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PURIFICATION AND PROPERTIES OF HUMAN SERUM ESTERASE

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Summary

Human serum esterase was purified by affinity column chromatography on a column of covalently linked *p*-trimethylammoniumanilinium dichloride to Sepharose 4B. The purified preparation hydrolysed both benzoylcholine and tributyrin. *p*-Trimethylammoniumanilinium dichloride inhibited non-competitively the hydrolysis of tributyrin and inhibited competitively the splitting of benzoylcholine.

The K_m value was $0.62 \cdot 10^{-3}$ M for tributyrin and $0.4 \cdot 10^{-3}$ M for benzoylcholine. Antiserum to this purified esterase was prepared in rabbit and it was found that the antiserum did not inhibit esterase activities of human liver, muscle and adipose tissue, although it could inhibit completely the esterase activities of human serum.

Introduction

Esterase found in the serum of animals can be classified into three main types: nonspecific esterases (ali-esterases, EC 3.1.1.1), arylesterases (EC 3.1.1.2) and pseudocholinesterase (EC 3.1.1.6) based upon the type of substrate and upon inhibition by eserine and organophosphates. The nonspecific esterases, which preferentially hydrolyze aliphatic esters, are sensitive to organophosphates but resistant to eserine. The arylesterases, which hydrolyze aromatic esters, are resistant to both organophosphates and eserine. The pseudocholinesterases which hydrolyze choline esters, are sensitive both to organophosphates and to eserine [1—3]. However, this classification is not absolute. For example, the pseudocholinesterases hydrolyze aliphatic esters and aromatic esters in addition to choline esters [3,4]. Muraoka and Okuda [5] investigated the relationship between pseudocholinesterase and nonspecific

esterase and found that activities of pseudocholine esterase and nonspecific esterase in human serum derived from the same enzyme molecule. Therefore, esterases in serum and tissues should now be classified by a new theory based on analysis of the enzyme molecule.

In the present investigation, human serum esterase was purified by affinity column chromatography and the relationship between pseudocholinesterase and nonspecific esterase was examined.

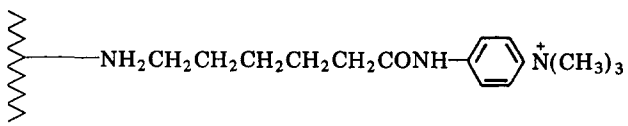
Materials and Methods

Materials

Benzoylcholine chloride and 6-amino-*n*-caproic acid were purchased from Wako Pure Chemical Industries (Osaka) and tri-*n*-butyrin from Nakarai Chemicals Ltd. (Kyoto). DEAE-Sephadex (A-50), CN-activated Sepharose 4B were the products of Pharmacia Fine Chemicals (Uppsala, Sweden) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was the product of Fluka (Switzerland). Other chemicals used were standard commercial products.

Synthesis of the affinity resin

p-Trimethylammoniumanilinium dichloride was prepared essentially according to the method of Traylar and Singer [6]. Gels of CN-activated Sepharose 4B (generally 25 ml in $NaHCO_3$ buffer, pH 8.3) was mixed with 6-amino-*n*-caproic acid and stirred at 4° C. After 15 h, remaining active groups on the gel were eliminated by stirring for 3 h with 1.5-ml ethanolamine. The gel was washed with 500 ml water, 500 ml 0.5 M NaCl and 1 l of water, successively. After adjusting the pH to 4.5, the gel was mixed with *p*-trimethylammoniumanilinium dichloride (223 mg) and EDC (260 mg). The gel was washed consecutively with 1 l water, 500 ml 0.5 M NaCl and 1 l water. The structure of resulting affinity resin was as follows.



Enzyme assay

Pseudocholinesterase activity was assayed with benzoylcholine as the substrate by Hesterin's method as modified by Roberts [7] and nonspecific esterase activity with tributyrin as the substrate by a modification [5] of the chromotropic acid method [8]. Protein was determined by the method of Lowry et al. [9] with bovine serum albumin as standard.

Electrophoresis

Polyacrylamide gel electrophoresis was performed in 3.5, 5 and 7% gels at a constant current using the Tris-glycine system of Davis [10]. Protein was detected by staining with Coomassie brilliant blue G-250 in 12.5% trichloroacetic acid and destained in 7% acetic acid [11]. Esterase activity was detected

by a slight modification of the method of Holmes and Masters [12], using α-naphthylacetate as the substrate.

Preparation of antiserum

Antiserum against the esterase was prepared by injecting a rabbit with purified human esterase with Freund's complete adjuvant [13]. Rabbit serum esterase activity in the antiserum was removed by gel filtration on Sephadex G-200 column. A 5 ml aliquot of rabbit antiserum was applyed to a Sephadex G-200 column (3.5 × 70 cm) equilibrated with 0.15 M NaCl (5-ml fractions). Active fractions of rabbit esterase were removed and the residual fraction was collected and concentrated. This latter solution was used as the esterase-free antiserum.

Immunochemical analysis

Human liver, muscle and adipose tissue were homogenized in 9 vols. 0.15 M NaCl. The homogenate was centrifuged at 700 × *g* for 10 min at 4° C and the supernatant was used as the enzyme preparation. The enzyme solution was mixed with an equal volume of the antibody solution. The mixture was left to stand overnight at 4° C and then centrifuged at 700 × *g* for 10 min. The esterase activity in the supernatant was examined. A control experiment was performed in the same way with the non-immunized rabbit serum.

Results

Purification of human serum esterase

A 200 ml aliquot of the serum was dialyzed against 10 mM potassium phosphate buffer (pH 8.0) at 4° C. The dialyzed serum was applied to the affinity column equilibrated with the same buffer. The esterase was eluted from the affinity column by 200 mM NaCl (Fig. 1).

The pooled esterase was dialyzed against the 10 mM potassium phosphate buffer (pH 8.0) and applied against to the affinity column. The elution was carried out with a linear gradient of 50—200 mM NaCl (Fig. 2).

The pooled esterase was dialyzed and then applied on a DEAE-Sephadex A-50 column equilibrated with 10 mM potassium phosphate buffer (pH 8.0). Fractions Nos. 55 to 64 were pooled and concentrated with collodion bags (Sartorius membrane filter, Göttingen) (Fig. 3).

The steps of the purification procedure and the yields of the enzyme at each step were summarized in Table I. With both substrates (benzoylcholine and tributyrin), the specific activity of the final material was increased approximately 3000-fold compared with that of the original. The ratio of pseudocholinesterase activity to nonspecific esterase activity was essentially constant during the purification procedures.

When the final purified enzyme (10 μl of a 10 mg/ml solution) was subjected to electrophoresis in 3.5, 5 and 7% polyacrylamide gels, respectively, it migrated as a single band, indicating that the enzyme was apparently electrophoretically homogeneous. It was found that the protein band on the electrophoresis was identical with that of esterase.

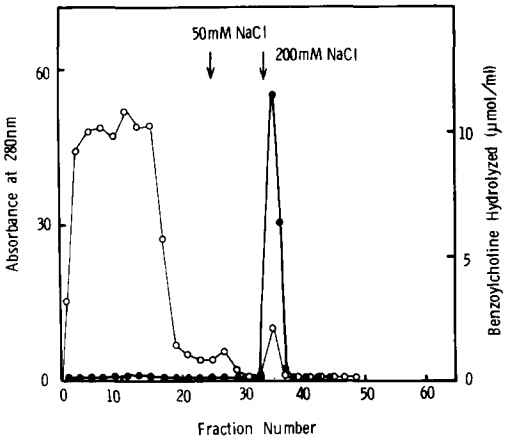


Fig. 1. The first affinity column chromatography of the human serum esterase. Human serum (200 ml) was dialyzed against 10 mM potassium phosphate buffer (pH 8.0) and the dialysate was applied directly to the affinity column (1.2 × 7.5 cm) previously equilibrated with the same buffer. The effluent from the column was then collected in 11.5 ml fraction and the absorbance at 280 nm (○———○) and the pseudocholinesterase activity (●———●) were measured. After washing with 200 ml of the same buffer and 100 ml of 50 mM NaCl in the above buffer, the activity appeared in the eluted fraction with 200 mM NaCl in the above buffer.

Enzymatic properties of the esterase

The effect of pH on the esterase activities was examined with the partially purified esterase obtained by the first affinity column chromatography. The pH optima for benzoylcholine and tributyrin were found to be 8.3 and 8.5, respectively (Fig. 4). The K_m values for benzoylcholine and tributyrin were $0.4 \cdot 10^{-3}$ and $0.62 \cdot 10^{-3}$ M, respectively. The hydrolysis of benzoylcholine was

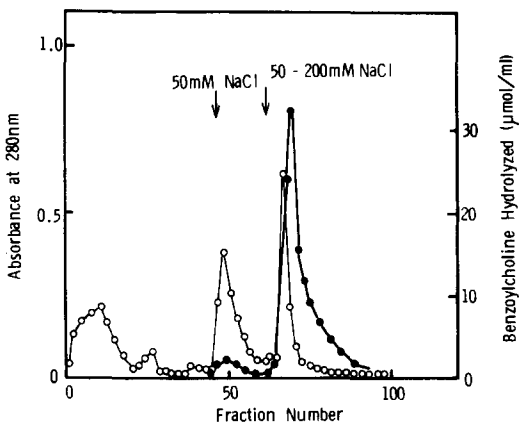


Fig. 2. The second affinity column chromatography of the human serum esterase. Fraction Nos. 35 and 36 chromatographed on the first affinity column were pooled, dialyzed against 10 mM potassium phosphate buffer (pH 8.0) and applied to the affinity column (1.2 × 7.5 cm) equilibrated with the same buffer. After washing 100 ml of the above buffer and 50 mM NaCl in the above buffer and the activity appeared with a linear gradient from 200 ml 50 mM to 200 ml 20 mM NaCl in the above buffer. 6-ml fractions were collected from the column and the absorbance at 280 nm (○———○) pseudocholinesterase activity (●———●) of each of the fractions were measured.

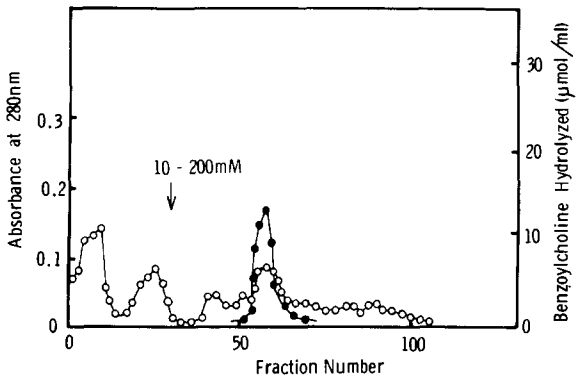


Fig. 3. The ion exchange chromatography of the human serum esterase on a DEAE-Sephadex column. The active fraction in the second affinity column chromatography (fraction Nos. 66—75) were pooled, dialyzed against 10 mM potassium phosphate buffer (pH 8.0) and applied to a DEAE-Sephadex column (1.2 X 16 cm) previously equilibrated with the same buffer. After washing with 200 ml of the same buffer, the activity appeared with a linear gradient from 200 ml of the same buffer to 200 ml of 200 mM potassium phosphate buffer (pH 8.0). 5-ml fractions were collected from the column and the absorbance at 280 nm (○———○) and the pseudocholine esterase activity (●———●) of each of the fractions were measured.

accompanied by substrate inhibition at concentrations greater than 0.6 mM.

It was found from the kinetic analysis that the affinity ligand (*p*-trimethylammoniumanilinium dichloride) inhibited non-competitively the hydrolysis of tributyrin, and competitively that of benzoylcholine.

Comparison with the liver esterase

It was observed that the antiserum against human serum esterase inhibited the activities of both pseudocholinesterase and nonspecific esterase. The control serum did not inhibit these activities. On the other hand, the antiserum did not show any effect on nonspecific esterase activities of human liver,

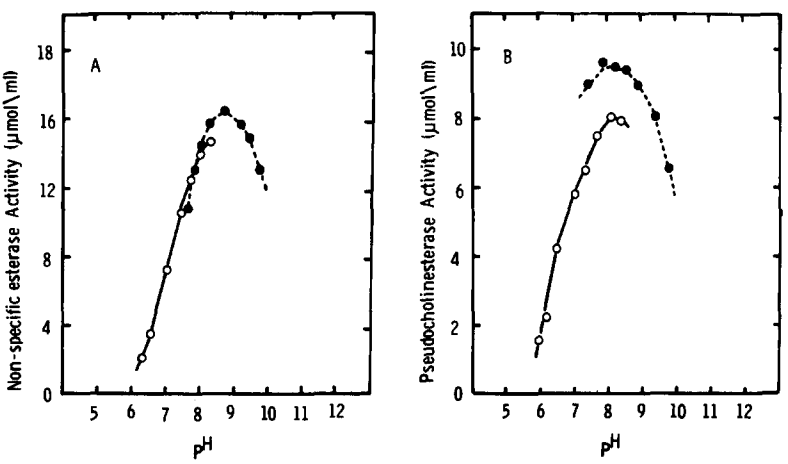


Fig. 4. The effect of pH on the activities of nonspecific esterase (A) and pseudocholinesterase (B). The phosphate (○) and the NaOH/glycine (●) buffer were used for the assays. The ionic strength was 0.1.

TABLE I

PURIFICATION OF PSEUDOCHOLINESTERASE AND NONSPECIFIC ESTERASE FROM HUMAN SERUM

Purification step	Total protein (mg)	Pseudocholinesterase				Nonspecific esterase			
		Total activity (μmol/min)	Yield (%)	Specific activity (μmol/mg per min)	Fold	Total activity (μmol/min)	Yield (%)	Specific activity (μmol/mg per min)	Fold
Serum	23 400	260	100	0.011	1	10.6	100	0.00045	1
First affinity chromatography	64	148	57.2	2.33	212	6.32	59.6	0.099	220
Second affinity chromatography	10.2	70	26.9	6.90	627	3.05	28.8	0.173	664
DEAE-Sephadex column chromatography	0.57	14.6	5.6	30.6	2782	0.67	6.3	1.42	3155

muscle and adipose tissue. In the immunoprecipitation with Ouchterlony's method, the antiserum reacted with human serum esterese, but not with the liver enzyme. In the course of this experiment, it was found that the liver pseudocholinesterase activity was considerably low as compared to the liver nonspecific esterase activity. The affinity ligand did not inhibit the liver non-specific esterase activity and did not adsorb the esterase in this affinity column.

Discussion

The result shown in Fig. 1 indicated that the Sepharose 4B coupled with *p*-trimethylammoniumanilinium dichloride behaved as an affinity absorbent for human serum esterase. The serum esterase was purified with this affinity column and DEAE-Seohadex. This purified enzyme was electrophoretically pure and its specific activity increased to approximately 3000-fold that of the initial enzyme preparation. During the purification procedure the ratios of pseudocholinesterase activities to nonspecific esterase activities were almost identical. In addition to this finding, the purified preparation in the present investigation hydrolyzed both benzoylcholine and tributyrin. These facts suggested that these two enzyme activities might be drived from the same enzyme molecule. However, it was impossible to find any hydrolyzing activity of emulsified triolein in this purified enzyme (unpublished data). At least this purified enzyme alone could not show any so-called lipase activity. Lately, Okuda and Fuji [14] had shown the possible interconversion of rat liver esterase and lipase, and postulated that the protein moieties of these enzymes were immunologically identical. Thus it is very important whether this is the case in human serum. Recently, Lombardo et al. [15] purified the esterase in human pancreatic juice and it was activated by the addition of bile acids. This fact also raises the question of whether the human serum esterase shows lipase activity by the addition of compounds such as bile salt. These questions were now under investigation. Muraoka and Okuda [5] reported that they purified the human esterase with a DEAE-cellulose, hydroxyapatite and Sepharose 4B column and that the specific activity of the final material was approximately 500-fold that of the initial crude enzyme preparation. In contrast to this procedure, the present procedure with the affinity column was simple, efficient and reproducible. The estimation of the molecular weight of this purified enzyme was done with the polyacrylamide gel electrophoresis, and it was about 315 000. This value was similar to that of the previous paper [5] which was estimated by Sepharose 4B gel filtration.

Affinity ligand inhibited competitively the hydrolysis of benzoylcholine and inhibited non-competitively the splitting of tributyrin. This might be explained in the hypothesis that there are two sites in the active center of human serum esterase, that is, catalytic and anionic sites (Fig. 5). And if the affinity ligand binds to the anionic site of the enzyme, and benzoylcholine associates with both the catalytic and anionic ones, then the ligand might inhibit competitively the hydrolysis of the substrate. On the other hand, if tributyrin binds only the catalytic site, then the ligand might inhibit non-competitively the splitting of the substrate.

Experiments are now in progress to clarify this possibility.

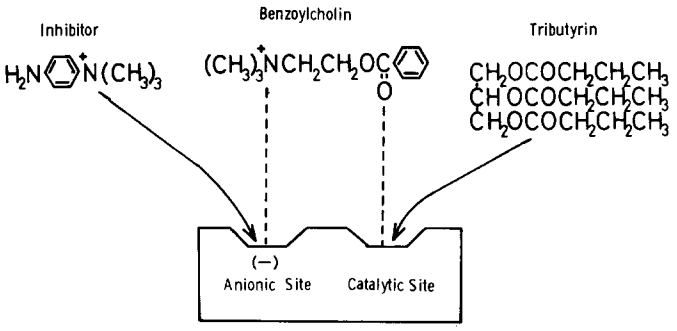


Fig. 5. Diagram of interaction of esterase with benzoylcholine, tributyrin and inhibitor.

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