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QUANTITATIVE MONITORING BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THE DISSOCIATION OF HUMAN SERUM CHOLINESTERASE BY LIMITED PROTEOLYSIS

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SUMMARY

Proteolytic action on human serum cholinesterase, a tetrameric enzyme, results in a partial disintegration which can be recorded only qualitatively by time-consuming electrophoretic techniques. In this study, a rapid high-performance liquid chromatographic method was used for the separation and determination of the active dissociation products. Separation of the cholinesterase subunits was accomplished by high-performance gel permeation chromatography on a combination of DIOL columns (Zorbax GF 450/GF 250) in 0.2 *M* phosphate buffer (pH 7.0). Detection and quantification of enzyme activity in the fractionated eluate were carried out using a Flexigem analyser (substrate, butyrylthiocholine). On limited tryptic digestion of partially purified human ChE, up to three peaks of enzyme activity could be identified. Their elution volumes corresponded to apparent molecular masses of 480 000, 270 000 and 120 000, indicating, in addition to the tetrameric holoenzyme, a dimeric and a monomeric form. Quantification of the relative amounts of individual enzyme activity peaks revealed that in the course of degradation, the dimer appeared first, followed by the monomer. This suggests that the first step in the sequence of dissociation is cleavage of the tetramer into a pair of dimers, then further into the monomeric subunit. During the incubation with trypsin, a significant change in the pattern of the different peaks had already occurred when the total enzyme activity was only slightly reduced.

INTRODUCTION

Human serum cholinesterase (EC 3.1.1.8) (for a review, see ref. 1) is a tetrameric enzyme with a molecular mass of approximately 350 000, composed of apparently identical subunits [2]. These are arranged as a dimer of dimers and attached to each other by non-covalent interactions and disulphide bridges [3]. Enzymatic activity is present even in the isolated monomeric subunit [2].

Cholinesterase present in human serum can be separated by starch gel electrophoresis into four bands of enzymatic activity, one major and three minor bands

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[4]. These multiple forms probably represent aggregates of different numbers of the monomeric subunit, i.e., oligomers of one to four polypeptide chains [5].

The pattern of these multiple forms of cholinesterase may be influenced in vitro by the action of proteolytic enzymes [6]. Limited proteolytic action on the enzyme results in the loss of bridging peptides by which the individual subunits are linked together [7]. During this process, the relative amounts of monomeric and dimeric aggregates appear to increase at the expense of the intact tetramer; further, the total enzyme activity decreases.

Up to now, the presence of multiple forms and the partial disintegration of human serum cholinesterase have usually been documented by electrophoretic techniques [4, 5, 7]. For the detection of enzyme activity in the gel matrix, an incubation with the substrate butyrylthiocholine and a counterstaining procedure applying copper ions and dithiooxamide were used [8]. Apart from being time-consuming and laborious, these methods suffer from the disadvantage that it is very difficult to quantify the distribution of the disintegration products by activity staining procedures. On the other hand, conventional chromatographic approaches to this particular separation problem which comprise the possibility of directly measuring enzyme activities in the eluate have been tried only occasionally using lengthy gel permeation analysis [9]. Therefore, high-performance gel permeation chromatography (HPGPC) was used in the present investigation for the development of a rapid separation and quantification of the multiple forms of ChE and its dissociation products.

EXPERIMENTAL

High-performance liquid chromatographic (HPLC) apparatus

A Model 6000A solvent delivery system was used in combination with a Model U6K injection system (Waters Assoc., Milford, MA, U.S.A.). The UV detector was a Schoeffel SF 770 spectrophotometric monitor (Schoeffel Instruments, Westwood, NJ, U.S.A.) equipped with a Servogor 210 recorder (Metrawatt, Nürnberg, F.R.G.). Proteins were detected at 280 nm. Fractions of the effluent were collected with an Ultrorac 7000 fraction collector (LKB, Bromma, Sweden).

HPLC columns

Chromatographic separations were carried out on a combination of two DIOL columns (Zorbax DIOL GF 450/GF 250, each 250×19 mm I.D.; DuPont, Wilmington, DE, U.S.A.). For their protection, a DIOL guard column was installed upstream of the analytical columns. During an earlier stage of this study, we also used TSK 3000SW columns (300×7.5 mm and 500×7.5 mm I.D.; Varian, Walnut Creek, CA, U.S.A.) in series, as previously described [10].

Mobile phase

Chromatography was performed using a sodium phosphate buffer (0.2 *M*, pH 7.0, containing 50 mg/l sodium azide,) at a flow-rate of 1 ml/min. The volume of the sample applied to the column was 50 μl.

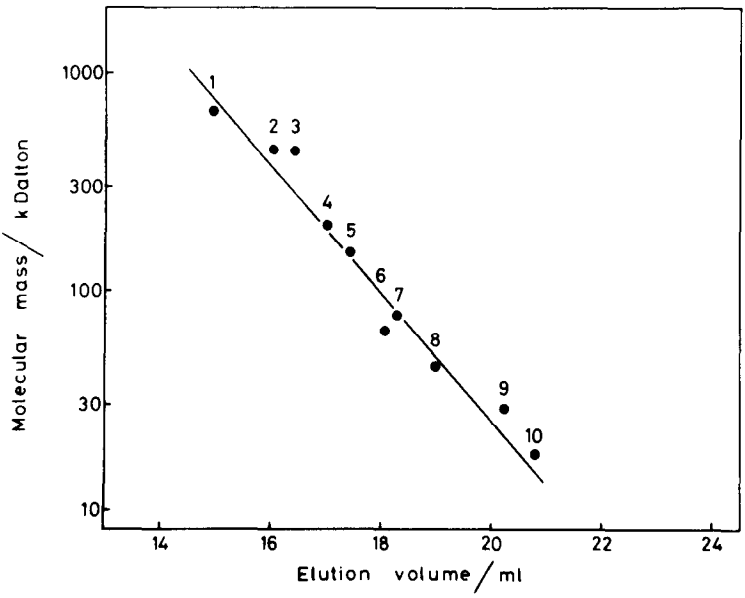

Fig. 1. Calibration graph for the determination of molecular mass by HPGPC. Retention volumes were derived from individual chromatograms of the isolated marker proteins. 1, Thyroglobulin; 2, ferritin; 3, apoferritin; 4, β-amylase; 5, alcohol dehydrogenase; 6, bovine serum albumin; 7, transferrin; 8, ovalbumin; 9, carbonic anhydrase; 10, myoglobin.

Determination of cholinesterase activity

Normally, the enzymatic activity of cholinesterase was determined automatically in a Flexigem centrifugational analyser (Electro-Nucleonics, Fairfield, NJ, U.S.A.) using the substrate butyrylthiocholine (6 m*M* in 0.05 *M* sodium phosphate, pH 7.7, 25°C), the thiocholine liberated by the enzyme being detected by reaction with Ellman's reagent. For measurement of the enzyme activity in the presence of thiol compounds, e.g., 2-mercaptoethanol, benzoylcholine (0.05 m*M* in 0.067 *M* sodium phosphate, pH 7.4, 37°C) was used as the substrate. The formation of free benzoate was monitored by measurement of the absorbance at 235 nm using a Zeiss PMQ II spectrophotometer (Zeiss, Oberkochen, F.R.G.).

Enzymes

Partially purified human serum cholinesterase (ca. 75 U/mg) was obtained from Behringwerke (Marburg, F.R.G.), and bovine pancreas trypsin, L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated (ca. 200 U/mg) from Cooper Biomedical (Malvern, PA, U.S.A.).

Determination of the relative molecular mass (M_r)

Molecular mass marker proteins were obtained from Serva (Heidelberg, F.R.G.), and Sigma (St. Louis, MO, U.S.A.). For calibration of the HPGPC columns, these proteins were chromatographed individually at a concentration of ca. 2 mg/ml. The linear relationship between log M_r and the elution volume obtained in our chromatographic system is shown in Fig. 1.

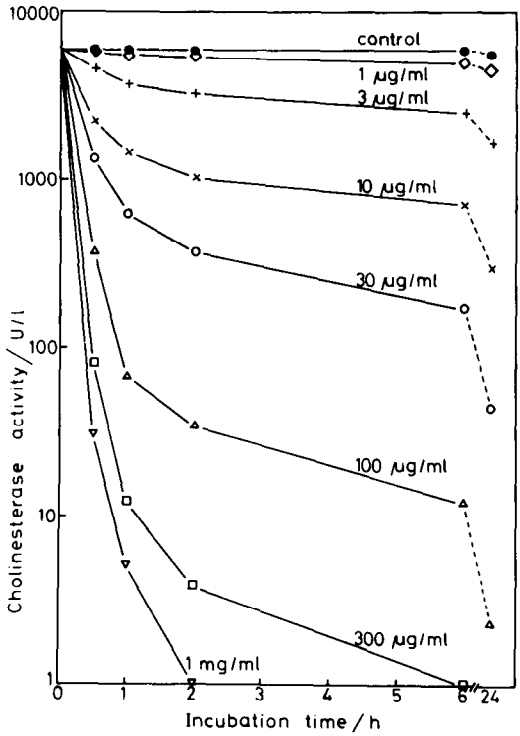

Fig. 2. Effect of tryptic digestion of partially purified cholinesterase on the enzyme activity. Human serum cholinesterase (ca. 60 U/ml) was incubated with trypsin (concentrations as indicated) in 0.1 *M* Tris-HCl (pH 8.1) at 37°C. Enzyme activity was determined immediately after termination of the proteolysis by dilution with ice-cold phosphate buffer (pH 7.0). The residual activity is shown on a logarithmic scale.

Limited tryptic digestion of cholinesterase

Lyophilized human serum cholinesterase was reconstituted with water (final concentration, 2 mg/ml) and diluted 1:2 with 0.1 *M* Tris-HCl (pH 8.1). A 0.4-ml volume of this solution was incubated with 0.1 ml of trypsin (freshly prepared; 0.005–5 mg/ml in 0.1 *M* Tris-HCl, pH 8.1) at 37°C. At several times during the incubation, aliquots of 0.05 ml were taken and diluted ten-fold with ice-cold sodium phosphate buffer (0.2 *M*, pH 7.0). Following the determination of the total residual activity, these aliquots were subjected to chromatographic analysis as described above.

RESULTS

Influence of trypsin on cholinesterase activity

Proteolytic action reduced the enzymatic activity of cholinesterase, but a significant reduction was observed only above a trypsin concentration of ca. 1 µg/ml (i.e., ca. 1.25 µg/mg of ChE), as shown in Fig. 2.

A 50% inhibition of enzymatic activity was caused by trypsin at a concentration of ca. 10 µg/ml after an incubation time of 30 min. The proteolytic process

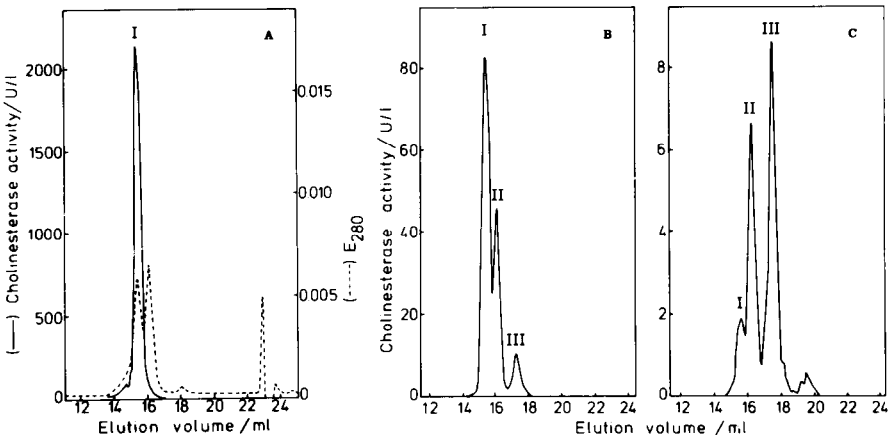

Fig. 3. HPGPC elution profiles of partially digested cholinesterase. Following the termination of the proteolytic process, aliquots of the diluted digest were immediately subjected to chromatographic analysis (incubation time with trypsin, 30 min). A, Native cholinesterase, prior to any proteolytic action; B, trypsin concentration 10 μg/ml; C, trypsin concentration 100 μg/ml. The lower enzyme activity scales in B and C partly result from the high dilution of the aliquots prior to chromatography.

appeared to be self-limited, most of the changes in the activity occurring during the first 60 min of incubation. A plot of the loss of enzymatic activity after 30 min versus the concentration of trypsin (not shown) revealed a direct proportionality of these two variables in the trypsin concentration range 1–30 μg/ml.

Chromatographic behaviour of native and partially digested cholinesterase

HPGPC of native, undigested cholinesterase on our diol-substituted silica gel columns revealed a single sharp peak of enzyme activity (peak I) coinciding with the first UV-absorbing peak eluting from the column (Fig. 3A).

Recoveries of the enzyme activity during chromatography were calculated by addition of the enzyme activities in the collected fractions and found to be in the range 80–90% of the enzyme applied to the column. The elution volume of the cholinesterase activity peak was 15.6 ± 0.15 ml, which corresponds to a molecular mass of ca. 480 000.

Chromatography of aliquots of the incubation mixture showed that in the course of tryptic digestion, two additional peaks of enzyme activity (II and III) were eluted from the column at a higher retention time than the native enzyme (Fig. 3B and C). According to their elution volumes of 16.4 and 17.6 ml, these fragments have molecular masses of ca. 270 000 and ca. 120 000, respectively.

In addition to these changes in the elution pattern of enzyme activity caused by the proteolytic process, alterations were also observed in the UV absorbance chromatogram. The intensity of the first of the two peaks originally recorded on chromatography of the native cholinesterase decreased markedly following the limited proteolysis, whereas the appearance of the second peak became significantly broader. Because the enzyme had to be diluted extensively prior to chromatography in order to stop the proteolytic reaction effectively, these changes in

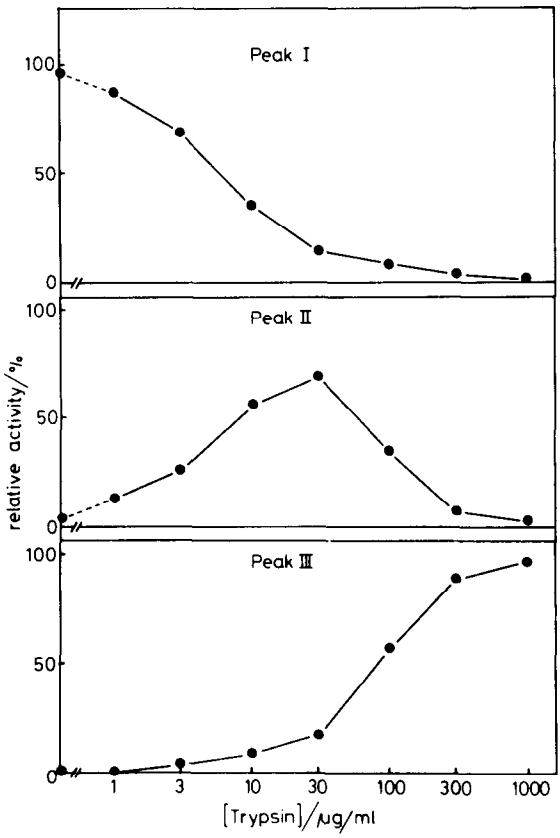

Fig. 4. Dependence of the pattern of cholinesterase dissociation products on the concentration of trypsin. Numbering of the enzyme activity peaks corresponds to that in Fig. 3. The enzyme activity eluted in the different peaks was summarized and normalized to the total activity recovered in the individual column runs.

the UV absorbance pattern could not be recorded with sufficient precision (consequently, UV absorbance traces are not shown in Fig. 3B and C).

Quantitative analysis of the pattern of cholinesterase dissociation products

A gradual increase in the trypsin concentration in the mixture for the limited proteolysis of cholinesterase led to a shift in the proportion of the enzyme activity peaks eluted during the subsequent chromatographic analysis. The activity of peak I, i.e., the intact tetrameric enzyme, decreased while that of peak III increased (Fig. 4).

Peak II appeared first during the limited proteolysis, and its relative amount rose at the expense of peak I, which suggests that the intact molecule was first cleaved into II. At a trypsin concentration of 10 μg/ml, the relative amount of peak II surpassed that of peak I. Peak III did not increase until peak I had nearly disappeared, which in turn suggests that III emerged from II.

The change in the pattern of the different peaks of cholinesterase activity, i.e., from the single peak observed with the native enzyme to the three peaks following

the digestion with trypsin, was already evident at a concentration of trypsin that lowered the total enzymatic activity only slightly. After 30 min of digestion using a trypsin concentration of 3 μg/ml, the relative activity of peak II included a significant portion (ca. 27%) of the total enzyme activity eluted from the column (Fig. 4), whereas the total enzyme activity was reduced by only 20% (Fig. 2).

DISCUSSION

In contrast to the membrane-bound acetylcholinesterase, whose participation in the process of transmitting nerve impulses within the synaptic cleft is well established, the function of the non-specific serum cholinesterase is still unknown. With regard to its genetic variability and its molecular structure, however, a considerable amount of information has accumulated in recent years [1].

From a clinician's point of view, the genetically determined variants of the enzyme [11] have raised particular interest. Prolonged apnoea following the administration of the muscle-relaxing agent succinylcholine have been attributed to the presence of the homozygotic "unusual" phenotype E^a [12], which may be distinguished from the normal variant E^u by a reduced sensitivity to the inhibitors fluoride and dibucaine [13, 14].

Irrespective of the genetically fixed variant of the enzyme, the cholinesterase present in human serum is not homogeneous. Using high-resolution polyacrylamide gel electrophoresis, Juul [8] demonstrated up to twelve bands of enzyme activity. Only four of these, mostly termed multiple forms, however, are present in significant amounts. Usually, they are referred to as C_1 to C_4 in order of their migrational speeds during electrophoresis. From the experiments of La Motta and Woronick [5], who demonstrated the interconvertability of these multiple forms, it could be concluded that the smaller components are derived from the largest species, C_4.

By applying the technique of limited proteolysis with trypsin, components identical with the naturally occurring multiple forms, with respect to electrophoretic mobility, have been produced by Saeed et al. [6] and later Lockridge and La Du [7]. These data suggested a partial proteolytic digestion as an ontogenetic principle for the existence of the enzyme's multiple forms.

Although up to now only a partial amino acid sequence of a peptide comprising the active centre of the enzyme has been established [15], it appears that it is made up of only one type of subunit.

The relative molecular mass of the enzyme has been determined to ca. 340 000 by applying ultracentrifugation [2], agarose gel electrophoresis [16] and gel chromatography [2, 17, 18]. In our experiments, considerably higher values were found for the intact molecule and for the disintegration products generated by the action of trypsin. These deviations might possibly be explained by a non-ideal behaviour of the protein during the chromatography on diol-substituted silica material. Further, the enzyme shows a highly asymmetric molecular shape according to the results of Masson [16], who reported an axial ratio of 1:8 for human serum cholinesterase. Therefore, a reduced elution volume in size-exclusion chromatography might not be an unexpected feature of the enzyme. On the

other hand, Lockridge and La Du [7] found molecular mass values corresponding to those obtained by ultracentrifugation methods when Sephacryl S300 gel permeation was used, whereas Harris and Robson [9] did not calibrate their gel permeation column and could not report any molecular mass data for the enzyme.

In an attempt to modify possible repulsive interactions between the enzyme and the HPLC solid phase, we performed chromatographic runs also in buffers of increasing ionic strength. However, up to a phosphate concentration of 0.5 *M*, no effects on the elution volumes were observed. Measurements of the molecular mass of partially denatured enzyme could not be performed because in the presence of SDS no enzymatic activity remained.

According to the results of Lockridge and La Du [7], proteolytic digestion of the native enzyme produces three new bands of enzyme activity appearing in agarose gel electrophoresis, which constitute a trimeric, dimeric and monomeric form. From our study, however, there is no evidence for the appearance of an active trimer during the tryptic digestion over a wide range of trypsin concentrations and incubation times. Not even a shoulder could be found on the first peak of enzyme activity, even when narrower fractions of the eluate were analysed for cholinesterase activity. However, polypeptides with molecular masses of 480 000 and 360 000 (the latter being calculated for a presumptive trimeric enzyme using our data for the intact molecule) should be sufficiently separated or at least partially resolved by our chromatographic system, as can be seen from the molecular mass calibration graph (Fig.1). From the elution volumes of the two additional peaks appearing during proteolysis, it is suggested that these enzyme species represent a dimeric and a monomeric form of the enzyme. Therefore, it might be concluded that under the conditions used in our study, only dimers and monomers of cholinesterase are produced.

The sequential course of the disintegration process induced by tryptic digestion of cholinesterase might be derived from the quantitative analysis of the relative amounts of the three active enzyme peaks as shown in Fig. 4. Here, we have evaluated the chromatographic enzyme activity pattern following proteolysis within a fixed time using trypsin in increasing concentrations, as an increase in protease concentration should be equal to the increase in digestion time. Further, this approach was also suggested by the non-linear time course of the loss of enzymatic activity by the tryptic digestion as illustrated in Fig. 2. Additionally, sequential analyses of the activity peak pattern during proteolysis with a fixed trypsin concentration revealed only slight variations after the first 30 min of incubation time (data not shown).

Provided that the specific activities of the three different enzyme species do not differ substantially from each other, it can be concluded that the first step in the degradation of intact cholinesterase by trypsin is cleavage of the tetramer into a pair of dimers followed by the splitting of the dimers into monomeric forms. Although we could not determine the specific activities of the three species in our study, it appears unlikely that they differ from each other significantly as the absolute enzyme activities of all three species concomitantly decline during the proteolytic process. The loss of activity is not confined to one isolated species; they all show total enzyme activities of the same order of magnitude.

The chromatographic pattern of the enzyme activity peaks and their elution volumes, including that of undigested cholinesterase, remained essentially unchanged when either the proteolytic digestion and/or the chromatographic analyses were performed in the presence of a disulphide-reducing agent (0.2 *M* 2-mercaptoethanol; data not shown). These findings, in accordance with those of Lockridge and La Du [7], indicate that intramolecular disulphide bonds are not the only forces responsible for the intact tetrameric structure of the holoenzyme. In addition to the interchain disulphide peptide [7], other peptides have to be removed which provide for hydrophobic interactions between the subunits, before a cleavage of cholinesterase can be demonstrated by chromatographic or electrophoretic techniques.

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