DIFFERENCES IN REACTIVITY OF FOUR BUTYRYLCHOLINESTERASE ISOZYMES TOWARDS SUBSTRATE AND INHIBITORS

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SUMMARY

Four isozymes of horse serum butyrylcholinesterase have been resolved by means of polyacrylamide gel electrophoresis combined with specific substrate staining and gel-scanning procedures. They differ 5-fold in their Km for butyrylthiocholine and 5 and 7-fold in reactivity with two organophosphates, malaoxon and Tetram.

INTRODUCTION

Multiple forms of serum cholinesterase (BuChE, E.C. 3.1.1.8) from different sources have become of considerable interest and been subject to extensive studies. Heilbronn (1) found that two components of horse serum BuChE purified by electrophoresis and chromatography showed no difference in their reactions with inhibitors and substrates. Later four components from human serum BuChE were fractionated by gel filtration and found to be different from one another in molecular size (2). Reiner et al (3) showed that two horse serum BuChE isozymes were inhibitied at different rates by organophosphates and further supported Lawler's hypothesis (4) that BuChE is an aggregate of similar subunits. LaMotta et al (5) reported that different molecular components were interconvertible in a concentration procedure using ammonium sulfate, suggesting that various forms of this enzyme were aggregates of a common polypeptide subunit. They subsequently demonstrated seven forms of human serum BuChE with different molecular size (6). Saeed et al (7) separated the same serum BuChE into five bands and further demonstrated that the major enzyme

component treated with various proteolytic enzymes could be converted to some other isozymes. By kinetic procedures, Main (8) has resolved several human and horse serum BuChE isozymes which showed different reactivity to certain organophosphates.

We have electrophoretically resolved four forms of horse serum BuChE by means of polyacrylamide gel combined with substrate staining and gel-scanning procedures. We report herein the differential reactivity of various isozymes, measured simultaneously in a single gel, towards the substrate butyrylthiocholine and two organophosphorus inhibitors. The interconvertibility of these isozymes by treating BuChE with trypsin was also investigated.

MATERIAL AND METHOD

Enzyme: Horse serum butyrylcholinesterase (BuChE) from Nutritional Biochemicals was freshly made (4 mg/ml) in 20 mM phosphate buffer at pH 7.0, containing 20% sucrose and a drop of bromphenol blue as a tracking dye. Fifty µl of this enzyme solution was applied to each gel for electrophoresis. The specific activity of this enzyme was 3.3 µmoles per min per mg of enzyme towards 1 mM butyrylcholine hydrolysis at pH 7.0, 25°C, assayed by a pH-stat titrimetric method.

Chemicals: Tetram, the oxalate salt of O,O-diethyl S-(2-diethylamino-ethyl) phosphorothiolate was synthesized in this laboratory by the method of O'Brien and Hilton (9). Malaoxon, (O,O-diethyl S-(1, 2-dicarbethoxy)ethyl phosphorothiolate was a gift from American Cyanamid Company. Butyrylthio-choline chloride (BuTCh) and trypsin were from Nutritional Biochemicals.

Treatment of BuChE with trypsin: Trypsin samples were prepared by dissolving 24 mg per ml of 20 mM phosphate buffer at pH 7.0, while BuChE solution was made 6 mg per ml of the above buffer. A series of 0.1 ml trypsin samples were added to 0.2 ml each of BuChE solution to start digestion. After incubation for various time intervals at 25°C, 0.04 ml of

80% sucrose solution and 0.01 ml of bromphenol blue were added to the trypsin-BuChE mixture. Also 50 µl of trypsin-treated or control BuChE was applied onto each gel for electrophoresis.

A gel-scanning method recently developed in our laboratory (10) was used for kinetic studies on catalysis and inhibition of the resolved isozymes of BuChE. The method was a combined polyacrylamide gel electrophoresis (11), histochemical staining method of Karnovsky and Roots (12) and spectrophotometric scanning of gels. The results from enzyme catalysis were calculated by a computerized version of the linear regression analysis of Wilkinson (13) and plotted by the double-reciprocal method, whereas the kinetic data of inhibition were calculated by a computerized version of the least square analysis according to Aldridge (14).

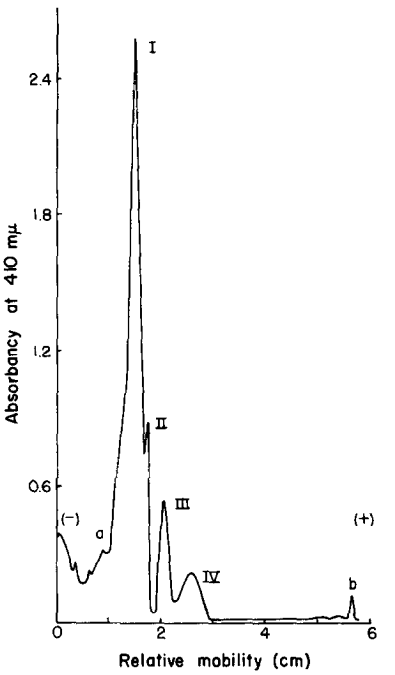

Fig. 1. An electropherogram of BuChE isozymes, showing the absorbancy and relative mobility of 4 isozymes. The gel was incubated with 1 mM BuTCh for 15 min. Enzyme conc., 200 µg/gel; gel scanner slit, 0.2 mm; scanning rate, 1.5 cm/min; a: junction of running and stacking gels. Note 3 minor bands in the stacking gel. b: front of tracking dye.

RESULTS

There were four distinctive bands of isozyme designated with numbers I through IV in the order of increasing anodic mobility in the separating gel, as shown in Fig. 1. Also present were three other minor bands which remained in the stacking gel. Only the four bands moving into the separating gel have been studied. Isozyme I was the predominant isozyme, and contributed at least two-thirds of the total enzyme activity, calculated on the basis of peak height absorbancy of the zymogram. The catalysis of these four isozymes towards BuTCh was directly measured in the gel and is illustrated in Fig. 2. The kinetic parameters (K_m and V_{max}), the % isozyme activity

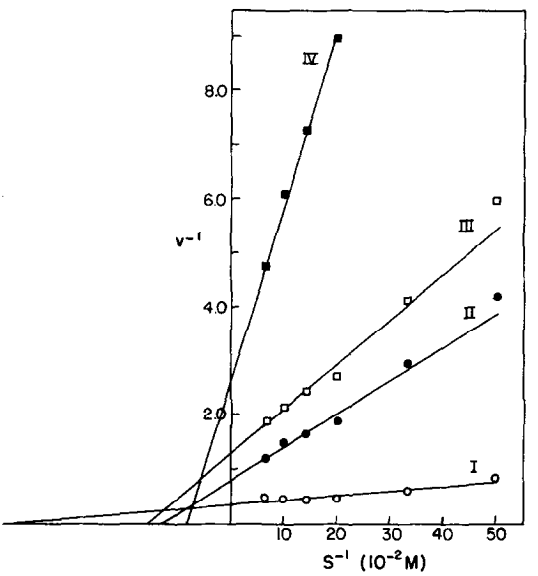

Fig. 2. Double-reciprocal plot of BuTCh for BuChE isozymes by the gel-scanning method. The designation of isozymes (I through IV) was corresponding to those shown in Fig. 1. The experimental conditions were the same as in Fig. 1.

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and the relative mobility (R_m) of each isozyme are summarized in Table 1. The four isozymes displayed a significant difference of up to 5-fold in K_m and of 8-fold in relative V_{max} values. As for inhibition, only three isozymes

Table 1. Michaelis constants (K_m) and V_{max} of BuTCh for BuChE isozymes by gel-scanning method

Isozyme	$K_m \times 10^4$ (M)	V_{max} (arbitrary units)	% Activity[a]	R_m[b]
I	2.46 ± 0.73	2.96 ± 0.29	63	0.25
II	7.62 ± 1.19	1.24 ± 0.10	20	0.30
III	6.21 ± 0.78	0.77 ± 0.04	12	0.36
IV	12.14 ± 1.64	0.38 ± 0.03	5	0.45

[a] Calculated on the basis of the relative absorbance from the stained zymogram with 1×10^{-3} M BuTCh.

[b] R_m is expressed as the relative mobility of each isozyme, calculated by the ratio of isozyme to the tracking dye migration.

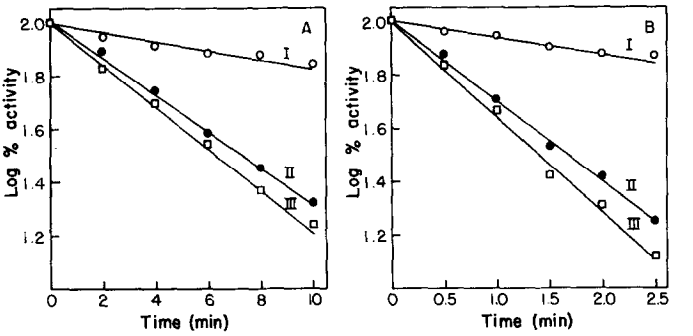

Fig. 3. Log % activity as a function of time for BuChE isozyme inhibition with 2×10^{-4} malaoxon (A) and 5×10^{-6} M Tetram (B), respectively. The residual enzyme activity after inhibition for various times was assayed by 5×10^{-4} M BuTCh with 15 min incubation. The isozyme IV was not studied because of poor resolution.

have been well resolved for both inhibitors used under the present experimental conditions. The time course of inhibition reaction is illustrated in Fig. 3

Table 2. Bimolecular rate constants (k_i) of BuChE isozymes with malaoxon and Tetram

Isozyme	Malaoxon (10^2 M^{-1} min^{-1})	Tetram (10^5 M^{-1} min^{-1})
I	1.64 ± 0.20	0.25 ± 0.02
II	7.90 ± 0.19	1.39 ± 0.04
III	8.80 ± 0.14	1.64 ± 0.06

and the bimolecular rate constants of inhibition of isozymes with malaoxon and Tetram are presented in Table 2. Although isozyme I showed a better affinity for BuTCh (if Km is a measure of affinity in this case), it displayed less reactivity to both inhibitors than the other two isozymes as expressed by the low k_i value.

The proteolytic digestion of BuChE with trypsin led to a 20% loss of activity for the major component, isozyme I, complete disappearance of isozyme II, and 80% loss of activity for both isozymes III and IV in two hrs.

DISCUSSION

Our previous communication has demonstrated that the k_i values for inhibitors of erythrocyte AChE are smaller for the enzyme in the gel than when measured on enzyme in solution (10). The same is true for horse serum BuChE. The latter showed even less sensitivity to both malaoxon and Tetram inhibitors as compared to erythrocyte AChE to the same inhibitors used. Furthermore it is worth noting that isozyme I is less susceptible than isozymes II and III to both malaoxon and Tetram. This result appears to parallel the findings of various investigators with the carbamate, eserine

(5, 7, 15). They all found that the major band of human serum BuChE resolved by both acrylamide and starch gels was less sensitive to eserine inhibition. However, the reverse is true for both housefly head and eel AChE in our recent findings (16).

Main (8) has reported, using kinetic evidence alone, that different isozymes have different reactivity to certain organophosphates. For example, with BuChE isozymes, k_i values differ from one form to the other in a magnitude of up to 1000 fold, with the major component showing the highest k_i value. The present result, however, shows that the major band as resolved by gel electrophoresis is less sensitive than other fast-moving isozymes to inhibition, and the k_i values differ only 5- and 7-fold for malaoxon and Tetram respectively.

The isozyme pattern of horse serum BuChE after trypsin digestion is different from that of human serum enzyme as reported by Saeed et al (7). They found that increasing the incubation time for the major isozyme with proteolytic enzymes led to an increase in the production of other isozymes. However, prolonging the incubation time for horse serum BuChE with trypsin produced no significant increase in other isozymes, when the activity of the major band, isozyme I, gradually decreased. The present results suggest that the major component of this enzyme is not as susceptible as other fast-moving bands to protease digestion, and that there is little evidence for conversion from one isozyme to another.

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