Optimization of Light Exposure on Superoxide Scavenger Test of Manganese (III)-Salen Acetate Complex

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Abstract

The light exposure optimization on superoxide scavenger test of manganese (III)-salenacetate complex, [Mn^{III}(salen)($C_2H_3O_2$)], has been studied through in-vitro non-enzymatic riboflavin photo reduction. The complex was synthesized from $Mn(C_2H_4O_2)_2.4H_2O$ and H_2salen in methanol solution, characterized by magnetic moment measurement, elemental analysis and infrared spectroscopy.

Superoxide scavenger of the complex was examined by mixing the complex at various concentrations with riboflavin, tetramethylethylenediamine (TEMED) and nitroblue tetrazolium (NBT). Then the mixture was lighted with 23-Watt tungsten lamp for 5 to 30 minutes in a closed box. The reduced NBT absorptions were measured at λ560 nm. The results showed that the optimum light exposure on the manganese (III)-salen acetate superoxide scavenger test is 10 minutes.

Keywords: Superoxide, scavenger, inhibition, [Mn^{III}(salen)($C_2H_3O_2$)]

Introduction

Manganese salen complexes have been studied widely due to their potential as a mimic enzyme superoxide dismutase (mSOD)[1–4] which catalyzed the disproportionation of superoxide anion radicals to oxygen and hydrogen peroxide molecules. These complexes have square pyramidal structures in which Mn is positioned in the center of the complexes coordinated equatorially by salen ligand. The axial positions of this complexes are usually coordinated by other neutral or anionicligands[5,6]. The oxidation state of Mn is three which is unstable in aqueous system. However, it is stabilized by the salen coordinated in the complexes.

The mSOD activities of manganese salen complexes can be observed by two methods: enzymatic and non-enzymatic. The most widely used method is enzymatic using xanthine/xanthine oxidase. Nevertheless, the lifetime of enzyme is usually not too long. The non-enzymatic method using riboflavin as superoxide generator, relatively stable depends on the light exposure time. This method has been applied to manganese salen complexes and allows relatively similar result compared to the test using enzymatic method[7] and it has demonstrated good activities[4]. In this research, the time of light exposure of riboflavin during the mSOD test of [Mn^{III}(salen)($C_2H_3O_2$)] complex has been determined using non-enzymatic assay of riboflavin photo reduction.

Material and Methods

Material: $Mn(C_2H_4O_2)_2.4H_2O$, H_2salen (N, N'-bis(salicylidene)ethylene-iamine), methanol, dimethylformamide, riboflavin, phosphate buffer saline, tetramethylethylenediamine (TEMED), nitroblue-tetrazolium (NBT), Whatmann papers.

Synthesis of [Mn^{III}(salen)($C_2H_3O_2$)]: The synthesis of this complex was carried out according to the previous method[4]. $Mn(C_2H_4O_2)_2.4H_2O$ (1.51 g, 6 mmol) and H_2salen (1.63 g, 6 mmol) were dissolved in methanol. The reaction mixture was stirred and heated for one hour. After the reaction was complete, the mixture was left at room temperature and dark brown crystal was obtained within one week. The crystal was washed with methanol and dried in a desiccator.

Physical measurements: The C, H and N were measured using Perkinelmer 240 analyzer. The functional groups of the ligand and the complex were observed by Shimadzu IR prestige-21. The magnetic susceptibility of the complex has been measured using Magnetic Susceptibility Balance Sherwood Scientific.

Superoxide scavenger test
Reagent preparation:
Working solution I: A mixture with quantity of 320 µL of phosphate buffer 0.016 MpH 7.4; 170 µL of85 µM NBT; 160 µL of 0.8 mMTEMED; and 240 µL of 12 µM riboflavin was prepared. The mixture were diluted in 19,110 µL of aquabidest and stored in a 50 mL tube.

Working solution II: It was prepared with the same procedure as working solution I, but riboflavin was not added to this solution and the volume of aquabidest is increased to become 19.350 µL.

Sample preparation: 20,000 µM sample solution was made from 0.0765 g of [Mn^{III}(salen)($C_2H_3O_2$)] dissolved in methanol. This solution is called stock solution for the preparation of various complex concentration ranging from 40 µM to 0.75 µM.

Measurement: Scavenging activities of the complex were measured indirectly using *in vitro* method. Microplate used for this assay was 12 x 8 wells. There were four kinds of tested solution. First, the samples solution, which contained

various concentrations of the complex as the sample was mixed with riboflavin from working solution I. Second, the blank 1 solution contained only methanol as the solvent and riboflavin. Blank 2 solutions became the standard for the sample and blank 1 solution respectively as no riboflavin in working solution II was added.

a. **b.**

Figure 1: The color changes (a) before and (b) after light exposure

The last is blank 3 contained only methanol and working solution II. The first until the fifth rows were for the sample solutions, the sixth and seventh rows were for the blank 2, the sixth first half of the last row was for the blank 1 and the rest were for the blank 3. These arrangements are shown in figure 1. The superoxide was generated from photoreduction riboflavin using ligh exposure varied between 5-30 minutes. The mechanism was NBT (yellow) attacked by superoxide to become formazan (dark purple). It was monitored at 560 nm spectrophotometrically[8,9].

Results and Discussion

Complex characterization: The dark brown [Mn^{III}(salen)($C_2H_3O_2$)] complex has been successfully synthesized from $Mn(C_2H_4O_2)])_2.4H_2O$ and H_2 salen in methanol. The change in oxidation number from Mn(II) to Mn(III) was observed visually through the change of color from pale pink to a dark brown solution. This oxidation by air occurred spontaneously[5]. The appearance of the complex is shown in figure 2.

a)

b)

Figure 2: [Mn^{III}(salen)($C_2H_3O_2$)] complex a) Visible and b) Under microscope

Based on the elemental analysis, the C, H and N contents are C: 56.69; H: 4.21; N: 7.52%, confirmed to the calculation presentation of C: 56.85; H: 4.51; N: 7.52% for [$MnC_{18}H_{17}N_2O_4$]. The complex was paramagnetic with the magnetic moment of µeff = 4.8 BM, indicating that there are four unpaired electrons in the *d*-orbital of Mn (III) ion.

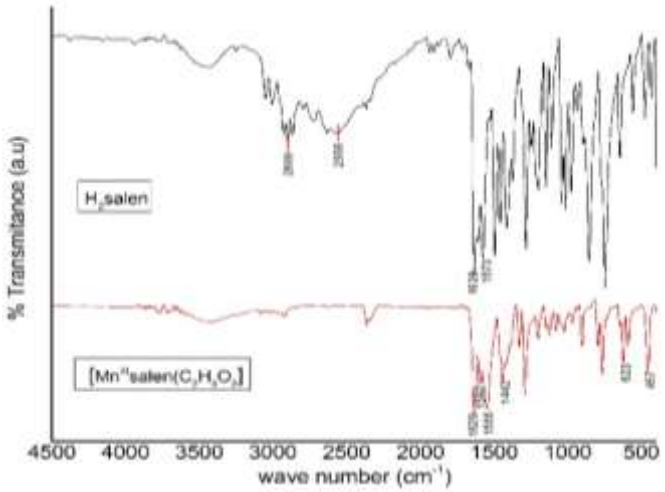

Figure 3: Infrared spectrum of H_2salen and [Mn^{III}(salen)($C_2H_3O_2$)]

The infrared (IR) spectrum of [Mn^{III}(salen)($C_2H_3O_2$)] complex is shown in figure 3. The sharp band at 1629 cm^{-1} indicates the C=N functional group. This band is slightly lower than the similar C=N from the H_2salen (1639 cm^{-1}), indicating that the nitrogen atom from the ligand has coordinated to the metal ion. Moreover, two bands at 623 and 457 $cm^{-1}$are assigned to Mn-N and Mn-O stretching modes. Two other bands at 1592 and 1442 $cm^{-1}$are related to antisymmetric and symmetric stretching vibration of acetate group ($C_2H_3O_2$) respectively indicating that acetate was coordinated to Mn(III) in the axial position oriented square pyramidal structure of the complex.

Optimization of light exposure on superoxide scavenger test: The riboflavin in solution was excited by a photon from the light source. In that condition, TEMED reduced the riboflavin to form semiquinone, which then reduced O_2 to $O_2^{\bullet-}$. Then, the complex and NBT (as a dye) would compete to attack the superoxide. When the NBT reduced the superoxide, it became dark purple formazan. But, if the complex reacted with the superoxide, the color change did not appear because the complex inhibits the dye's reduction (Figure 1) and the colors remained yellow. The mechanism of superoxide scavenging is shown in figure 4.

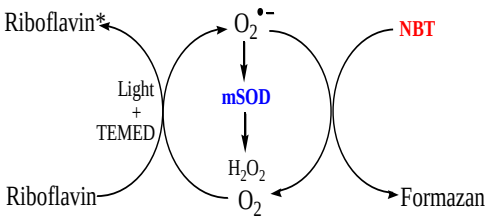

Figure 4: Superoxide radical scavenger mechanism

Figure 1(b) shows that the color changes corresponded to the concentration of the complex. The higher is the concentration of the complex, the higher is the percentage of inhibition. This means in the higher concentration, the complex attacks the superoxide rather than the NBT. The obtained absorbances were calculated using equation 1 where B1 is the absorbance for blank 1, B2 is the absorbance for blank 2, B3 is the absorbance for blank 3 and S is the absorbance for the sample.

$$\% I = \frac{(B1 - B3) - (S - B2)}{(B1 - B3)} \qquad (1)$$

Optimization of the light exposure was obtained by turn on the lamp in the light box for about 10 minutes before applied to the test solution.

From the data above in figure 5, the optimum exposure time if riboflavin photoreduction is 10 minutes, the numbers of the superoxide anion radicals generated were maximum, therefore the scavenging activities of the complex have reached its limit after the light exposure time optimization.

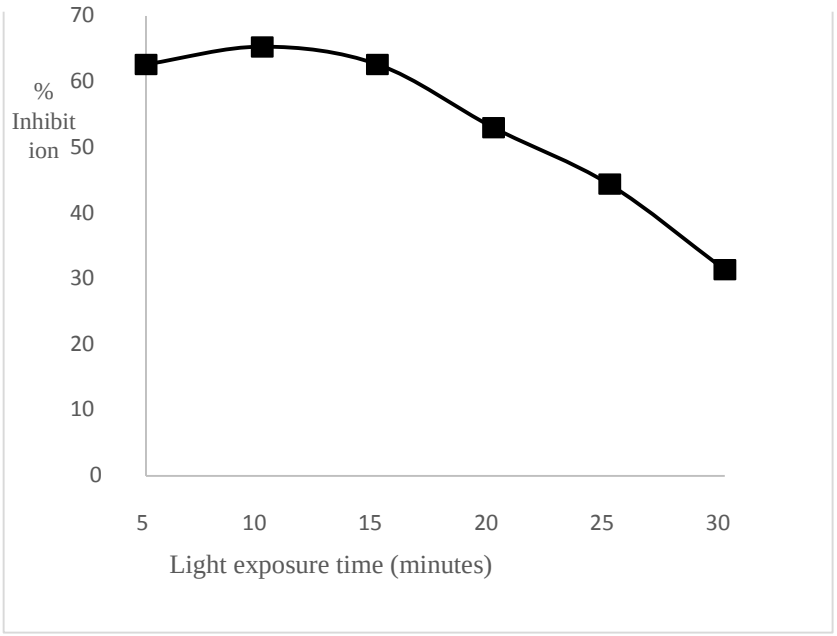

Figure 5: Inhibition percentage of 1,5 µM [Mn^{III}(salen)($C_2H_3O_2$)] versus light exposure time.

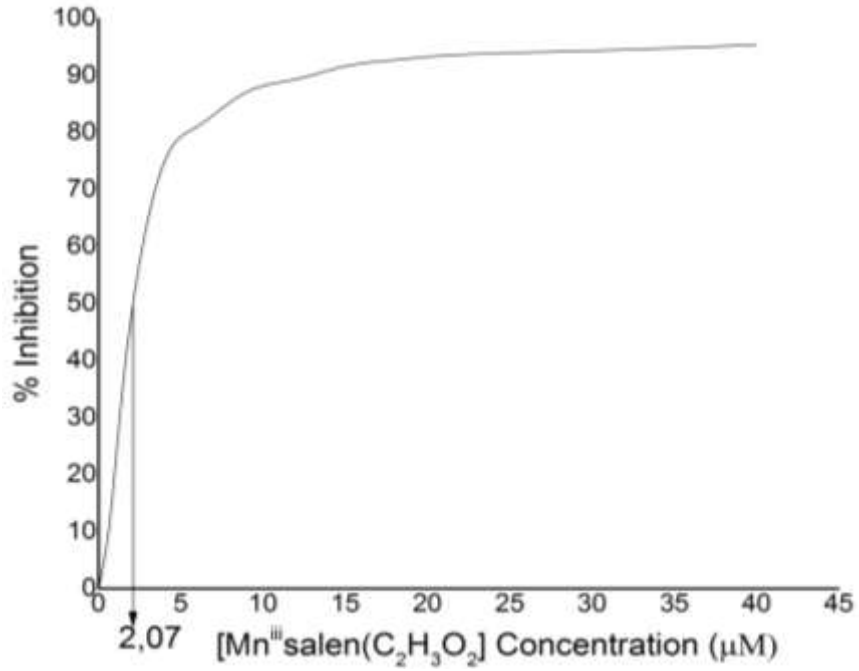

Figure 6: Superoxide radical scavenging activity (% inhibition) of various [Mn^{III}(salen)($C_2H_3O_2$)] concentrations

Scavenging activity of this complex determined as IC_{50} correspond for 50% activity of the complex to inhibit superoxide radical. Thus, from data plotted in figure 6, the $[Mn^{III}(salen)(C_2H_3O_2)]$ concentration determined was 2.07µM.

Conclusion

The optimization of light exposure on the riboflavin photoreduction superoxide scavenger $[Mn^{III}(salen)(C_2H_3O_2)]$, has been investigated and determined as 10 minutes. With optimal light exposure, the inhibition percentage value increased significantly. sssThis indicated that this manganese salen acetate complexe is potential for mimic enzyme superoxide dismutase (mSOD).

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