sugar.

Immunological studies

Anti-BuChE serum was raised in a rabbit and purified as described earlier [4]. The purified antibody was free of any detectable BuChE or AAA activity. Immunoprecipitation was performed by cross-reacting different dilutions (0.025–0.2 mg) of antibody with the enzyme in 20 mM potassium phosphate, pH 7.2 followed by incubation at 4°C for 24 h. After centrifugation at 10000 × *g* for 30 min, the BuChE and AAA activities were determined in the supernatant.

Amino-terminal-sequence analysis

Partial manual sequencing of the amino acids was performed by the method of Chang [20], using the dimethylaminoazobenzene isothiocyanate/phenyl isothiocyanate double-coupling technique.

Enzyme assay and protein estimation

BuChE activity was assayed with butyrylthiocholine iodide as substrate according to Ellman et al. [21]. One unit of BuChE activity was defined as the amount of enzyme causing a change in absorbance of 1/min at 412 nm, under standard assay conditions. Aryl acylamidase was assayed as described earlier [4]. One unit of aryl acylamidase hydrolyzes 1 µmol *o*-nitroacetanilide/h. Protein was determined according to Lowry et al. [22] with crystalline bovine serum albumin as standard, or by the absorbance at 280 nm (for column effluents).

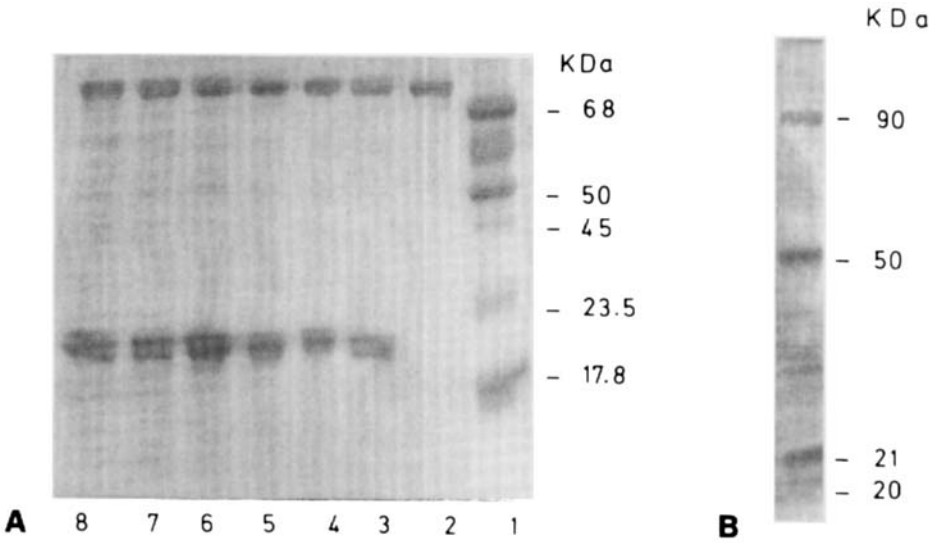

Fig. 2. *SDS/polyacrylamide gel electrophoresis of purified BuChE digested with α-chymotrypsin for various time periods.* (A) Purified BuChE (14 μg) was digested in 100 mM Tris/HCl, 1 mM EDTA, pH 7.5 at 37°C with α-chymotrypsin (0.14 μg) for different periods. At the end of each period, protein-dissociating buffer was added and the mixture was boiled for 3 min and subjected to SDS-gel electrophoresis on 10% polyacrylamide slab gels. Staining was with Coomassie blue R. Lane 1, molecular mass markers: bovine serum albumin (68 kDa), immunoglobulin G (IgG) heavy chain (50 kDa), ovalbumin (45 kDa), IgG light chain (23.5 kDa) and myoglobin (17.8 kDa); lane 2, purified BuChE containing chymotrypsin and chymotrypsin inhibitor. The chymotrypsin and chymotrypsin inhibitor were not detectable at the concentrations used with this staining procedure; lanes 3–8, purified BuChE digested with chymotrypsin for 1 h, 2 h, 4 h, 6 h, 12 h and 14 h, respectively. (B) SDS-gel electrophoresis pattern of an 8-h chymotryptic digest of purified BuChE, after silver staining

RESULTS

Effect of α-chymotrypsin treatment on BuChE and AAA activity

Fig. 1 shows the changes in BuChE and AAA activity when the purified enzyme was digested with α-chymotrypsin (BuChE/chymotrypsin = 1:0.01, by mass) for various periods. There was a slow decline in both enzyme activities with time. The percentage loss of BuChE and AAA activities was the same at each time interval studied. After chymotryptic digestion for 5 h there was a 50% loss in activity. Even after digestion for 18 h the enzyme retained about 20% of BuChE and AAA activity.

When the chymotrypsin digestion was performed with a BuChE/chymotrypsin ratio of 1:0.02 (by mass), a similar loss in BuChE and AAA activity with respect to time was observed except that the loss was slightly higher (56% loss at 5 h; not shown).

SDS/polyacrylamide gel electrophoresis of BuChE digested with α-chymotrypsin

Purified BuChE gave a major protein band of ≈ 90 kDa on SDS gel electrophoresis under reducing conditions. Fig. 2A shows the SDS gel electrophoresis pattern of protein bands obtained at varying periods (1–14 h) of α-chymotrypsin digestion (BuChE/chymotrypsin ratio 1:0.01; by mass). In addition to the uncleaved enzyme band of ≈ 90 kDa, two bands close to each other (≈ 20 kDa and 21 kDa) and a diffuse 50-kDa band which was stained poorly by Coomassie blue were seen at all periods of digestion. Fig. 2B shows an 8-h digest of BuChE on SDS/gel electrophoresis, after silver staining of the gel, which clearly indicated the 50-kDa band in addition to the other bands. No additional protein bands were seen at progressive time intervals. These results suggested that ≈ 20-kDa, ≈ 21-kDa and ≈ 50-kDa proteins that were observed by silver staining at all periods of digestion were the major products arising from α-chymotrypsin digestion of BuChE.

A similar pattern of protein bands at different periods was observed when the digestion was performed with a BuChE/chymotrypsin ratio of 1:0.02 (by mass; not shown).

RCA_1-Sepharose affinity chromatography

The galactose-specific lectin, RCA_1, linked to Sepharose was used as an affinity matrix to examine the glycosylated nature of purified BuChE, before and after chymotrypsin digestion. The purified BuChE alone or in the presence of both chymotrypsin and chymotrypsin inhibitor could fully bind to the RCA_1-Sepharose column and 97% of the bound BuChE was eluted from the column by lactose. However, after chymotryptic digestion for 6 h about 15–20% of the total BuChE or AAA activity could not bind to the RCA_1-Sepharose column and was recovered in the run-through fractions (Fig. 3). Rechromatography of the enzyme in the run-through fractions again showed its inability to bind to RCA_1-Sepharose column, suggesting that the enzymatically active fragment in the run-through fractions was devoid of galactose residues.

Sephadex G-75 gel filtration

Sephadex G-75 chromatography of the purified BuChE before chymotrypsin digestion showed the cholinesterase activity emerging as a sharp peak in the void volume (Fig. 4A). After digestion for 6 h with α-chymotrypsin, two peaks of cholinesterase activity were observed when the digestion mixture was chromatographed on Sephadex G-75, one corre-

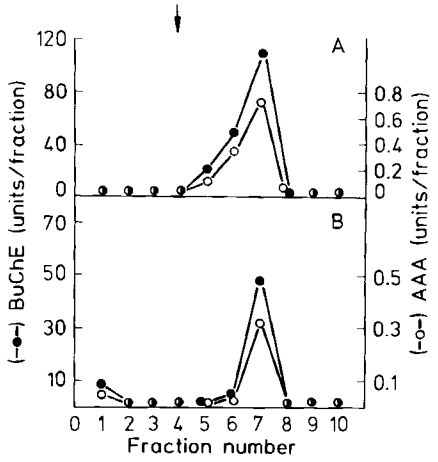

Fig. 3. *RCA_1-Sepharose chromatography of purified BuChE (A) and BuChE after chymotryptic digestion for 6 h (B).* (↓) Indicates the fraction where elution with lactose was started. Both BuChE (●) and AAA (○) activities were measured. Details of RCA_1-Sepharose chromatography are given in Materials and Methods

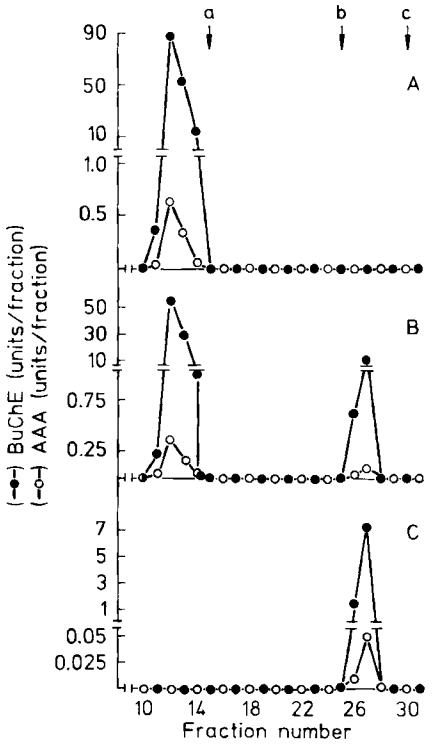

Fig. 4. *Sephadex G-75 chromatography.* Elution patterns of (A) purified BuChE; (B) purified BuChE digested with α-chymotrypsin for 6 h and (C) BuChE fragment which did not bind to RCA_1-Sepharose, recovered in the run-through fractions after α-chymotrypsin digestion and RCA_1-Sepharose chromatography. Details of chromatography and assay procedures are given in Materials and Methods. Each fraction was assayed for BuChE (●) and AAA (○) activity. The molecular mass standards used were bovine serum albumin, 68 kDa (a); carbonic anhydrase, 29 kDa (b) and cytochrome *c*, 12.4 kDa (c)

sponding to that found in the void volume and another lower-molecular-mass form of approximately 20 kDa (Fig. 4B). Both BuChE and AAA activities co-eluted during chromatography. In further experiments it was observed that the low-

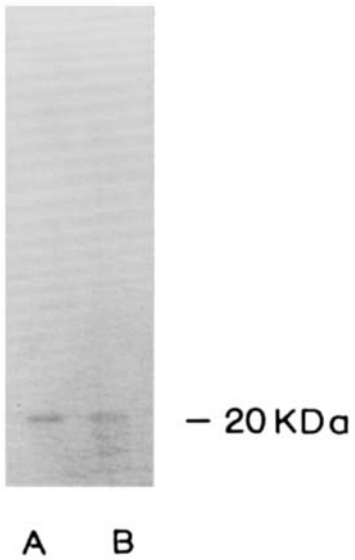

Fig. 5. *SDS-gel electrophoretic pattern of the chymotryptic fragment of purified BuChE (10 μg) after a 6-h digestion.* (A) Isolated by RCA_1-Sepharose chromatography; (B) RCA_1-Sepharose chromatography followed by Sephadex G-75 chromatography

molecular-mass BuChE from the Sephadex G-75 column did not bind to RCA_1-Sepharose. Alternatively, when the BuChE, from a chymotryptic digest of purified BuChE and which did not bind to RCA_1-Sepharose, was subjected to Sephadex G-75 chromatography it was eluted as a low-molecular-mass, 20-kDa peak (Fig. 4C). These results suggested that chymotryptic digestion of purified BuChE resulted in a low-molecular-mass form of enzyme that was the same as the BuChE form which did not bind to RCA_1-Sepharose observed in the earlier experiment.

SDS-gel electrophoresis of the BuChE form which did not bind to RCA_1-Sepharose and the Sephadex G-75 low-molecular-mass BuChE obtained after chymotryptic digestion of BuChE

The BuChE activity that did not bind to RCA_1-Sepharose and the BuChE that emerged as the low-molecular-mass fragment on Sephadex G-75 chromatography, following RCA_1-Sepharose chromatography, were subjected to SDS-gel electrophoresis. Each showed a single protein band of ≈ 20 kDa (Fig. 5). These results again confirmed their identity and suggested that the enzymatically active product obtained by chymotryptic digestion of BuChE had a molecular mass of ≈ 20 kDa.

Polyacrylamide gel electrophoresis under non-denaturing conditions of BuChE after chymotryptic digestion

Purified BuChE before chymotryptic digestion migrated as a single band containing both BuChE and AAA activity on polyacrylamide gel electrophoresis under non-denaturing conditions. After chymotryptic digestion of BuChE, the digest was subjected to gel electrophoresis and the gel slices assayed for enzyme activity. In addition to the gel slice corresponding to the undigested BuChE, an additional gel slice was also found to have both BuChE and AAA activity. Under the electrophoresis conditions used, the intact BuChE migrated

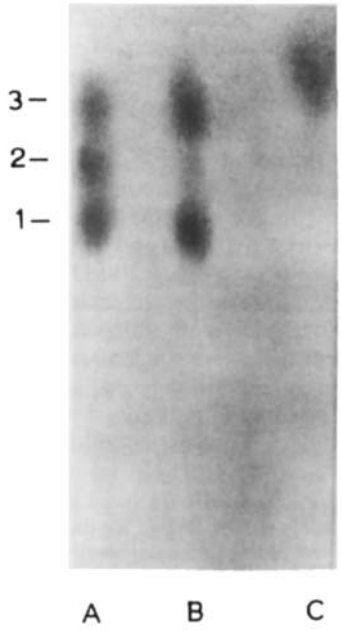

Fig. 6. *Paper chromatographic pattern of neutral sugars from purified human serum BuChE (B) and the 20-kDa BuChE fragment (C).* The conditions of hydrolysis, isolation of neutral sugars and paper chromatography are described in Materials and Methods. Standard sugars (A) used were (1), D-galactose; (2), D-glucose and (3), D-mannose. The mannose spot in C is slightly ahead of the standard mannose because of the faster migration of the solvent front in that region

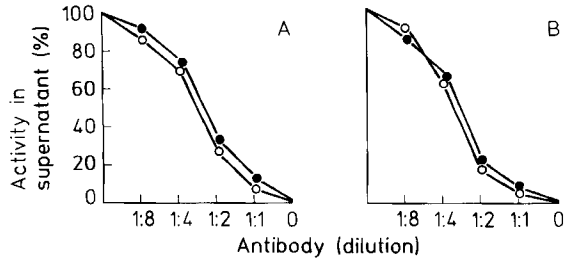

Fig. 7. *Immunoprecipitation of purified BuChE (A) and the 20-kDa BuChE fragment (B) at different dilutions of the antibody raised against purified BuChE.* Details of the experiment are given in Materials and Methods. Both BuChE (●) and AAA (○) activities were measured

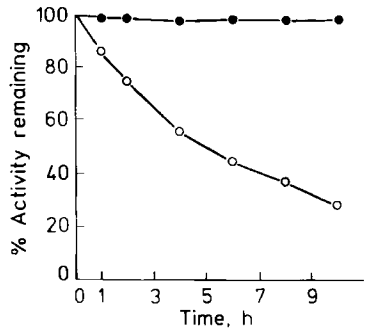

Fig. 8. *Thermal stability of purified BuChE and the 20-kDa BuChE fragment at 37°C.* Purified BuChE (●) and the 20-kDa BuChE fragment (○), isolated by RCA_1-Sepharose chromatography, were kept at 37°C and at different time intervals measured for enzyme activity

1.5 mm from the origin while the additional activity migrated 12.5 mm from the origin. The enzyme from this latter gel slice was extracted and subjected to SDS-gel electrophoresis. This gave a single protein band of ≈ 20 kDa (not shown). This experiment also confirmed that the ≈ 20-kDa fragment was the cleavage product which retains enzyme activity.

Neutral sugar analysis

The neutral sugar content of the purified BuChE was 0.088 mg/mg protein corresponding to about 8.8% sugar. This value agrees well with that of 9.3% (galactose plus mannose) reported for human serum cholinesterase [3]. The ≈ 20-kDa enzyme which did not bind to RCA_1-Sepharose contained approximately 0.07 mg neutral sugar/mg protein. Neutral sugar analysis by paper chromatography showed the presence of both mannose and galactose in the purified enzyme whereas the ≈ 20-kDa fragment showed only mannose (Fig. 6).

Immunoprecipitation studies

An antibody raised against purified BuChE could immunoprecipitate the enzyme activities from the purified BuChE as well as the ≈ 20-kDa fragment. Both BuChE and AAA activities were co-precipitated by the antibody at different dilutions (Fig. 7).

Thermal stability of the cholinesterase activity present in the 20-kDa BuChE and the parent BuChE

A comparison of the thermal stability at 37°C of cholinesterase activity present in the ≈ 20-kDa fragment and the parent BuChE is shown in Fig. 8. The cholinesterase activity of the 20-kDa fragment was less stable than that of the parent BuChE; it lost about 55% activity in 6 h compared to the parent BuChE which lost less than 5% activity during the same period.

Amino-terminal-sequence analysis of the ≈ 20-kDa BuChE fragment

The ≈ 20-kDa BuChE fragment obtained after chymotryptic digestion of purified BuChE was subjected to Sephadex G-75 chromatography followed by RCA_1-Sepharose chromatography. The RCA_1-Sepharose run-through fractions were pooled, dialyzed against water and used for amino-terminal-sequence analysis. The following sequence of five amino acids was obtained: Arg-Val-Gly-Ala-Leu. This sequence is identical to amino acid residues 147–151 in the BuChE sequence reported by Lockridge et al. [3].

DISCUSSION

The complete amino acid sequence of human serum BuChE has been provided by Lockridge et al. [3]. The protein contains 574 amino acids/subunit and nine carbohydrate chains attached to asparagine residues. Earlier studies on limited trypsin digestion of human serum BuChE [1] have shown the removal of a peptide containing the interchain disulfide bond. The 90-kDa subunit of BuChE, after trypsin digestion, did not show any apparent change, as observed on SDS-gel electrophoresis, because the cleavage took place about seven amino acids away from the C-terminus [23].

In the present study, limited α-chymotrypsin digestion of purified human serum BuChE shows the formation of three

major fragments, two in the molecular mass range ≈ 21 – 20 kDa and another of ≈ 50 kDa, as observed on SDS-gel electrophoresis. The two low-molecular-mass fragments are stained well by Coomassie blue whereas the larger-molecular-mass fragment is poorly stained, presumably because it contains the major proportion of carbohydrate chains. Of the two low-molecular-mass fragments, one (≈ 20 kDa) is enzymatically active and exhibits both BuChE and AAA activities. This fragment which can be separated by Sephadex G-75 gel filtration, does not bind to RCA_1-Sepharose and contains only mannose as a neutral sugar. This is in contrast to the uncleaved BuChE, that contains both galactose and mannose and binds fully to RCA_1-Sepharose. Sequencing of amino acids from the amino terminal shows that the ≈ 20-kDa fragment has the sequence Arg-Val-Gly-Ala-Leu. This sequence agrees with amino acid residues 147 – 151 in the BuChE sequence given by Lockridge et al. [3]. The chymotryptic cleavage generating the N-terminus of the ≈ 20-kDa fragment has therefore taken place between Tyr^{146} and Arg^{147} of the BuChE molecule. Considering a molecular mass of ≈ 20 kDa, the C-terminus of this fragment can be somewhere between residues Phe^{290} and Leu^{306} of the BuChE subunit. There are three possible chymotryptic cleavage sites in this region, Phe^{290}-Gly^{291}, Phe^{298}-Leu^{299} and Leu^{306}-Leu^{307}. It is not certain at which of the three sites the cleavage might have taken place. The following scheme summarizes these conclusions and indicates the three major possible fragments generated from BuChE by limited α-chymotrypsin digestion:

Glu^1 ----------	Tyr^{146}	Inactive low-molecular-mass fragment (≈ 21 kDa)
Arg^{147} -------	Phe^{290} or Phe^{298} or Leu^{306}	Enzymatically active low-molecular-mass fragment devoid of galactose (≈ 20 kDa)
Gly^{291} or Leu^{299} ------- or Leu^{307}	Leu^{574}	High-molecular-mass fragment containing major proportion of carbohydrate (≈ 50 kDa)

The enzymatically active ≈ 20-kDa fragment that also contains the active-site serine residue was studied in detail. The fragment possessed BuChE and AAA activities in the same ratio as the intact enzyme. The BuChE and AAA activity in this fragment could be immunoprecipitated by antibody raised against the purified parent BuChE. The BuChE activity in this fragment was less stable than the parent BuChE when kept at 37 °C for different periods. The gradual loss in BuChE and AAA activity observed during chymotryptic digestion shown in Fig. 1 can be attributed to the instability at 37 °C of the ≈ 20-kDa enzymatically active fragment generated from the parent BuChE. The additional possibility of a lowered catalytic efficiency in the fragment also cannot be excluded. Further detailed studies on the comparative kinetics, substrate and inhibitor specificities and active-site labelling of the intact BuChE and ≈ 20-kDa fragment are in progress.

Another interesting aspect of the enzymatically active ≈ 20-kDa fragment was the complete absence of galactose, as evidenced by RCA_1-Sepharose chromatography and neutral sugar analysis. A total of nine asparagine residues containing carbohydrate chains have been proposed for the BuChE subunit [3]. Of these, two chains are present in the enzymatically active ≈ 20-kDa fragment. The present studies would suggest that these two chains at Asn^{241} and Asn^{256} contain only mannose, but no galactose, residues.

Finally, limited chymotryptic digestion did not separate the BuChE and AAA activities and suggested the co-localization of their active sites in the ≈ 20-kDa enzymatically active fragment. The isolation of an enzymatically active fragment by chymotryptic digestion should permit further studies on the localization of the active-site amino acid residues of AAA and BuChE. The present studies also suggest that the carbohydrate chains on Asn^{241} and Asn^{256} of BuChE are of the high-mannose type and not the complex type.

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