

Mei-Hsien Lee<sup>1</sup>  
Mao-Te Chuang<sup>2</sup>  
Wen-Chi Hou<sup>1</sup>

<sup>1</sup>Graduate Institute of  
Pharmacognosy Science,  
Taipei Medical University,  
Taipei, Taiwan, R.O.C.

<sup>2</sup>St. Martin De Porres Hospital,  
Chiayi, Taiwan, R.O.C.

## Activity staining of plasma amine oxidase after polyacrylamide gel electrophoresis and its application to natural inhibitor screening

Plasma amine oxidase (plasma AO, EC 1.4.3.6) is a copper-containing AO which converts benzylamine (BZ) to benzaldehyde, generating hydrogen peroxide and ammonia. The peroxidase was used as an ancillary enzyme to couple hydrogen peroxide to 3-amino-9-ethylcarbazole (AEC) to achieve plasma AO activity after electrophoresis on native polyacrylamide gels. It was confirmed that plasma AO is inhibited by semicarbazide but neither by clorgyline nor by deprenyl. We also used plasma AO activity staining for the screening of natural inhibitors. This fast and sensitive method can be used in the process of plasma AO purification, characterization, and inhibitor screening.

**Keywords:** Activity staining / Amine oxidase / Native polyacrylamide gel electrophoresis / Screening  
EL 5045

### 1 Introduction

Amine oxidases (AOs) have traditionally been divided into two main groups, based on the chemical nature of the attached cofactor [1]. One is the flavin adenine dinucleotide (FAD)-containing enzymes (monoamine oxidase A (MAO-A), MAO-B and polyamine oxidase) [1, 2]. MAO-A and -B are well-known mitochondrial enzymes that have firmly established roles in the metabolism of neurotransmitters (noradrenaline) [2]. The other contains a cofactor possessing one or more carbonyl groups (diamine oxidase, lysyl oxidase or semicarbazide-sensitive AO (SSAO)) [3–5]. SSAO (EC 1.4.3.6) is a common name for a group of heterogeneous enzymes widely distributed in nature, including plants, microorganisms, and organs of mammals (vasculature, dental pulp, eye and plasma) [6]. SSAO converts primary amines into the corresponding aldehydes, generating hydrogen peroxide and ammonia. Benzylamine (BZ) appears to be a good substrate for all SSAOs and MAO-B [5], and a variety of other amines (e.g., serotonin, tyramine, tryptamine, polyamine, dopamine) have been reported to be substrates for some, but not all, SSAOs. The endogenous compounds aminoacetone and methylamine are good substrates for most SSAOs [1, 7–8]. In recent researches, it was found that plasma SSAO is raised in diabetes mellitus and heart failure and is implicated in atherosclerosis, endothelial damages and glucose transport in adipocytes [9–14].

**Correspondence:** Prof. Wen-Chi Hou, Graduate Institute of Pharmacognosy Science, Taipei Medical University, No. 250, Wu-Hsien Street, Taipei 110, Taiwan, R.O.C.

**E-mail:** wchou@tmu.edu.tw

**Fax:** +886-2-2378-0134

**Abbreviations:** AEC, 3-amino-9-ethylcarbazole; AO, amine oxidase; BZ, benzylamine; SSAO, semicarbazide-sensitive AO

Several spectrophotometric methods are used to measure the different types of AO activities based on the use of specific inhibitors. MAO-A is inhibited by clorgyline and MAO-B is inhibited by deprenyl or pargyline [2]. SSAO is inhibited by semicarbazide [1], hydroxylamine or other hydrazide derivatives [5]. Holt *et al.* [15] used *p*-tyramine as a substrate to measure MAO-A activity on liver homogenates following inhibition of MAO-B with pargyline (500 nM), and to measure MAO-B activity following inhibition of MAO-A with clorgyline (500 nM). The end product, hydrogen peroxide, was further metabolized by peroxidase and coupled with 4-aminoantipyrine to detect MAO activities. Lizcano *et al.* [16] used methylamine as a substrate to measure SSAO activity. The end product, formaldehyde, was further metabolized by formaldehyde dehydrogenase and coupled with NAD<sup>+</sup> to detect SSAO activities by the increase of the absorbance at 340 nm.

Some reports concern AO activity staining on polyacrylamide gel. Falk [17] used nitroblue tetrazolium and phenazine methosulfate as staining agents for pig plasma AO. Paz *et al.* [18] used a redox cycling staining method (nitroblue tetrazolium/glycinate assay at pH 10.0) for quinoprotein. Lizcano *et al.* [19] adopted the method from Paz *et al.* [18] for SSAO from bovine lung. However, the Paz *et al.* [18] method was suitable only for quinoproteins (such as SSAO). This method was not suitable for MAO-A and MAO-B and polyamine oxidase which contained the cofactor of FAD instead of topaquinone. However, all of the different types of AO produced hydrogen peroxide after catalysis. Therefore, the common staining method for hydrogen peroxide could achieve AO activity staining. In this research, we used BZ as substrate for commercial plasma AO activity staining. The end product, hydrogen peroxide, was further metabolized by horseradish peroxidase and coupled with 3-amino-9-ethylcarbazole (AEC)

to detect plasma AO activities after electrophoresis on native polyacrylamide gels. AEC was cheaper than that of nitroblue tetrazolium or phenazine methosulfate. We also used this method for plasma AO natural inhibitor screenings from *Melastoma candidum* D. Don and the hooks of *Uncaria rhynchophylla* (Miq.) Jack. Both of them were medicinal plants used in China. This activity staining method can be used for the characterization of different types of AO, purity determinations, and natural inhibitor screenings.

## 2 Materials and methods

### 2.1 Materials

Commercial plasma AO from bovine plasma (0.1 unit, 2.8 units/g solid, M-9643), horseradish peroxidase (148 units/mg solid, type I, P-8125), AEC, BZ, clorgyline, deprenyl, semicarbazide, *N,N*-dimethylformamide were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Electrophoresis-grade acrylamide and Bis, TEMED and APS were obtained from E. Merck (Darmstadt, Germany).

### 2.2 Polyacrylamide gel electrophoresis of plasma AO

Native gel electrophoresis on 10% acrylamide was performed according to Davis [20]. The vertical mini Protean 3 system (Bio-Rad Hercules, CA, USA) with 0.75 mm thickness was used. A 75  $\mu$ L aliquot of commercial plasma AO was mixed with 25  $\mu$ L of 60 mM Tris buffer (pH 6.8) containing 14.4 mM 2-mercaptoethanol, 25% glycerol, and 0.1% bromophenol blue.

### 2.3 Activity staining of plasma AO on native PAGE gels

When native PAGE was finished, the gels were equilibrated twice for 20 min in 50 mM phosphate buffer (pH 7.5) before activity staining. Twenty mg BZ and 10 mg AEC were dissolved in 3 mL dimethylformamide and then added to 50 mL of 50 mM phosphate buffer (pH 7.5) as the substrate solution, in which the gel was submerged and shaken for 5 min. Then, 200  $\mu$ L horseradish peroxidase (5 mg/mL) was added. The gel was gently shaken in darkness at room temperature for 20–60 min. The gel was then destained with 10% acetic acid and washed with distilled water.

### 2.4 Screening of natural inhibitors of plasma AO on native PAGE gels

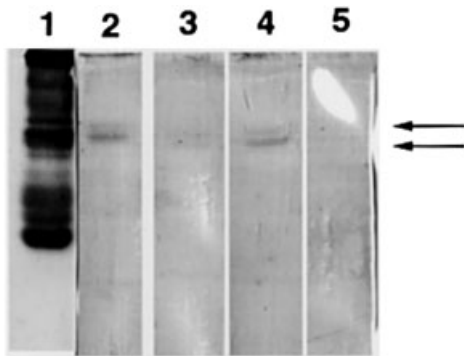
Several pure flavonoids (isoquercetin, quercetin, rutin) from *Melastoma candidum* D. Don were isolated from 70% acetone crude extracts according to the method of Lee *et al.* [21]. The hooks of *Uncaria rhynchophylla* (Miq.) Jack. were cut into pieces and extracted with two volumes of 50% ethanol at 50°C for 6 h. The extract was concentrated under reduced pressure, freeze-dried and stored in a closed container until use. Each pure compound (10 mg/mL) and crude extracts (50 mg/mL) of *Melastoma candidum* D. Don and hooks of *Uncaria rhynchophylla* (Miq.) Jack. were dissolved in DMSO. Ten  $\mu$ L natural products were mixed with 224  $\mu$ U plasma AO at 50 mM phosphate buffer (pH 7.5) overnight and then electrophoresis on native PAGE for activity staining was performed according to the methods described above.

## 3 Results and discussion

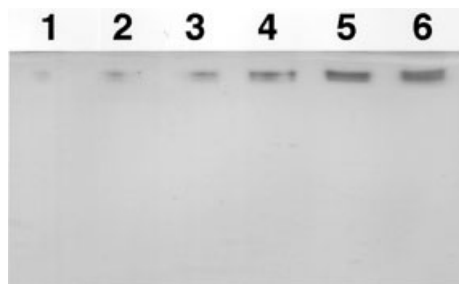
The problems of aging-related diseases, such as neurodegenerative diseases (*e.g.*, Alzheimer, Parkinson, and Huntington diseases), have been emphasized recently. The intricate causes of the aging process are still a matter of extensive speculation giving rise to many theories; in particular, the role of the reactive oxygen species is a prerequisite nowadays for understanding this process [22–25]. Another prominent feature accompanying aging, an increase in catecholamine metabolism, has also attracted attention, and AO, a key enzyme in this process, has been studied extensively.

The idea of the present method for plasma AO activity staining on gels came from Shimoni [26] using 3-amino-9-ethylcarbazole (AEC) to detect peroxidase activity. For plasma AO activity staining, the peroxidase could be used as an ancillary enzyme to further metabolize the hydrogen peroxide by coupling AEC after electrophoresis on native polyacrylamide gels. It was necessary to check the suitable pH conditions for plasma AO and horseradish peroxidase in this activity staining method. Figure 1 shows plasma AO activity staining on native PAGE gels at pH 7.5, 7.0, 6.5, and 6.0 (lanes 2 to 5) comparison with its protein staining (lane 1). It was clear that plasma AO exhibits highest activity at pH 7.5. This activity staining method demonstrated a low purity for commercial plasma AO preparations.

Figure 2 shows the sensitivity of the activity staining method of commercial plasma AO at pH 7.5 on a native PAGE gel. Lanes 1 to 6 correspond to loads of 1.6, 4.8, 8, 16, 24, and 32  $\mu$ U plasma AO, respectively. It was found



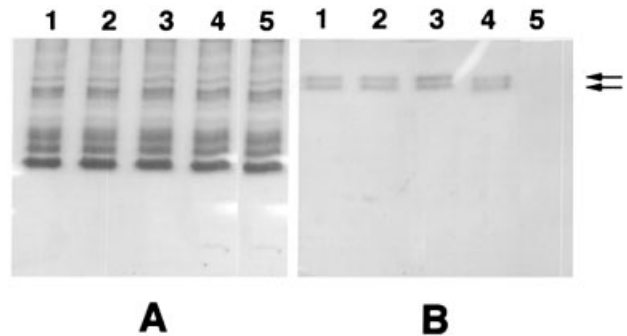
**Figure 1.** Protein staining (lane 1) and activity staining of commercial plasma amine oxidase at pH 7.5 (lane 2), 7.0 (lane 3), 6.5 (lane 4) and 6.0 (lane 5), respectively, from bovine plasma in 10% polyacrylamide gels after native PAGE. Protein loading: for protein staining 7.27  $\mu$ g protein, for activity staining 24  $\mu$ U.



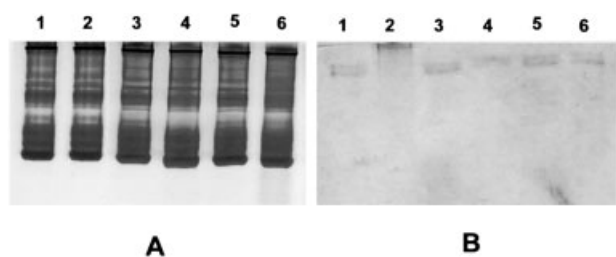
**Figure 2.** The sensitivity of plasma amine oxidase activity staining at pH 7.5 on a 10% native PAGE gel. Lanes 1–6, 1.6, 4.8, 8, 16, 24, and 32  $\mu$ U plasma amine oxidase.

that the sensitivity of this activity staining method was 24  $\mu$ U (lane 5). Figure 3 shows the protein staining (A) and activity staining at pH 7.5 (B) of commercial plasma AO on 10% native PAGE after treatment with different inhibitors at 4°C overnight. Lane 1, plasma AO as controls; lane 2, plasma AO plus clorgyline (1 mM); lane 3, plasma AO plus deprenyl (1 mM); lane 4, plasma AO plus clorgyline (1 mM) and deprenyl (1 mM); lane 5, plasma AO plus semicarbazide (1 mM). Compared with protein staining (Fig. 3A), plasma AO treated with semicarbazide (lane 5, Fig. 3B) lost its activity. Therefore, it was concluded that commercial plasma AO belonged SSAO using BZ as substrates.

The plasma AO activity staining method was used for screening its natural inhibitors from medicinal plants. We used pure flavonoids [21] and crude extracts (50 mg/mL) of *Melastoma candidum* D. Don and of hooks of *Uncaria rhynchophylla* (Miq.) Jack as testing materials for SSAO inhibitory activity screenings. Figure 4 shows protein staining (A) and the inhibitory activity staining (B) on plasma AO by different natural compound treatments.



**Figure 3.** (A) Protein staining and (B) activity staining at pH 7.5 of commercial plasma amine oxidase (224  $\mu$ U) on 10% native PAGE under different inhibitor treatments at 4°C overnight. Lane 1, plasma AO as controls; 2, plasma AO plus clorgyline (1 mM); 3, plasma AO plus deprenyl (1 mM); 4, plasma AO plus clorgyline (1 mM) and deprenyl (1 mM); lane 5, plasma AO plus semicarbazide (1 mM).



**Figure 4.** (A) Protein staining and (B) inhibitory activity staining of plasma amine oxidase (224  $\mu$ U) on 10% native PAGE under different natural compound treatments. Lane 1, plasma AO as controls; 2, plasma AO plus crude extracts of *Melastoma candidum* D. Don (50 mg/mL); 3, plasma AO plus isoquercitrin (10 mg/mL); 4, plasma AO plus quercetin (10 mg/mL); 5, plasma AO plus rutin (10 mg/mL); 6, plasma AO plus crude extracts of hooks of *Uncaria rhynchophylla* (Miq.) Jack (50 mg/mL).

Lane 1, plasma AO as controls; lane 2, plasma AO plus crude extracts of *Melastoma candidum* D. Don; lane 3, plasma AO plus isoquercitrin; lane 4, plasma AO plus quercetin; lane 5, plasma AO plus rutin; lane 6, plasma AO plus crude extracts of hooks of *Uncaria rhynchophylla* (Miq.) Jack. From Fig. 4A it can be seen that the protein staining was not affected by different natural compound treatments. The crude extracts of *Melastoma candidum* D. Don (lane 2) had apparently inhibitory activity toward plasma AO, while the pure flavonoids, isoquercitrin (lane 3), rutin (lane 5), isolated from *Melastoma candidum*, did not show inhibitory activity against plasma AO, however, quercetin (lane 4) isolated from *Melastoma candidum* and the crude extracts of hooks of *Uncaria rhynchophylla* (Miq.) Jack (lane 6) showed partially inhibitory activities against plasma AO. In lane 2 (Fig. 4B), some dark precipitations were observed at the top of the gel. Compared to

protein staining (Fig. 4A), it was possible that some compounds in the 70% acetone crude extracts of *Melastoma candidum* were precipitated during electrophoresis which could interact with AEC dye and resulted in dark staining in the stacking gel. Lee *et al.* [21] reported that the flavonoids of isoquercitrin, quercetin and rutin showed MAO-B inhibitory activity. It might be possible that the SSAO inhibitory activity in crude extracts of *Melastoma candidum* might come from quercetin and other compounds other than isoquercitrin and rutin. The compounds in crude extracts of hooks of *Uncaria rhynchophylla* (Miq.) exhibited plasma AO inhibitory activities.

In conclusion, the fast and sensitive activity staining on native PAGE gels for plasma AO presented in this report could be applied to any type of amine oxidase which could produce hydrogen peroxide after different physiological substrates metabolism (such as putrescine or cadaverine for diamine oxidase). It also allows screening the potential candidates for AO inhibitors from herbal or medicinal plants, and performing purity tests and activity staining in one gel; thus it can be used during enzyme purification and for characterization of AO from different sources.

*The authors greatly acknowledge the financial support (NSC 90-2313-B038-001) from the National Science Council, Republic of China (R.O.C.).*

Received March 8, 2002

#### 4 References

- [1] Jalkanen, S., Salmi, M., *EMBO J.* 2001, 20, 3893–3901.
- [2] Shih, J. C., Chen, K., Ridd, M. J., *Annu. Rev. Neurosci.* 1999, 22, 197–217.
- [3] Klinman, J. P., Mu, D., *Annu. Rev. Biochem.* 1994, 63, 299–344.
- [4] Klinman, J. P., *J. Biol. Chem.* 1996, 271, 27189–27192.
- [5] Lyles, G. A., *Int. J. Biochem. Cell Biol.* 1996, 28, 259–274.
- [6] Boomsma, F., van Dijk, J., Bhaggoe, U. M., Bouhuizen, A. M. B., van den Meiracker, A. H., *Comp. Biochem. Physiol. Part C* 2000, 126, 69–78.
- [7] Precious, E., Gunn, C. E., Lyles, G. A., *Biochem. Pharmacol.* 1988, 37, 707–713.
- [8] Lyles, G. A., Chalmers, J., *Biochem. Pharmacol.* 1992, 43, 1409–1414.
- [9] Yu, P. H., Zuo, D. M., *Diabetes* 1993, 42, 594–603.
- [10] Yu, P. H., Zuo, D. M., *Atherosclerosis* 1996, 120, 189–197.
- [11] Yu, P. H., Zuo, D. M., *Diabetologia* 1997, 40, 1243–1250.
- [12] Boomsma, F., Derkx, F. H. M., van den Meiracker, A. H., Manint Veld, A. J., Schalekamp, M. A. D. H., *Life Sci.* 1995, 88, 675–679.
- [13] Boomsma, F., van Veldhuisen, D. J., de Kam, P. J., Manint Veld, A. J., Mosterd, A., Lie, K. I., Schalekamp, M. A. D. H., *Cardiovasc. Res.* 1997, 33, 387–391.
- [14] Boomsma, F., van den Meiracker, A. H., Winkel, S., Aanstoot, H. J., Batstra, M. R., Manint Veld, A. J., Bruining, G. J., *Diabetologia* 1999, 42, 233–237.
- [15] Holt, A., Sharman, D. F., Baker, G. B., Palcic, M. M., *Anal. Biochem.* 1997, 244, 384–392.
- [16] Lizcano, J. M., Unzeta, M., Tipton, K. F., *Anal. Biochem.* 2000, 286, 75–79.
- [17] Falk, M. C., *Biochemistry* 1983, 22, 3740–3745.
- [18] Paz, M. A., Fluckiger, R., Boak, A., Kagan, H. M., Gallop, P. M., *J. Biol. Chem.* 1991, 266, 689–692.
- [19] Lizcano, J. M., Tipton, K. F., Unzeta, M., *Biochem. J.* 1998, 331, 69–78.
- [20] Davis, B. J., *Ann. N. Y. Acad. Sci.* 1964, 121, 404–427.
- [21] Lee, M. H., Lin, R. D., Shen, L. Y., Yang, L. L., Yen, K. Y., Hou, W. C., *J. Agric. Food Chem.* 2001, 49, 5551–5555.
- [22] Bickford, P. C., Gould, T., Briederick, L., Chadman, K., Pollock, A., Young, D., Shukitt-Hale, B., Joseph, J., *Brain Res.* 2000, 866, 211–217.
- [23] Schulz, J. B., Lindenau, J., Seyfried, J., Dichgans, J., *Eur. J. Biochem.* 2000, 267, 4904–4911.
- [24] Abrass, I. B., *West. J. Med.* 1990, 153, 641–645.
- [25] Geokas, M. C., Lakatta, E. G., Makinodan, T., Timiras, P. S., *Ann. Intern. Med.* 1990, 113, 455–446.
- [26] Shimoni, M., *Anal. Biochem.* 1994, 220, 36–38.