Short Communication

Modified TLC bioautographic method for screening acetylcholinesterase inhibitors from plant extracts

The present work describes modifications to an existing TLC bioautographic method for detecting acetylcholinesterase inhibitors from plant extracts. The basic principle of the method is that the enzyme converts 1-naphthyl acetate into naphthol which reacts with Fast Blue B salt to make a purple-colored background on the TLC plates. Inhibitors of acetylcholinesterases produced white spots on the background. Our modifications involve changes in the concentration of the enzyme, the reagents, and the time of the reaction. With these changes, the consumption of the enzyme was reduced by 85% and the detection limits were decreased remarkably.

Keywords: Acetylcholinesterase inhibitor / Alzheimer’s disease / Bioautography / Huperzine A / TLC

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1 Introduction

Alzheimer’s disease (AD) is more and more prevalent in the older population from worldwide sense. A promising treatment strategy for AD has been the use of acetylcholinesterase inhibitors, whose basis is the cholinergic hypothesis [1]. Some acetylcholinesterase inhibitors have been developed to drugs. However, to date, only tacrine, donepezil, rivastigmine, and galanthamine have been approved by the Food and Drug Administration in the United States [2]. These drugs, except for galanthamine, are all synthetic drugs and have side effects [3]. Therefore, it is desirable to find natural acetylcholinesterase inhibitors with less side effects.

To screen the almost inexhaustible sources of natural products, an effective and fast assay system is needed. Ellman’s method is a classical method in detecting acetylcholinesterase inhibitors [4, 5]. However, the results will be uncertain when testing colored samples or hydrophobic samples. TLC bioautographic method has arisen in recent years [6, 7]. As a simple and rapid method, it has been used to screen and bioassay-guidedly isolate acetylcholinesterase inhibitors from plant extracts. But there are some limitations with the existing method. First, the great consumption of enzyme makes the procedure expensive, which is an obstacle to the scientific research centers lacking enough funds. Second, the detection limits are high which make the traces of inhibitors undiscovered from plant extracts.

To overcome the disadvantages, we modified Marston’s method [7] by reducing the concentration of the enzyme to 1.0 U/mL and changing the operations, the concentration of the substrate and the chromogenic reagent, and the time of the reaction correspondingly. As a result, the consumption of the enzyme was reduced by 85% and the detection limits were also decreased remarkably.

2 Materials and methods

2.1 Chemical reagents

Acetylcholinesterase (EC3.1.1.7, Sigma product No. C2888), Fast Blue B salt, physostigmine, and huperzine A were purchased from Sigma (St. Louis, MO, USA). BSA was from Roche (Shanghai, China) and 1-naphthyl acetate was obtained from Sinopharm Chemical Reagent (Shanghai, China). Silica gel G plates and silica gel (200–300 mesh) were purchased from Qingdao Haiyang Chemical (Qingdao, China), with the thickness between 0.2 and 0.25 mm. Macroporous resin (HPD-100) was obtained from Hebei Cangzhou Chemical (Baoding, China). Other reagents were of analytical grade.

2.2 Plant materials

Salvia miltiorrhiza was purchased from Huanghe Herb Market in Lanzhou, which was identified by Associate Professor Lin Yang, School of Life Science and Engineering, Lanzhou University of Technology, Lanzhou, China.
2.3 Isolation of acetylcholinesterase inhibitor from *S. miltiorrhiza*

Dried root of *S. miltiorrhiza* (5 kg) was powdered and extracted with 95% ethanol (5 L × 2) to obtain ethanol extract (100 g). Ethanol extract were fractionated by macroporous resin column chromatography in various ethanol–water ratios, 0:1, 3:7, 5:5, 7:3, and 1:0, to generate 5 major fractions which were SM-1 (43.56 g), SM-2 (12.32 g), SM-3 (37.39 g), SM-4 (47.97 g), and SM-5 (44.37 g).

2.4 Modified TLC bioautographic assay

Acetylcholinesterase (500 U) was dissolved in 500 mL Tris–hydrochloric acid buffer (0.05 mol/L, pH = 7.8) with 500 mg BSA added to stabilize the enzyme during the bioassay. The solution remains active for 6 months at 4°C. 1-Naphthyl acetate (150 mg) was dissolved in ethanol (40 mL) and then diluted by distilled water (60 mL). Fast Blue B salt (50 mg) was dissolved in distilled water (100 mL). Samples were applied to silica gel TLC plate and migrated by proper solvent. Then the plate was dried absolutely with a hair dryer. The enzyme solution and 1-naphthyl acetate solution were sprayed to TLC plate subsequently. After each solution was sprayed, TLC plate was blown quickly with cold wind from a hair dryer (800 W) until no free liquid flowing on it. The plate could not be dried absolutely otherwise the enzyme could be inactivated. However, ethanol should be removed because it might inhibit acetylcholinesterase activity [5]. The TLC plate was put in a closed vessel containing a little water for humidity whereas plate should not contact with water directly. The plate was kept in this closed vessel at 37°C for 20 min to make the enzyme react with 1-naphthyl acetate completely. Then the solution of Fast Blue B salt was sprayed onto the TLC plate, the inhibited acetylcholinesterase spot would appear white and other parts purple.

2.5 Test false positive result due to inhibition of 1-naphthol reaction with Fast Blue B salt

1-Naphthol (150 mg) was dissolved in ethanol (40 mL) and then diluted by distilled water (60 mL). Fast Blue B salt (50 mg) was dissolved in distilled water (100 mL).

Samples were applied to silica gel TLC plate and migrated by proper solvent. When the plate was dried by a hair dryer, the solution of 1-naphthol and Fast Blue B salt were sprayed to TLC plate subsequently. It indicated false positive result if white spot appeared.

3 Results and discussion

To reduce the consumption of the enzyme in Marston's method, we selected a known acetylcholinesterase inhibitor, huperzine A, as a positive control and did the following experiments: (1) We reduced the concentration of enzyme in Marston's method (6.7 U/mL) to 1.0 U/mL, while keeping other conditions. After spraying mixed solution of chromogenic reagent (Fast Blue B salt) and substrate (1-naphthyl acetate), we observed that the color of TLC plate turned into light purple and disappeared immediately. This may be explained by producing an unstable azo-product that decomposed at once in the chromogenic process. In other words, 1-naphthol produced from hydrolyzing substrate did not react with Fast Blue B salt completely. As we all know, the structure of Fast Blue B salt has two active diazoamino groups that can both react easily with 1-naphthol, but here the molecule number of 1-naphthol is far less than Fast Blue B salt. Therefore, 1-naphthol reacted with one diazoamino group only to produce an unstable azo-product that still had one free diazoamino group. This unstable azo-product could cause the unusual phenomenon above. (2) Accordingly, the concentration of chromogenic reagent was reduced for increasing the relative molecule number of 1-naphthol reacting with Fast Blue B salt. When the final concentration of Fast Blue B salt was reduced to 0.5 mg/mL, the color of the plate in TLC bioautographic assay no longer disappeared. The experiment was successful, but the background of TLC plate still appeared very light purple, which may be caused by only minor azo-dyes being produced in the assay. (3) To solve this problem, we prolonged the reaction time of the enzyme with the substrate and increased the concentration of the substrate (up to 1.5 mg/mL) and obtained a satisfied purple background. After spraying 1.5 mg/mL solution of 1-naphthyl acetate, TLC plate was incubated at 37°C for 20 min and then sprayed the solution of Fast Blue B salt, instead of spraying the mixed solution of Fast Blue B salt and 1-naphthyl acetate immediately.

Two known acetylcholinesterase inhibitors, huperzine A and physostigmine were selected to confirm the effec-
tiveness of this modified method. Both compounds showed antiacetylcholinesterase activity on the TLC plates. In order to establish detection limits for the bioautographic assay, huperzine A and physostigmine were applied at varying amounts onto the TLC plate, and the lowest amount with the observable white spot was determined. Thus, the amount of huperzine A inhibiting the enzyme was down to $1 \times 10^{-10}$ g (Fig. 1), while the least amount of physostigmine required was $1 \times 10^{-5}$ g (Fig. 2) which was far lower than published values [7].

Ethanolic extract of *S. miltiorrhiza* was also analyzed since the plant was reported to contain acetylcholinesterase inhibitors [8]. Two white spots were observed on TLC plate (Fig. 3). Fraction SM-5 was submitted to chromatography on a silica gel column chromatography (petroleum ether–acetone, 20:1 v/v) repeatedly by a bioactivity-guided fractionation approach to obtain reported acetylcholinesterase inhibitor: dihydrotanshinone I (80 mg).

While proceeding modified TLC bioautographic assay, a key point must be concerned. The TLC plate should not be dried thoroughly with hot wind from a hair dryer after spraying enzyme and substrate. Otherwise the experiment might fail, because high temperature could make the enzyme lose activity.

However, there were two weak points when 1-naphthol and Fast Blue B salt were employed as reagents. The first was that a false positive result might appear due to some compounds inhibiting the 1-naphthol reaction with Fast Blue B salt to produce white spot, which could be tested by a control experiment (Section 2.5). The second was phenolic compounds in extracts would react with Fast Blue B salt to produce colored spot that was an obstacle to observe white spot. When the problem encountered, we proposed other methods to detect anti-acetylcholinesterase activity such as Ellman’s method [4].

Attempts to further modify TLC bioautographic assay by reducing the concentration of enzyme to 0.5 U/mL and changing the according condition of experiment were unsuccessful.

### 4 Concluding remarks

The TLC bioautographic method for screening acetylcholinesterase inhibitors was modified by changing its operations, the concentration of the substrate, the chromogenic reagent, and the time of the reaction. The expense of the assay was reduced remarkably and sensitivity was improved simultaneously, which was benefit to research groups pursuing for natural acetylcholinesterase inhibitors.

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### 5 References