ACTIVITY OF SUPEROXIDE DISMUTASE MIMIC OF [Mn(SALEN)OAc] COMPLEX COMPOUND NON-ENZYMATICALLY *IN VITRO* THROUGH RIBOFLAVIN PHOTOREDUCTION

AKTIVITAS MIMIK SUPEROKSIDA DISMUTASE SENYAWA KOMPLEKS [Mn(SALEN)OAc] SECARA *IN VITRO* NON-ENZIMATIK MELALUI FOTOREDUKSI RIBOFLAVIN

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ABSTRACT

The complex compound of [Mn(salen)OAc] can serve as mSOD and its activity has been determined non-enzymatically *in vitro* through riboflavin photoreduction. The complex was synthesized from Mn(OAc)₂·4H₂O and H₂salen. Based on the elemental analysis, the C=56.69%; H=4.21%; and N=7.52% content are corresponding to the chemical formula of MnC₁₈H₁₇N₂O₄. The functional groups and ionic species in the complex have been analyzed by infrared spectroscopy and ESI-MS. SOD activity was determined by mixing complex at various concentrations with riboflavin and nitroblue tetrazolium (NBT), then the mixture was lighted with 20 watts tungsten lamp for 15 minutes in a closed box. Then the reduced NBT absorptions were measured at λ 560 nm. The difference of absorbance between standard and sample solutions (without and with riboflavin, respectively) was multiplied by 100% to obtain %inhibition of each various sample concentration against NBT. SOD activity was obtained from IC₅₀ data defined as a 50% inhibition of the plot curve of % inhibition to the concentration of the complex. The result obtained for this compound is IC₅₀ = 2.7 ± 0.1 μ M as well as enzymatic method. Therefore, this method can be used to determine the SOD activity by giving more stability and accuracy of IC₅₀ value.

Keywords: [Mn(salen)OAc], SOD, non-enzymatic, photoreduction, riboflavin

ABSTRAK

Senyawa kompleks [Mn(salen)OAc] dapat berfungsi sebagai mSOD dan aktivitasnya telah ditentukan secara *in vitro* non-enzimatik melalui fotoreduksi riboflavin. Kompleks ini disintesis dari Mn(OAc)₂·4H₂O dan H₂salen. Berdasarkan analisis unsur diperoleh kadar unsur C=56,69%; H=4,21%; dan N=7,52% yang sesuai dengan rumus kimia MnC₁₈H₁₇N₂O₄. Gugus fungsi dan spesi ion dalam kompleks tersebut telah dianalisis dengan spektroskopi inframerah dan ESI-MS. Aktivitas SOD ditentukan dengan mencampurkan kompleks pada berbagai konsentrasi dengan riboflavin dan nitroblutetrazolium (NBT), yang kemudian campuran tersebut disinari oleh lampu wolfram 20 watt selama 15 menit pada kotak tertutup. Setelah itu serapan NBT tereduksi diukur pada λ 560 nm. Perbedaan absorbansi antara larutan standar dan sampel (masing-masing tanpa dan dengan adanya riboflavin) dikalikan dengan 100% untuk mendapatkan % inhibisi dari setiap variasi konsentrasi sampel terhadap NBT. Aktivitas SOD diperoleh dari data IC₅₀ yang ditentukan sebagai 50% inhibisi pada kurva aluran persen inhibisi terhadap konsentrasi kompleks. Hasil yang diperoleh untuk kompleks ini adalah IC₅₀ = 2,7 ± 0,1 μ M sama seperti pada metode enzimatik. Oleh karena itu metode ini dapat digunakan untuk menentukan aktivitas SOD dengan memberikan nilai IC₅₀ yang lebih stabil dan akurat.

Kata kunci: [Mn(salen)OAc], SOD, non-enzimatik, fotoreduksi, riboflavin

INTRODUCTION

Superoxide radical (O₂^{•-}) is one of the reactive oxygen species (ROS) which the most dangerous in comparison with other

ROS (Vecchio & Lanza, 2009). The existence of excessive radicals in the body can cause tissue damage associated with the emergence of various diseases, such as rheumatoid

arthritis, cancer, neurodegenerative disorders, complications of diabetes, stroke, inflammation and reperfusion injury.

Superoxide reactivity could be overcome by a metalloenzyme known as superoxide dismutase (SOD), that catalyze the dismutation of $O_2^{\cdot-}$ reactive species to O_2 and H_2O_2 (Vecchio & Lanza, 2009). But the limited number of metalloenzyme in the body, the use of supplements containing SOD from plants extract or other living beings or synthetic SOD become an alternative. Synthetic SOD is known as SOD mimic or mSOD (Miriyyala et al., 2012).

Manganese (III)-salen complexes compound are one of an mSOD type which the activities much-studied. The complex compounds have high stability and relatively low toxicity (Haberle, Reboucas, & Spasojevic, 2010). Several compounds of Mn(III)-salen, with variety salen ligand structure, generally have monomeric structures (Doctrow et al., 2002; Meftah & Yazdanparast, 2013).

The *in vitro* indirect SOD test is an initial test conducted to determine a potential of a complex compound as an mSOD (Iranzo, 2011). The test principle is competition between mSOD and active redox indicator to react with $O_2^{\cdot-}$ species. The activity of an mSOD of a complex compound shown by the ability of the complex compound to inhibit reduction process of the active redox indicator by $O_2^{\cdot-}$ species. Through this indirect SOD test, SOD activity is obtained as IC_{50} value, that is a quantity used to express one unit of SOD activity. IC_{50} is a quantity that describes the amount of SOD to inhibit the reduction reaction of an active redox indicators by species $O_2^{\cdot-}$ as much as 50%.

Based on the source of $O_2^{\cdot-}$ species, there are two types of SOD assay, that is enzymatic and non-enzymatic methods. In the enzymatic method (McCF: McCord-Fridovich), xanthine and xanthine oxidase are used as $O_2^{\cdot-}$ species source, while in the non-enzymatic method, $O_2^{\cdot-}$ species sources which often used are derived from flavin photoreduction (Kostyuk, Potapovich, Kostyuk, & Cherian, 2007; Retnoningrum et al., 2016), organic compound autooxidation (Li, 2012) and gamma irradiation (Barnese, Gralla, Cabelli, & Valentine, 2008). The non-enzymatic method through riboflavin photoreduction is currently used because it is easier to use, relatively cheaper, and accurate.

The complex compound of [Mn(salen)OAc] is one of manganese(III)-salen which is a pioneer as mSOD. The patent name of the compound is EUK-108, provided by a pharmaceutical company in the United States (Eukarion, Inc.) (Doctrow et al., 2002). There are two studies that reported IC_{50} values of [Mn(salen)OAc] complex compound tested with McCF (indicator: cyt c) enzymatic method, those are $0.7 \pm 0.2 \mu M$ (Doctrow et al., 2002) and $2.54 \pm 0.2 \mu M$ (Vecchio & Lanza, 2009). Both the IC_{50} values differ greatly, which indicate that the SOD test by the McCF enzymatic method gives inconsistency IC_{50} values of a compound. This may be caused by enzyme activity decreased or unstable.

The non-enzymatic method through riboflavin photoreduction (indicator: NBT)/rb-NBT has been widely used to determine SOD activity of various types of natural SOD, such as SOD-manganese recombinant hybrid of *Staphylococcus equorum* and *S. saprophyticus* with *Escherichia coli* (Retnoningrum et al., 2016) and SOD-manganese from *Staphylococcus equorum* (Indrayati, Asyarie, Suciati, & Retnoningrum, 2014). However, there were only found 9 SOD mimics of manganese complex compound whose activities were determined by this rb-NBT non enzymatic method, those are: 1 complex of manganese(II)-bovine serum albumin (Yan et al., 2016), 2 complexes of manganese(III)-salen derivatives: $Na[Mn^{III}(5-SO_3-salpnOH)(H_2O)] \cdot 5H_2O$ dan $Na[Mn^{III}(5-SO_3-salpn)(MeOH)] \cdot 4H_2O$ (Moreno, Daier, Palopoli, Tuchagues, & Signorella, 2010), 2 complexes of manganese (IV)-salen derivatives: $[Mn^{IV}(salbutO)N_3]$ and $[Mn^{IV}(salbutO)SCN]$ (Daier, Moreno, Duhayon, Tuchagues, & Signorella, 2010), 1 complex of manganese(II)-bacitracin (Piacham et al., 2006), 2 complexes of manganese (II)-macrocyclic: $[Mn^{II}(1-(benzimidazol-2-ylmethyl)-1,4,7-triazacyclononane)Cl_2]$ and $[Mn^{II}(1,4-bis(benzimidazol-2-ylmethyl)-1,4,7-triazacyclononane)Cl]ClO_4 \cdot MeOH$, and 1 complex of manganese(III)-multidentate acyclic $[Mn^{III}(bpb)(H_2O)Cl]$ (Iranzo, 2011).

New in this research, the non-enzymatic method through riboflavin photoreduction using NBT indicator was used to determine SOD activity (IC_{50}) of the

[Mn(salen)OAc]. The purpose of this study is to evaluate the ability of this rb-NBT non-enzymatically method in analyzing SOD activity of the [Mn(salen)OAc] complex compound.

EXPERIMENTAL SECTION

In this research, the [Mn(salen)OAc] complex has been synthesized, characterized, and SOD activity tested by the *in vitro* non-enzymatic method through riboflavin photoreduction using NBT indicator (rb-NBT)

Materials

In this study, chemicals that used are the high purity grade from Sigma-Aldrich, those are ethylenediamine 99%, salicylaldehyde 98%, absolute ethanol 99.5%, manganese(II) acetate tetrahydrate 99%, distilled water, and methanol 99.8%. The chemicals used in the SOD test are nitroblue tetrazolium chloride (NBT) 98% from Thermoscientific, tetramethylethylenediamine (TEMED) 99% from Biorad, riboflavin 98%, the solution of 1.0 M phosphate buffer pH 7.4 (25°C), and aqua bidest.

Instrumentation

Tools and instruments used in this study include glasswares, Perkin-Elmer 240 elemental analyzer, FTIR Nicolet Magna IR 750 series II spectrophotometer, ESI-MS Bruker Daltonics esquire3000 plus spectrometer, Sherwood Scientific magnetic susceptibility balance (MSB), and HANNA Instruments HI 8819W Professional ATC conductometer. While, equipment used in the SOD activity test include micropipette 1000 and 30-300 µL, 96-well microplate, light box (25 x 25 x 25 cm³) coated by aluminum foil and using a 20 watts tungsten lamp, and Multiskan Go Thermofisher Scientific UV/vis double beam microplate reader spectrophotometer.

Procedures

Synthesis of [Mn(salen)OAc] Complex

The [Mn(salen)OAc] complex was prepared using a published procedure (Boucher & Farrell, 1973) with using glass beakers instead of reflux and methanol as a solvent for recrystallization instead of petroleum. The H₂salen (N,N'-bis(salicylidene)ethane-1,2-diamine) that used as a ligand was prepared from salicylaldehyde and ethylenediamine by the reported method

(Haikarainen, Sibila, Pietikainen, Pajunen, & Mutikainen, 2001). The dark brown crystals of the [Mn(salen)OAc] complex were obtained in 45-67% yield.

SOD Assay: Riboflavin Photoreduction Test of [Mn(salen)OAc] Complex

The [Mn(salen)OAc] complex was tested for SOD activity using a previously described method (Kostyuk et al., 2007). The SOD activity measurement of the complex compound was based on the reduction of nitroblue tetrazolium by superoxide anions generated in riboflavin photoreduction, which was carried out at room temperature (25°C) under fluorescent lighting (20 w, 20 cm). The sample solution was prepared in 12 variations of concentration and each 40 µL of them was added to a solution (200 µL) containing 6 µM of riboflavin, 0.8 mM of N,N,N',N'-tetramethylethylenediamine (TEMED) in 16 mM phosphate buffer (pH 7.4), and 85 µM of nitroblue tetrazolium (NBT) in a microplate. The sample mixtures were subjected to light exposure as mentioned above for 15 min and the colorimetric activity assay by a microplate reader spectrophotometer follows the generation of NBT-diformazan, which absorbs at 560 nm (Janknegt, Rijstenbil, van de Poll, Gechev, & Buma, 2007).

The absorbance of sample blank mixture (blank 1), without riboflavin, was measured as well. In the standard mixture and its blank (blank 2), methanol solvent was added instead of the sample solution. The difference of absorbance between standard and samples (without and with riboflavin, respectively) was multiplied by 100% to obtain % inhibition of each various sample concentration against NBT (Retnoningrum et al., 2016).

RESULTS AND DISCUSSION

Characterization of H₂Salen Ligand and [Mn(salen)OAc] Complex

Elemental analysis of the H₂salen (C₁₆H₁₆N₂O₂) and [Mn(salen)OAc] complex compound (MnC₁₈H₁₇N₂O₄) resulted from synthesis show that each compound has C, H, and N elements percentage which fit the theoretical calculations (**Table 1**). These results indicate that H₂salen and [Mn(salen)OAc] complex compound have been successfully synthesized.

Table 1. Elemental analysis of C, H, and N for H₂salen and [Mn(salen)OAc] complex

Compound	Empirical Formula	Relative Molecular Mass	Calculation (%)			Found (%)		
			C	H	N	C	H	N
H ₂ Salen	C ₁₆ H ₁₆ N ₂ O ₂	268.31	71.62	6.01	10.44	71.60	6.05	10.44
[Mn(salen)OAc]	MnC ₁₈ H ₁₇ N ₂ O ₄	380.28	56.85	4.51	7.52	56.69	4.21	7.52

Figure 1. Infrared spectra of: H₂salen (above), [Mn(salen)OAc] (bottom)

In the infrared spectrum of the [Mn(salen)OAc] complex (**Figure 1** (below)), several different wave numbers are observed compared with the H₂salen spectrum (**Figure 1** (above)). The differences are observed in the vibration of O-H, C-H, C-O-H, Mn-N, and Mn-O functional groups. In the complex compounds, the vibration of O-H and C-H functional groups occurs successively in the wave numbers of 3426-3775 and 2611-3084 cm⁻¹, while the ligands occurred successively in the wave numbers of 3425-3789 and 2610-3052. The vibration of C-O-H which appeared at the wave numbers of 1717-1942 cm⁻¹ in the H₂salen spectrum, did not appear in the spectrum of [Mn(salen)OAc] complex. This

fact indicates that after H₂salen bound to Mn(III), the vibration of C-O-H did not happen again due to the release of the H atom of the -OH group of H₂salen. Metal-ligand bond in the [Mn(salen)OAc] complex can be observed in the fingerprint region at the wave number of 593-624 cm⁻¹ (Mn-N) and 452 cm⁻¹ (Mn-O).

Measurement of the ESI-MS mass spectrometry of [Mn(salen)OAc] complex was carried out in methanol-acetonitrile. In the positive ionization spectrum (**Figure 2** (left)), a cationic species with a high-intensity peak was observed at m/z 320.9 which corresponding to the four-coordinated cation [Mn(salen)]⁺. This suggests that the

[Mn(salen)]⁺ cation is the parent species of the [Mn(salen)OAc] complex compound. Besides, a moderate intensity peak also observed at m/z 269.0, which corresponding to a cationic free ligand species (salen + H)⁺. On the other side, in the negative ionization spectrum (**Figure 2** (right)), a peak indicating two acetate ions which coordinated to the Mn(III) ion was observed, although the intensity was very low, that is at m/z 441, corresponding to the anion species of [Mn(salen)(OAc)₂]⁻. In addition, the negative ionization spectrum is also observed three peaks at m/z 255.0, 265.0, and 283.1, which corresponding to the (salen - N)⁻, (salen - H)⁻ and (salen + N)⁻. Meanwhile, a peak at m/z 319 which corresponding to the [Mn (salen) - H]⁻ species showed that the acetate ion binding to the Mn(III) ion could be broken). Based on the results of the ESI-MS, the [Mn(salen)OAc] complex compound have been successfully synthesized.

The conductometry measurement results of the [Mn(salen)OAc] complex compound can be seen in **Table 2**. The

methanolic solution of the complex was provided a molar conductivity of 0.17 mS. This molar conductivity is smaller than the value of the smallest molar conductivity of NaCl ionic compound. These results indicate that the complex is a neutral [Mn(salen)OAc] complex compound, where the acetate bound as ligands to Mn(III) or in other words it is not as a free anion or a counter ion. The results obtained from this molar conductivity value measurements in accordance with the molecular formula of the ionic species that appear on the ESI-MS spectrum and the content of C, H, N on elemental analysis.

Based on the result of magnetic measurement, the [Mn(salen)OAc] complex compound is paramagnetic with the experimental magnetic susceptibility of 4.93 BM. This value indicates that the solution reaction of Mn(II) with H₂salen caused oxidation of Mn(II) to Mn(III) ion. This result are consistent with the theoretical magnetic susceptibility of Mn³⁺, which has four unpaired electrons ($\mu_{\text{theory}} = 4.90$ BM).

Figure 2. The ESI-MS spectrum of [Mn(salen)OAc] complex compound in a solution of methanol-acetonitrile for: positive ionization region (left) and negative ionization region (right)

Table 2. The results of the molar conductivity measurements

Solution	Solvent	Λ (mS)	Number of ions
Methanol	-	0	-
H ₂ Salen	-	0	-
NaCl	Methanol	0.21	2
Mn(OAc) ₂ ·4H ₂ O	Methanol	0.33	3
[Mn(salen)OAc]	Methanol	0.17	-

Activity of SOD of [Mn(salen)OAc] Complex Compound

The potential as mSOD of the [Mn(salen)OAc] complex compound was determined by IC_{50} value, that is the concentration of the complex which is able to inhibit the reduction of NBT by superoxide anion as well as 50%. The SOD activity was tested *in vitro* non-enzymatically through riboflavin photoreduction and NBT is used as an indicator that can be reduced to NBT-diformazan when it reacts with superoxide anions generated from photoreduction of riboflavin. This indirect method includes several reactions: the photochemically excited

riboflavin was first reduced by TEMED into semiquinone, which donated an electron to oxygen to form superoxide anion source. The superoxide readily converted a yellow NBT into a purple formazan product. The SOD activity was inversely related to the amount of formazan formed

The measurement results of the inhibition of the [Mn(salen)OAc] complex against NBT reaction with superoxide in the form of absorption values before and after irradiation are shown in **Table 3**. An empty microplate before being filled by any mixture gives the same absorption, about 0,060, either before or after lighting.

Table 3. The absorbance data of SOD activity test of [Mn(salen)OAc] complex

No.	[Mn(salen)OAc]	No. of Data	A_{sample}		Inhibition (%)		SD
			I	II	I	II	
Standard	0	-	0.755	0.788	0	0	-
1	0.5 μM	1	0.758	0.796	0	-1	3.4
		2	0.721	0.755	5	5	
		3	0.739	0.774	2	2	
		4	0.77	0.733	-2	8	
		Rate			2		
2	1 μM	1	0.593	0.685	24	14	3.0
		2	0.608	0.655	21	18	
		3	0.592	0.64	24	20	
		4	0.623	0.647	19	20	
		Rate			20		
3	1.5 μM	1	0.528	0.564	33	31	2.7
		2	0.555	0.572	29	30	
		3	0.528	0.524	33	37	
		4	0.556	0.542	29	34	
		Rate			32		
4	2 μM	1	0.507	0.518	36	37	2.4
		2	0.484	0.516	39	38	
		3	0.479	0.487	40	42	
		4	0.478	0.539	40	34	
		Rate			38		

No.	[Mn(salen)OAc]	No. of Data	A_{sample}		Inhibition (%)		SD
			I	II	I	II	
5	3 μM	1	0.392	0.409	53	53	0.8
		2	0.385	0.393	54	55	
		3	0.387	0.395	53	54	
		4	0.387	0.402	53	54	
		Rate			54		
6	4 μM	1	0.356	0.369	58	58	1.8
		2	0.342	0.352	60	60	
		3	0.342	0.331	60	63	
		4	0.328	0.354	62	60	
		Rate			60		
7	5 μM	1	0.339	0.324	60	64	5.5
		2	0.333	0.325	61	64	
		3	0.374	0.306	55	67	
		4	0.396	0.3	52	68	
		Rate			62		
8	6 μM	1	0.271	0.277	70	71	1.4
		2	0.259	0.262	72	73	
		3	0.243	0.257	74	74	
		4	0.262	0.272	72	72	
		Rate			72		

No.	[Mn(salen)OAc]	No. of Data	A_{sample}		Inhibition (%)		SD
			I	II	I	II	
9	7 μM	1	0.247	0.25	74	75	1.9
		2	0.22	0.228	78	78	
		3	0.21	0.236	79	77	
		4	0.215	0.225	78	78	
		Rate			77		
10	10 μM	1	0.185	0.181	83	84	0.9
		2	0.176	0.183	84	84	
		3	0.165	0.184	86	84	
		4	0.167	0.184	85	84	
		Rate			84		
11	15 μM	1	0.147	0.151	88	88	1.2
		2	0.141	0.151	89	88	
		3	0.135	0.135	90	91	
		4	0.137	0.162	90	87	
		Rate			89		
12	20 μM	1	0.124	0.162	92	87	1.8
		2	0.126	0.132	91	91	
		3	0.126	0.122	91	92	
		4	0.121	0.14	92	90	
		Rate			91		

Notes:

No. of Data	A_{standard}		$A_{\text{blank 1 \& 2}}$	
	I	II	I	II
1	0.770	0.792	0.064	0.066
2	0.727	0.792	0.062	0.067
3	0.768	0.787	0.083	0.088
4	-	0.779	0.078	0.072
5	-	-	0.062	0.057
6	-	-	0.055	0.06
7	-	-	0.058	0.06
Rate	0.755	0.788	0.066	0.067

SOD Activity (IC_{50})		
No. of Data	IC_{50} (μM)	
	I	II
1	2.8	2.8
2	2.7	2.7
3	2.7	2.6
4	2.7	2.8
Rate	2.7	
SD	0.05

Figure 3. The curve of %inhibition and various sample concentration solutions of [Mn(salen)OAc] complex

From the data of **Table 3**, some information are obtained, first, without lighting, no absorption of reduced NBT formazan that can be observed, which suggests that the superoxide anions were not formed so as not to react with NBT and there is no inhibition process. Similarly, what happened in the mixture without riboflavin (blanks 1 and blanks 2) after lighting. Second, the smaller the concentration of the complex compound in the solution (from left to right), the lower the inhibition ability of the complex against the NBT which indicated by the deeper of purple color of NBT formazan. Third, the absorptions of blanks 1 those are relatively common around 0,060 for all various concentration of sample beginning from deep brown to pale color from left to right, indicating that although colored, the [Mn(salen)OAc] complex compound does not provide absorption at λ 560 nm.

The curve of %inhibition and various sample concentration solutions of [Mn(salen)OAc] is shown in **Figure 3**. The extrapolation result of 50% inhibition was obtained from the complex compound with the concentration of $2.7 \pm 0.1 \mu\text{M}$. Based on this result, the assay showed that the [Mn(salen)OAc] complex with the concentration of $2.7 \pm 0.1 \mu\text{M}$ can inhibit as much as 50% of NBT works. The IC_{50} value obtained corresponds to the IC_{50} value in the previously reported study using the enzymatic method (Vecchio & Lanza, 2009).

CONCLUSIONS

The activity of superoxide dismutase of the [Mn(salen)OAc] complex compound has been determined by *in vitro* non-enzymatic method through riboflavin photoreduction. Its potential as an SOD mimic was known from a high and accurate IC_{50} value of $2.7 \pm 0.1 \mu\text{M}$ that obtained as well as IC_{50} obtained by the enzymatic method reported previously. Therefore, the *in vitro* non-enzymatic method of riboflavin photoreduction with NBT as an indicator can be used to determine the SOD activity of an SOD mimic of a metal complex compound by giving more stability and accuracy of IC_{50} value.

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