Communications to the Editor

A CHOLINESTERASE INHIBITOR PRODUCED BY *ASPERGILLUS TERREUS*

Sir:

Various types of synthetic organic compounds that are able to inhibit cholinesterase (ChE) have been identified[1], and as a result some compounds have found utility as insecticides and as chemotherapeutic agents. However, we know of only one report concerning the production of ChE inhibitors by microorganisms[2]. Indeed no systematic attempt has been made to examine microorganisms as sources of inhibitors.

Recently, acetyl ChE (AcChE)[3,4] and butyryl ChE (BuChE)[4] were isolated from bacterial cells and the enzymes were characterized. Consequently, we were interested in screening microorganisms for the presence of inhibitors using these easily obtainable enzymes. From a number of cultures examined, *Aspergillus terreus* was selected for further study because it produced a metabolite of low molecular weight inhibitory to AcChE. In this communication, production, purification and biological properties of the AcChE inhibitor from *A. terreus* are described.

AcChE solution containing 6.24 units activity/ml in 0.1 M potassium phosphate buffer, pH 7.4 (one unit was defined as the amount of protein that hydrolyzed 1 μmole acetylthiocholine in 1 minute at 30°C), was prepared from *Pseudomonas aeruginosa* A-16[3]. The screening procedure followed for identification of AcChE inhibitors was as follows: 0.1 ml of AcChE solution, 300 μmoles of potassium phosphate buffer, pH 7.4, and 0.2 ml of the microbial culture filtrate (obtained after 2~3 days cultivation) were mixed in a total volume of 0.6 ml. After preincubation for 30 minutes at 30°C, the activity in 0.1 ml of the mixture was measured on a Shimazu UV-200 spectrophotometer at 412 nm with additions of 1 μmole of 5:5-dithio-bis-2-nitrobenzoate and 0.075 μmole of acetylthiocholine iodide and 300 μmoles of potassium phosphate buffer, pH 8.0[5]. One unit of inhibitor was defined as that amount required to give 50% inhibition of AcChE activity under the above conditions.

Among a number of microorganisms tested, several strains of *A. terreus* produced the desired inhibitory activity in culture filtrates. Since the active substances in the filtrates of the strains appeared identical based on similar chromatographic behavior, the inhibitor (I-6123) produced by *A. terreus* IFO 6123 was selected for further investigation. At present, I-6123 is not completely pure, since an amount of culture broth sufficient for large-scale purification could not be obtained because of the highly variable yield of the inhibitor in fermentations. The organism was transferred to CZAPEK-DOX medium, pH 4.0, containing 0.35% yeast extract and the inoculated medium was cultured aerobically at 28°C for 4~6 days. The culture filtrate containing 3~5 units inhibitor/ml was passed through an ion exchange column containing Amberlite XE-100 (Na^+). The active fraction was eluted with 1 N HCl, adjusted to pH 3.5 with Amberlite IRA-400 (OH^-), and finally lyophilized to yield a brownish powder. The ethanol extract of the powder was evaporated to dryness *in vacuo* and dissolved in *n*-butanol-acetic acid-water (4:1:2). This solution was applied on a cellulose powder column and developed with the same solvent mixture. Further purification was carried out on a Sephadex G-10 column developed with deionized water. The active fractions were then lyophilized. The white powder thus obtained gave 50% inhibition in the anti-AcChE test at about 20 μg/ml. The stability of the broth filtrate was tested over a range of pH values at 70°C and 100°C as shown in Table 1. I-6123 appeared stable at pH 2.5 and

Table 1. Stability of I-6123 in culture filtrate

pH	Activity remaining (%)	
	70°C, 20 min.	100°C, 10 min.
2.5	100	100
4.5	100	90
7.0	8	5
9.0	0	0

The activity was determined following procedure described in the text.

Fig. 1. Kinetics of inhibition by I-6123 in *P. aeruginosa*-AcChE-Acetylthiocholine system (A) and *E. electricus*-AcChE-Acetylthiocholine system (B)

The reaction was carried out with (○) or without (●) I-6123, 1.12 units/ml under the condition described in the text with substrate concentration varied.

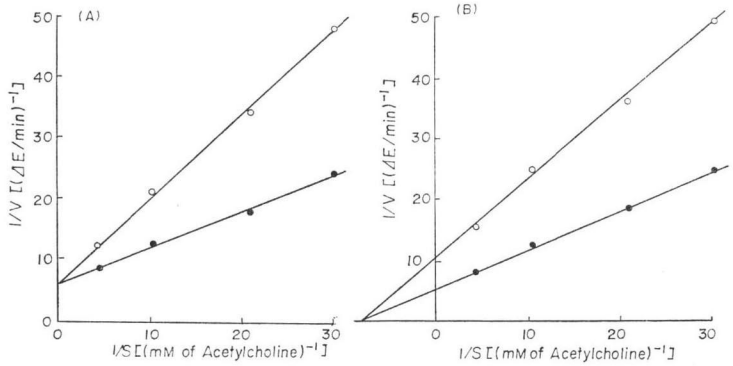

Table 2. Effect of I-6123 on various enzyme systems

Enzyme	Substrate (concentration used)	ID_{50} (units/ml)
P. aeruginosa-AcChE	Acetylthiocholine (3×10^{-4} M)	1.07
E. electrics-AcChE	Acetylthiocholine (3×10^{-4} M)	1.25
Horse serum-ChE	Butyrylthiocholine (3×10^{-4} M)	1.79
Pig liver-esterase	*p*-Nitrophenylacetate (1.32×10^{-4} M)	0.09

The reaction condition was the same as described in Fig. 1 with substrate concentration as noted.

pH 4.5 even at elevated temperatures, but was extremely labile in neutral and alkaline solutions. Even as a dry powder, the potency gradually disappeared in a desiccator at room temperature over several days. Thin-layer chromatography on cellulose powder sheet (Eastman Kodak Co., Ltd.) conducted with I-6123 gave Rf values of 0.55 with *n*-butanol-acetic acid-water (4 : 1 : 2) and 0.85 with methanol-water (9 : 1). A single active spot, weakly positive to ninhydrin, was observed on the chromatogram.

The kinetics of the effects of I-6123 on the hydrolysis of acetylthiocholine by AcChE from *P. aeruginosa* or *Electrophorus electricus* (Boehringer Mannheim Co., Ltd.), and of *p*-nitrophenylacetate by esterase from pig liver (Boehringer Mannheim Co., Ltd.) were studied. Typical results are shown in the LINEWEAVER-BURK plot in Fig. 1. Inhibition of the *P. aeruginosa*-AcChE was competitive, while that of *E. electricus*-AcChE was noncompetitive. Competitive inhibition was also observed in a system containing horse serum-ChE and butyrylthiocholine, and non-competitive inhibition in the system of pig liver-esterase and *p*-nitrophenylacetate. A comparison of the inhibitory effects of I-6123 on the four esterases under conditions where substrate inhibition does not occur was made (Table 2). I-6123 was strongly inhibitory of the non-specific pig-liver esterase and a weakly inhibitory of the others.

KOICHI OGATA
KOZO UEDA*
TORU NAGASAWA
YOSHIKI TANI

Department of Agricultural Chemistry,
Kyoto University, Kyoto, Japan
*Central Research Institute,
Kurare Co., Ltd., Kurashiki, Japan

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