Appl Biochem Biotechnol (2011) 165:69–74
DOI 10.1007/s12010-011-9233-z

Reversible Inhibition of Esterase Activity After Separation and Immobilization

Takahiro Sakikawa · Youji Shimazaki

Received: 7 September 2010 / Accepted: 23 March 2011 /
Published online: 7 April 2011

Abstract An inhibitor, 9-amino-1,2,3,4-tetra hydroacridine (tacrine), is a reversible inhibitor of esterases. The reversible inhibition of the enzyme activity is thought to be examined after separation and immobilization of the enzyme under non-denaturing conditions. Hydrolytic changes of phosphatidylcholine by carboxylesterase were obtained using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry after the esterase was separated by non-denaturing two-dimensional electrophoresis, was immobilized to membranes and was stained by Ponceau S. The changes were inhibited after the enzyme on the membrane was treated by tacrine. Furthermore, the hydrolytic activity of the esterase was recovered after the inhibitor was washed with aspartic acid solution. These results indicate that the phosphatidylcholine hydrolysis activity of the isolated and immobilized enzyme is reversibly inhibited under non-denaturing conditions. Furthermore, this method can be developed to the production of an enzyme reactor able to regulate amounts of lipids.

Keywords Electrophoresis · Ponceau S · MALDI-TOF MS · Tacrine · Phosphatidylcholine

Abbreviations

2-DE	Two-dimensional electrophoresis
PVDF	Polyvinylidene fluoride
Tacrine	9-Amino-1,2,3,4-tetra hydroacridine
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
CBB	Coomassie brilliant blue

T. Sakikawa · Y. Shimazaki (✉)
Graduate School of Science and Engineering (Science Section), Ehime University,
Matsuyama 790-8577, Japan
e-mail: shimazaki.yoji.my@ehime-u.ac.jp

T. Sakikawa · Y. Shimazaki
Venture Business Laboratory, Ehime University, Matsuyama 790-8577, Japan

Introduction

It has been reported that tetrahydroaminoacridine (tacrine) is a reversible inhibitor of esterases [1–3]. Non-specific esterase activity is observed in human and other mammalian tissues including mouse liver [4]. However, there are no evidences that esterases obtained from mouse liver are inhibited by tacrine. To examine whether inhibitors are removed from enzymes or they are transformed on enzymes, it is preferable that targeted enzymes are separated and immobilized under native conditions. We have reported that a spot at p*I* 5.5/120,000 is identified as carboxylesterase 1 after proteins in mouse liver cytosol are separated by non-denaturing two-dimensional electrophoresis (2-DE), and the esterase is immobilized onto membranes [5]. Furthermore, hydrolytic changes of phosphatidylcholine by the esterase are analyzed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) after the enzyme immobilization [6]. The esterase activity is thought to be inhibited by the tacrine, and the tacrine is thought to be removed by the addition of native chemicals such as aspartic acid.

Here, we describe that phosphatidylcholine hydrolysis activity of the isolated and immobilized enzyme is reversibly inhibited under non-denaturing conditions. The method can be developed to the production of an enzyme reactor able to regulate amounts of lipids.

Materials and Methods

Sample Preparation, Non-Denaturing 2-DE, Electrotransfer to Membranes, Ponceau S Staining, and Esterase Activity Staining

Mouse livers (Swiss Webster) were purchased from Rockland Inc. (MA) and homogenized in 100 mM Tris–HCl buffer at pH 7.2. The homogenate was centrifuged for 5 min at 10,000×*g* to obtain the cytosolic fraction. Sucrose was added to the liver cytosolic fraction at a concentration of 40%. Proteins in the cytosolic fraction (100–300 μg) were subjected to microscale non-denaturing 2-DE by a method previously reported [7]. First dimensional isoelectric focusing (IEF) was performed on rod gels (35×1.3 mm ID) containing 4% acrylamide, 0.2% bisacrylamide, 3% pharmalyte pH 3–10, 0.05% ammonium persulphate, and 0.029% N,N,N′,N′-tetramethylenediamine. The electrode solutions comprised 0.04 M NaOH (cathode) and 0.01 M H_3PO_4 (anode). After IEF, the gel was placed on top of the second-dimension slab gel, which was then run on a 4–17% acrylamide linear gradient (0.2–0.85% bisacrylamide gradient). The electrode buffer comprised 0.05 M 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) and 0.38 M glycine (pH 8.3). For Coomassie brilliant blue (CBB) staining, the gels were stained with 0.1% CBB, 7% acetic acid, and 50% methanol for 15 min and destained in 20% methanol and 7% acetic acid for 2 h. In order to immobilize proteins on polyvinylidene fluoride (PVDF) membranes, proteins were transferred to a PVDF membrane by a semi-dry type transblotting apparatus using electrode buffers of 0.05 M Tris and 0.38 M glycine (pH 8.3) with a constant current of 23 mA per gel for 4 h. For detection of proteins on the PVDF membrane, the membrane was soaked in 0.5% Ponceau S in 10 mL of 0.1 M acetate buffer (pH 5.1). For analysis of esterase activity, PVDF membranes were incubated in 10 mL of 0.2 M phosphate buffer (pH 7.1), containing 0.2 mL of 1% α-naphthylacetate and 4 mg of 4-chloro-2-methylbenzene diazonium salt (Fast Red TR salt). For inhibition of esterase activity on the PVDF membrane, membrane was incubated in a solution of 20 mM tacrine for 2 h. To remove tacrine from esterase on the membrane, the membrane was washed with 5 mL of 10 mM aspartic acid for 10 s.

Hydrolytic Changes of Phosphatidylcholine by MALDI-TOF MS Analysis

To analyze the hydrolysis of phosphatidylcholine by MALDI-TOF MS, carboxylesterase on the membrane was incubated with 15 μL of 1.9 mg mL^{-1} phosphatidylcholine for 1 h at 37 °C. For inhibition of the esterase activity, the esterase on membrane was incubated in a solution of 1.5 mM tacrine for 15 min. To remove tacrine from esterase, the esterase on the membrane was washed with 400 μL of 10 mM aspartic acid for 3 min. After washing, esterase on the membrane was incubated with 15 μL of 1.9 mg mL phosphatidylcholine for 1 h at 37 °C. The hydrolytic changes of phosphatidylcholine by the esterase on membrane were examined after incubation. After incubation, all liquid was collected. Then, 1 μL of the liquid was mixed with 1 μL of a solution containing saturated α-cyano 4-hydroxycinnamic acid, 0.1% trifluoroacetic acid, and 70% acetonitrile. The sample mixture and matrix were put onto a stainless steel sample plate (sample plate for Voyager DE PRO; Applied Biosystems, Framingham, MA, USA) and dried. Mass analysis was performed using MALDI-TOF MS (Voyager DE PRO; Applied Biosystems) in positive ion reflector mode.

Results and Discussion

Separation and Immobilization of Esterase Under Non-Denaturing Condition

Figure 1 shows CBB staining of cytosolic proteins in mouse liver that were separated by non-denaturing 2-DE (a), Ponceau S staining (b), and esterase activity staining (c) after cytosolic proteins in mouse liver were separated by non-denaturing 2-DE and were electroblotted onto membranes. Esterase activity was retained even after the esterase was separated, immobilized, and stained by Ponceau S in 0.1 M acetate buffer (pH 5.1). The activity was also retained after staining in 0.1 M acetate buffer (pH 4.8), whereas it was lost after staining in 0.1 M acetate buffer (pH 2.7). We have reported that malate dehydrogenase is also retained even after staining by Ponceau S in 0.1 M acetate buffer (pH 5.1) [8]. Therefore, many enzyme activities are thought to be retained even after treatment with 0.1 M acetate buffer (pH 5.1). Figure 2 shows changes of esterase activity on the

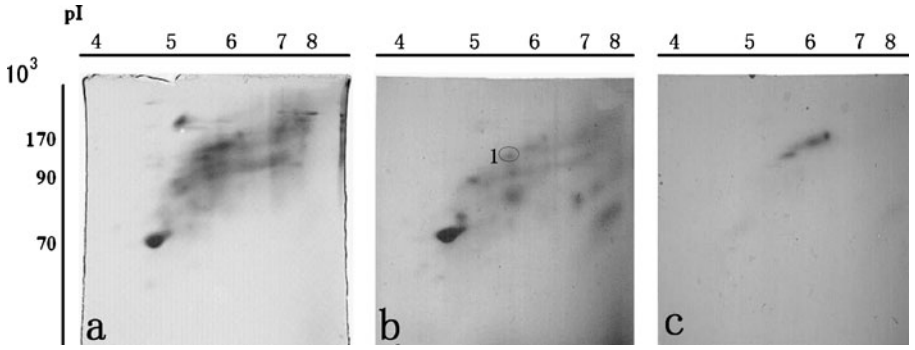

Fig. 1 CBB staining of cytosolic proteins from mouse liver after separation by non-denaturing 2-DE (**a**), Ponceau S staining in 0.1 M acetate buffer solution (pH 5.1) (**b**) and esterase activity staining following Ponceau S staining (**c**) after separation by non-denaturing 2-DE and electroblotting onto membrane. The excised spot is indicated as spot *1* (**b**)

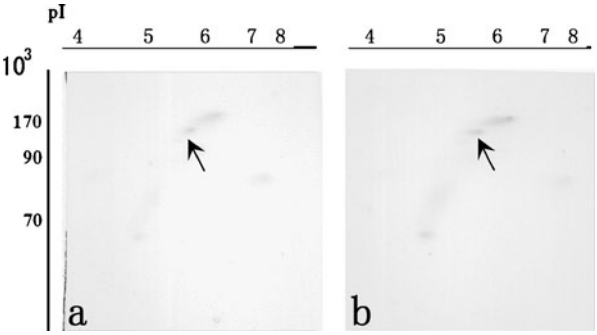

Fig. 2 Esterase activity staining when the membrane was treated by tacrine (**a**), and washed by aspartic acid solution following treatment by tacrine (**b**) after separation by non-denaturing 2-DE and electroblotting onto membrane. Enzymatic activities of carboxylesterase 1 are indicated as *arrows*

electroblotted membrane when the membrane was treated by the inhibitor, tacrine (a), and washed by aspartic acid solution following treatment by tacrine (b) after separation by non-denaturing 2-DE and electroblotting onto membrane. The esterase activity was inhibited by treatment of tacrine, and the activity was recovered after the removal of tacrine by rinsing with aspartic acid solution for 10 s. Further, esterase activity was obtained within several seconds using α-naphthylacetate and Fast Red TR salt. Since the recovery of the enzyme activity is instantaneous after the inhibitor is washed within several seconds, the inhibition is thought to be a reversible inhibition.

Reversible Inhibition of Lipid Hydrolysis by Esterase on Membrane

Figure 3 shows MALDI-TOF MS spectra of phosphatidylcholine and lysophosphatidylcholine when phosphatidylcholine was incubated with spots 1 (Fig. 1b) for 1 h at 37 °C after the proteins were separated, electrotransferred to the membrane, and stained. Since it has been reported that the peaks of phosphatidylcholine and lysophosphatidylcholine are examined by MALDI-TOF MS, the peaks obtained in the present study are compared with evidences in the report [9]. The main peak (m/z=761) corresponds to phosphatidylcholine ($C_{16:0}/C_{18:1}$) in Fig. 3. The peaks of m/z=496 and 518 correspond to the H^+ and Na^+ adducts of lysophosphatidylcholine ($C_{16:0}$), respectively, as shown in Fig. 3b. The peaks of m/z=496 and 518 were not obtained when the esterase on the membrane was treated with 1.5 mM of tacrine, as shown in Fig. 3c. The peaks of m/z=496 and 518 were obtained after esterase on the membrane was washed with aspartic acid solution for removal of the inhibitor (Fig. 3d). The hydrolysis of phosphatidylcholine by esterase was inhibited by 1.5 mM of tacrine, whereas it was not inhibited by 15 μM of tacrine (data not shown). It has been reported that human carboxylesterase is inhibited by more than 100 μM of tacrine [2]. Therefore, for the inhibition of carboxylesterase, it is believed that a large amount of tacrine is needed. Figure 4 shows the relative amounts of lysophosphatidylcholine (m/z= 496) when pure phosphatidylcholine was applied to the esterase on membrane (b); the inhibitor, tacrine, was treated with esterase before pure phosphatidylcholine was applied to esterase on membrane (c), and tacrine was removed by aspartic acid solution before pure phosphatidylcholine was applied to esterase on membrane (d). Lysophosphatidylcholine is synthesized from phosphatidylcholine by the esterase on the membrane even after staining by Ponceau S (Figs. 3b and 4b), and the synthesis is inhibited by the inhibitor, tacrine (Figs. 3c and 4c). The synthetic reaction is clearly recovered after the inhibitor is washed from the esterase on the membrane by aspartic acid solution (Figs. 3d and 4d). We have reported that the excised spot at p*I* 5.5/120,000 contains mouse carboxylesterase 1 [5]. Therefore, the results indicate that tacrine inhibits carboxylesterase activity, and the

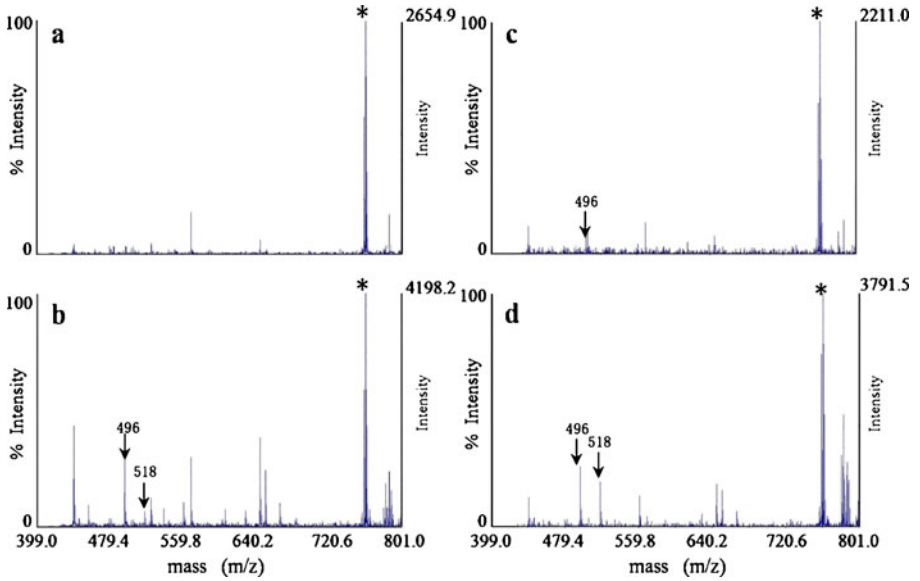

Fig. 3 MALDI-TOF MS spectra of phosphatidylcholine and lysophosphatidylcholine before (**a**) and after phosphatidylcholine was incubated with esterase on spot *1* (Fig. 1b) for 1 h at 37 °C (**b**, **c**, and **d**). Before incubation of phosphatidylcholine with esterase, the esterase on spot *1* was treated by tacrine for 15 min (**c**), and washed by aspartic acid solution after treatment by tacrine (**d**). Peaks of phosphatidylcholine and lysophosphatidylcholine are indicated by *asterisks* and *arrows*, respectively

inhibition is reversible. It has been reported that four or five tacrine molecules are bound within the catalytic pocket of human carboxylesterase 1 monomer, and its activity is inhibited [2]. After washing by aspartic acid solution, the activity of carboxylesterase on the membrane is recovered. The explanation of this is that the tacrine molecules are thought to be removed from the catalytic pocket of the enzyme by washing with aspartic acid solution. When the enzyme is immobilized on membranes, it is easy to wash out inhibitors from the

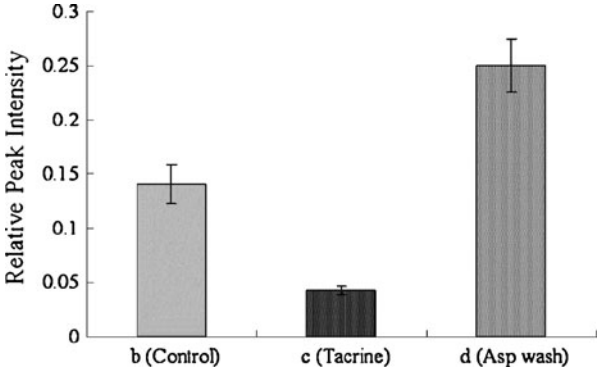

Fig. 4 The relative amounts of lysophosphatidylcholine (m/z=496) when pure phosphatidylcholine was applied to esterase on membrane (**b**), tacrine, was treated with esterase before pure phosphatidylcholine was applied to esterase on membrane (**c**) and tacrine was removed by aspartic acid solution before pure phosphatidylcholine was applied to esterase on membrane (**d**). Each datum indicates mean±standard error obtained from more than 19 individual measurements

immobilized enzyme. It has been reported that esterase activity in the esterase reactors is examined after immobilization of esterases [10, 11]. We present that the esterase activity on the membrane is reversibly inhibited and recovered, and this method can be developed to the production of an esterase reactor able to regulate the amounts of lipids. In Fig. 4, the relative amount of lysophosphatidylcholine with the restored enzyme (d) is more than that with the original enzyme (b). The explanation of this is that not only tacrine, but also Ponceau S can be completely removed by washing with aspartic acid solution, and the enzyme activity can increase. However, further investigation is necessary to solve this problem.

Conclusion

Phosphatidylcholine hydrolysis by immobilized mouse liver carboxylesterase is reversibly inhibited by tacrine. This method can be applied to the production of enzyme reactors able to regulate amounts of lipids.

References

1. Marquis, J. K. (1990). *Biochemical Pharmacology, 40*, 1071–1076.
2. Bencharit, S., Morton, C. L., Hyatt, J. L., Kuhn, P., Danks, M. K., Potter, P. M., et al. (2003). *Chemistry & Biology, 10*, 341–349.
3. Smith, C. P., Bores, G. M., Petko, W., Li, M., Selk, D. E., Rush, D. K., et al. (1997). *Journal of Phamacology and Experimental Therapeutics, 280*, 710–720.
4. Lund-Pero, M., Jeppson, B., Arneklo-Nobin, B., Sjorgen, H. O., Holmgren, K., & Pero, R. W. (1994). *Clinica Chimica Acta, 224*, 9–20.
5. Shimazaki, Y., & Watanabe, S. (2008). *Clinica Chimica Acta, 390*, 145–147.
6. Shimazaki, Y., & Kuroda, T. (2009). *Biotechnology Letters, 31*, 1545–1549.
7. Shimazaki, Y., Sugawara, Y., Ohtsuka, Y., & Manabe, T. (2003). *Proteomics, 3*, 2002–2007.
8. Shimazaki, Y., & Sakikawa, T. (2010). *Protein and Peptide Letters, 17*, 1048–1052.
9. Zschornig, O., Pietsch, M., Suß, R., Schiller, J., & Gutschow, M. (2005). *Journal of Lipid Research, 46*, 803–811.
10. Sathishkumar, M., Jayabalan, R., Mun, S. P., & Yun, S. E. (2010). *Bioresource Technology, 101*, 7834–7840.
11. He, P., Davies, J., Greenway, G., & Haswell, S. J. (2010). *Analytica Chimica Acta, 659*, 9–14.