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Biochemical Characterization of Recombinant Cu–Zn SOD from *Citrus limon* Fused to Gliadin Peptides

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ABSTRACT

Two fusion proteins of SOD *Citrus limon* (SOD_Cl) with gliadin peptides, QQPYPQPQPF (GliSOD_P61) and LGQQQPFPPQQPYPQPQPF (GliSOD_P51) were constructed to enhance their permeability through epithelial cells. The introduction of gliadin peptide might alter the biochemical properties of the protein. In the present study, the effect of using synthetic codon-optimized gene to the level of SODs production in *Escherichia coli* BL21(DE3) and the effects of the introduction of gliadin peptides to the biochemical character of SOD_Cl were investigated including their enzyme kinetics and stability. All proteins were overproduced and affinity purified into homogeneity. Residual SOD activity was determined based on nitro blue tetrazolium-riboflavin oxidation-reduction. All recombinant SODs shared similar enzymatic properties including activity over a broad pH range from 2.0–8.0 and were relative tolerance to chemical agents tested at low concentration. Inhibition by KCN and H_2O_2 confirmed that SOD_Cl belonged to Cu/Zn-SOD. The stability of the SODs increased with increasing concentrations of KCl, $MgCl_2$ and $MgSO_4$. Of the three enzymes, SOD_Cl exhibited the highest specific activity and its kinetic parameters (K_m and V_{max}). In conclusion, the introduction of gliadin peptides to SOD_Cl decreases specific activity, thermal stability and stability at alkaline pH (9-11).

INTRODUCTION

The use of superoxide dismutase (SOD) has become a rational therapeutic approach in efforts to minimize oxygen radical-induced damage due to its role in the metabolism of superoxide anionic radicals and its potent antioxidant known in nature. Over the years, SODs have attracted many researches and are widely used in the pharmaceutical, cosmetic, food, supplementary products (Bafana *et al*., 2011a; Gopal and Elumalai, 2017) since some clinical studies showed positive and promising results (Bafana *et al*., 2011b; Carillon *et al*., 2013).

Several administration routes of SOD have been studied (Carillon *et al*., 2013). However, oral administration of SODs is limited by their degradation in the gastrointestinal tract due to acidic pH in the stomach and the presence of digestive enzymes. The hydrophilic nature and large molecular weight of SODs are also responsible for poor intestinal permeation (Aungst, 2012; Yun *et al*., 2013). Our work was concerning a recombinant Cu/Zn-SOD from *Citrus limon* (SOD_Cl) that was reported to be acid stable (Lin *et al*., 2002). In our previous study, a fusion of SOD_Cl to a gliadin peptide, QQPYPQPQPF (GliSOD_P61) but not SOD_Cl was found to penetrate Caco2 cells indicating the peptide enhanced SOD permeability (Utami *et al*., 2017). Another fusion SOD protein, GliSOD_P51 was also constructed with a gliadin peptide, LGQQQPFPPQQPYPQPQPF to be tested for its permeability.

The recombinant SODs were overexpressed from codon-optimized coding sequence as histidine-tagged proteins in *Escherichia coli* BL21(DE3) and affinity purified to obtain highly purified soluble SOD_Cl, GliSOD_P51 and GliSOD_P61 proteins. In this work, the level of SODs production in *E. coli* BL21(DE3) using synthetic gene with codon optimized was studied also a number of characterization to access their biochemical properties and stabilities was presently determined to study whether the addition of gliadin peptide to SOD_Cl

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affected the properties of protein fusions, GliSOD_P51 and GliSOD_P61. The results of this study will provide information for further *in vitro* permeability study, their potential use in the pharmaceutical field, formulation purposes, and storage of the recombinant SODs.

MATERIAL AND METHODS

Bacterium and plasmids

E. coli strains BL21(DE3) (Invitrogen, USA) for protein expression is maintained at the Laboratory of Pharmaceutical Biotechnology, the School of Pharmacy, Institut Teknologi Bandung, Indonesia. The plasmids for overexpression were pJExpress416 (ATUM, https://www.atum.bio/) for GliSOD_P61 and pET16b (Genscript, http://www.genscript.com/) for SOD_Cl and GliSOD_P51. The recombinant *E. coli* was grown in selective medium containing 100 µg/mL ampicillin for clone selection and protein overproduction.

DNA construction

The gliadin peptide sequence was fused to the amino terminus of a complete amino acid sequence of SOD_Cl (accession no. AF318938, GenBank, NCBI) i.e. LGQQQPFPPQQ PYPQPQPF and QQPYPQPQPF to create GliSOD_P51 and GliSOD_P61, respectively using genetic engineering approach. Analysis of SOD *C. limon* sequence (accession no. AF318938.1, GenBank, NCBI) using Rare Codon Calculator (http://nihserver.mbi.ucla.edu/RACC/) revealed the presence of AGG and AGA, codons rarely employed in *E. coli*. Codon Adaptation Index (CAI) analysis was performed using CAI Calculator (http://ppuigbo.me/programs/CAIcal/). In an attempt to increase the level of recombinant SOD_Cl, GliSOD_P51 and GliSOD_P61 production in *E. coli*, codon optimization strategy performed by OPTIMIZER software (http://genomes.urv.es/OPTIMIZER) based on *E. coli* B preferred codons (http://www.kazusa.or.jp/codon/). The coding regions were each commercially synthesized and cloned to create pET16b_SODCl, pET16b_GliSODP51 by GenScript and pJX416_GliSODP61 by ATUM.

Protein overexpression and purification

A single colony of recombinant *E. coli* was inoculated into 5 ml Terrific Broth (TB) supplemented with ampicillin 0.1 mg/mL. This culture was grown overnight at 37°C and sub-cultured for protein expression with induction by Isopropyl β-D-1-thiogalactopyranoside (IPTG) final concentration of 0.5 mM for 4h for GliSOD_P61 and 22°C without IPTG induction for 24h for SOD_Cl and GliSOD_P51. The cell pellets were harvested by centrifugation (4500 *g*, 4°C, 20 min), resuspended in Lysis-Equilibration-Wash Buffer containing 1 mM Phenylmethylsulfonyl Fluoride and lysed by sonication using Ultrasonic Homogenizers CY-500 (Optic Ivymen System, Italy). Cell debris were removed by centrifugation (4500 x *g* 4°C, 20 min) and the supernatant was collected by decantation. Protein purification was performed at room temperature using cOmplete™ His-tag Purification Resin (Roche Applied Science, Mannheim, Germany) and the recombinant SODs were eluted by 250 mM imidazole. All fractions containing purified protein were concentrated using a 10 kDa Nanosep devices by cold centrifugation. The concentrated purified protein was analyzed by a Coomassie brilliant blue stained 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE).

Superoxide dismutase assay

SOD activity was determined by measuring its ability to inhibit photochemical reduction of nitro blue tetrazolium (NBT), following the published procedure (Kostyuk *et al*., 2004) with slight modifications. Assay mixture consisted of 12 µM Riboflavin, 0.8 mM Tetramethylethylenediamine (TEMED), 85 µM NBT in 0.016 M phosphate buffer of pH 7.8. Riboflavin was then added last and mixed by shaking. One set of tubes was illuminated under fluorescent light source for 5 min. Mixture without SOD was similarly exposed to the light as well as in dark and used as controls. Absorbance was measured at 560 nm. Activity staining was performed as published procedure (Retnoningrum *et al*., 2017) where samples containing the enzyme were first separated in a 10% native PAGE gel for 2 h at 80 V. The gel containing duplicate lanes was sliced into two parts. One part was stained for SOD activity and the other part was stained with Coomassie Blue. Gels were soaked in 1.23 mM NBT for 20 min followed by immersion for 15 min in a solution containing 28 mM TEMED, 0.028 mM riboflavin and 100 mM potassium phosphate buffer of pH 7.8. The gel was then placed on a dry white illumination tray for 15 min. The intensity of activity indicated as clear band.

One unit activity (U) of SOD is defined as that amount of protein that inhibits NBT reduction by 50% and specific activity is defined as the rSOD activity per milligram protein (U/mg) and was determined using purified protein at varying concentrations. A graph was drawn by plotting the percent inhibition versus log rSOD concentrations (Indrayati *et al*., 2014). The kinetic parameters of the purified enzyme were determined by using riboflavin as a substrate at varying concentrations (0.52 µM–0.36 mM). The activity assay was performed as described earlier then a Lineweaver-Burk plot was drawn in the coordinates (1/V on 1/S). S is the concentration of the substrate, and V is the rate of product formation at a substrate concentration (Kumar *et al*., 2014).

Stability assay

Thermo- and pH-stability of the three SODs was determined in this study. The pH stability was determined in pH 2-11 using following buffers (50 mmol L^{-1}): HCL-KCl (pH 2), citric acid-Na_3citrate (pH 3-5), phosphate (pH 6-7), Tris-HCl (pH 8-9) and glycine/NaOH (pH 10-11) at 37°C for 60 min in a microcentrifuge tube. In the thermostability, SOD was pretreated at different temperatures (from 37°C to 80°C) for 60 min at optimum pH. The residual SOD activity was determined as in the colorimetric SOD assay. The effect of some chemicals on SOD activity was determined at optimum pH buffer containing 1, 10 and 20 mM KCN, EDTA, $CaCl_2$, KCl, $MgCl_2$, H_2O_2, $ZnSO_4$, and $MgSO_4$. The residual SOD activity was assayed after treatment for 60 min at 37°C.

Statistical analysis

Kinetic and statistical calculations of the results were carried out using the MS Office software and Minitab 17 programs (https://www.minitab.com). Statistical analysis of the research results determining differences between groups was carried out by analysis of variance followed by an unpaired two-tailed Student's t-test.

RESULTS & DISCUSSION

Gene construction, overproduction and purification of SOD *Citrus limon*

In the current study, we optimized the coding sequence for expression SOD_Cl, GliSOD_P51 and GliSOD_P61. CAI values of optimized sequences were significantly improved and considered to be high (Table 1). Coding regions with high CAI value were reported to be accounted for a high-level of protein expression (Kudla *et al*., 2009) and protein with high level production was more easily obtained from a gene with high CAI (Gustafsson *et al*., 2012). GC contents of optimized sequence increased from 47.5% to 54.7% and they were more similar with that of *E. coli* genome. In several studies, high expression levels (35-90% of total proteins) were observed from codon-optimized genes i.e ProFalcaipain-2, the human recombinant Interferon2b fusion protein and bone morphogenetic protein-2 in *E. coli* systems (Retnoningrum *et al*., 2010, 2012; Sarduy *et al*., 2012). Our results showed high expression of three recombinant proteins was produced at 39.84%–42.38% of total intracellular host proteins (Figure 1A). The three proteins were obtained with purity level higher than >95% based on Coomassie blue stained SDS PAGE analysis (Figure 1C). Introduction of gliadin sequence to SOD_Cl in GliSOD_P51 and GliSOD_P61 retained the dismutase activity based on zymography assay (Figure 1B and 1D).

Table 1: The values of CAI and %GC of SOD coding sequences.

Coding sequences	CAI	%GC
SOD_Cl*	0.543	47.5
SOD_Cl**	0.875	53.6
GliSOD_P51**	0.821	52.6
GliSOD_P61**	0.865	52.4

*Before or **after codon optimization.

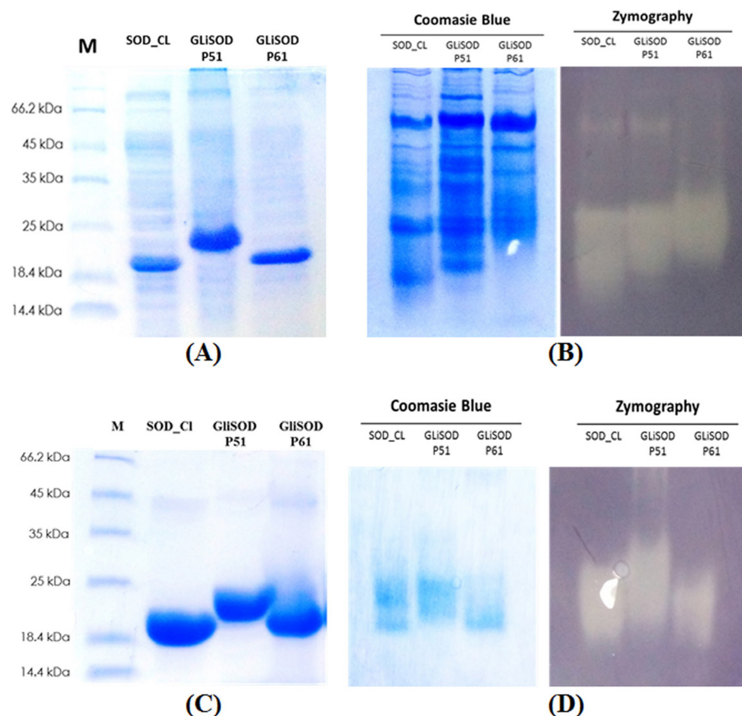

Fig. 1: Profile of recombinant SODs in crude extract *E. coli* and in purified forms. Total soluble protein profile analysis by SDS PAGE (A) NATIVE PAGE with coomasie blue staining and zymography (B) purified protein profile analysis by SDS PAGE (C) NATIVE PAGE with coomasie blue staining and zymography (D).

Specific activity and kinetic parameters of purified SOD

The specific activity of SOD_Cl was previously reported i.e 7456 units/mg (Lin *et al*., 2002) but its kinetic parameters (K_m and V_{max} values) has not been determined. In the present study, specific activity and kinetic parameters were determined for the three purified protein. In our study, the specific activity of SOD_Cl was 6832 units/mg and the introduction of gliadin peptide to SOD_Cl decreased the specific activity i.e. GliSOD_P51 (5191 units/mg) and GliSOD_P61 (4790 units/mg) (Figure 2). However, the specific activities of our SODs were higher than those of others reported SODs such as Cu/Zn-SOD from *Cicer arietinum*, 157.5 U/mg (Singh *et al*., 2013), Cu/Zn-SOD *Cucurbita moschata*, 1794 U/mg (Qin *et al*., 2012), Cu/Zn-SOD *Allium sativum*, 2867 U/mg (Liu *et al*., 2010), 4619 U/mg (Wang *et al*., 2012), and Cu/Zn-SOD from *Black soybean*, 4124 U/mg (He *et al*., 2008). The high specific activity of SODs allows the use of low dose for their efficacy resulting low toxicity and less side effect.

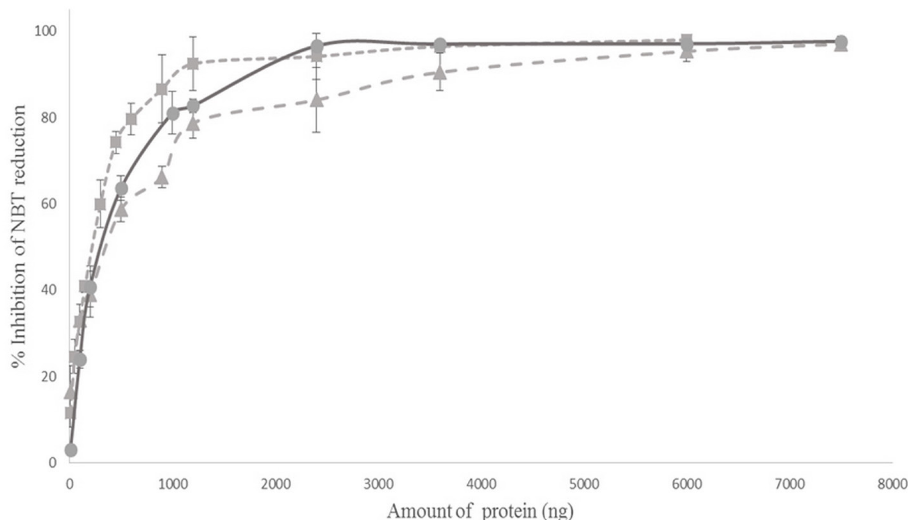

Fig. 2: Inhibition NBT reduction by recombinant SOD_Cl, GliSOD_P51 and GliSOD_P61. Specific activity is defined as the rSOD activity per milligram of total protein (U/mg) calculated by plot of percent inhibition versus log rSOD concentration. ( ) SOD_Cl, ( ) GliSOD_P51 and ( ) GliSOD_P61.

Table 2: Kinetic parameters of recombinant SOD_Cl, GliSOD_P51, GliSOD_P61 and others SOD from different sources.

Source	K_m	V_{max}	References
SOD_Cl	264.8 µM	2.461 µMs^{-1}	This research
GliSOD_P51	243.9 µM	2.111 µMs^{-1}	This research
GliSOD_P61	74.4 µM	1.03 µMs^{1}	This research
Cu,Zn SOD *Cicer arietinum*	10.16 ± 2.5 µM	-	(Singh *et al*., 2013)
Cu,Zn SOD *Curcuma aromatica*	47 ± 8 µM	1250 ± 24 units/mg	(Kumar *et al*., 2014)
Mn SOD *Stemona tuberosa*	2738.9 ± 32 µM	38.167 ± 0.021 µmol.min^{-1}mg^{-1}	(Niyomploy *et al*., 2014)
Mn SOD *Curcuma aeruginosa*	1510 ± 14 µM	254.1 ± 0.022 µmol.min^{-1}mg^{-1}	(Moon-Ai *et al*., 2012)

We also studied the kinetic properties the three SODs. The introduction of gliadin peptide in GliSOD_P51 only slightly altered the K_m and V_{max} values. On the other hand, the K_m and V_{max} values of GliSOD_P61 decreased about 3 and 2 fold, respectively than that of SOD_Cl. The inclusion of QQPYPQPQPF gliadin peptide may have reduced the number of active sites in the enzyme molecule. The three SODs displayed different K_m and V_{max} values than those Cu/Zn-SOD from different sources (Table 2).

As a therapeutic-enzymes, low K_M and high V_{max} values are preferable in order to maximize the efficiency at the very low enzyme and substrate concentrations as occurred in the human body. A low K_M means less substrate required for the enzymatic reaction to occur (a high affinity of the enzyme for the substrate). Since *in vivo* substrate levels usually are too low, reaction velocities are strongly affected by enzyme-substrate affinity (K_M) (Kumar *et al*., 2014). Compared to Cu/Zn-SOD from *C. arietinum* and *Curcuma aromatica*, the three SODs displayed higher K_M but lower than Mn SOD from *Stemona tuberosa* or *Curcuma aeruginosa*. However the three SODs still have good K_M value since for most enzymes, K_M lies between 10^{-1} and 10^{-7} M (Berg *et al*., 2002).

pH and thermal stability

Many environmental factors include temperature, pH, ionic strength, metal ions from physical and chemical processes can lead to proteins destabilization. Temperature and pH have the greatest influence on protein stability since the activity and stability of proteins, in general, are pH and temperature dependent (Talley and Alexov, 2010). In the present study, the effect of incubation in various pHs on superoxide dismutation activity was studied. Our results showed that the three SODs displayed equal stability in pH 2-8. The residual activity of GliSOD_P51 significantly decreased at higher pHs (9-11) and GliSOD_P61 at pH 10-11 compared to that of SOD_Cl (Figure 3A). These results did not corroborate with Lin studies at 2008 (Lin *et al*., 2002) showed that equal stability of SOD_Cl at pH 9-11, probably due to different assay used in this study.

The enzyme activity decreased in alkaline pH might be due to deprotonation of amino acid residue near active site that leads to inhibition (Kumar *et al*., 2016) or dimer dissociation due to disruption of hydrogen bonds that plays role in interactions between side chains of each monomer (Retnoningrum *et al*., 2017). The difference in residual activity for the three proteins in alkaline pH especially at pH 9-11 for GliSOD_P51 and GliSOD_P61 at pH 10-11 may be due to the presence of the gliadin peptide at the amino-terminus of the GliSOD_P51 and GliSOD_P61 that resulted in structure and conformational changes leading to decrease in enzyme activity.

Table 3: Effects of chemical reagents on the activity of purified SOD_Cl, GliSOD_P51 and GliSOD_P61.

Chemicals	Residual Activity (%)								
	1 mM			10 mM			20 mM		
	SOD_Cl	GliSOD_P51	GliSOD_P61	SOD_Cl	GliSOD_P51	GliSOD_P61	SOD_Cl	GliSOD_P51	GliSOD_P61
KCN	56.57 ± 6.07	58.17 ± 9.27	25.53 ± 5.45	26.87 ± 4.69	35.03 ± 11.97	18.31 ± 6.02	6.77 ± 1.88	28.81 ± 9.95	0 ± 0
EDTA	71.78 ± 7.57	92.37 ± 6.95	93.60 ± 1.87	39.58 ± 2.51	81.70 ± 4.61	78.48 ± 4.84	18.17 ± 3.47	51.77 ± 12.49	68.22 ± 1.72
$CaCl_2$	91.45 ± 7.38	94.99 ± 5.75	91.44 ± 3.05	76.49 ± 5.48	99.48 ± 2.85	87.03 ± 1.73	65.70 ± 4.10	101.89 ± 4.48	73.91 ± 4.47
KCl	71.53 ± 5.51	94.01 ± 5.53	97.67 ± 1.94	97.03 ± 2.31	96.62 ± 2.33	97.83 ± 1.89	104.10 ± 1.96	97.66 ± 1.84	100.91 ± 3.25
$MgCl_2$	79.32 ± 9.52	92.36 ± 5.91	94.45 ± 2.81	101.40 ± 0.98	96.72 ± 2.57	92.53 ± 3.62	104.76 ± 1.83	98.19 ± 4.34	91.20 ± 1.54
H_2O_2	45.20 ± 3.78	98.90 ± 8.37	96.95 ± 1.68	32.43 ± 5.29	93.06 ± 6.21	80.58 ± 4.78	14.61 ± 8.07	87.75 ± 13.79	64.77 ± 8.76
$ZnSO_4$	74.64 ± 9.41	90.45 ± 3.76	54.75 ± 7.98	27.00 ± 2.30	74.85 ± 7.87	18.65 ± 2.03	18.29 ± 0.70	46.12 ± 5.06	0 ± 0
$MgSO_4$	50.44 ± 2.72	93.88 ± 6.75	65.48 ± 7.42	76.19 ± 4.04	93.23 ± 7.06	75.94 ± 9.36	101.53 ± 0.65	98.38 ± 4.88	73.51 ± 5.12

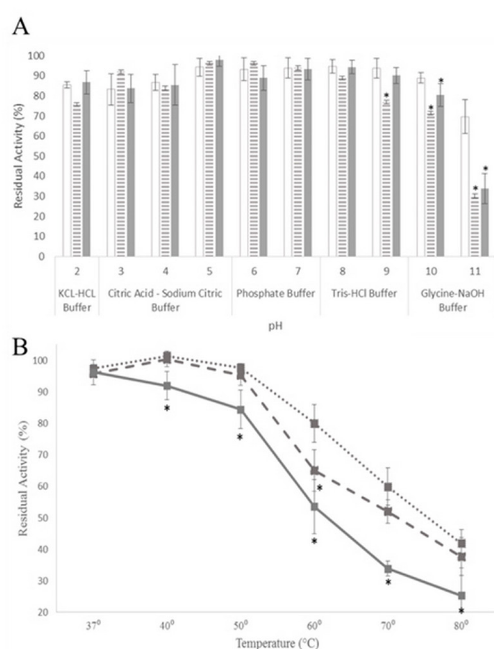

Fig. 3: Stability of the recombinant SOD_Cl, GliSOD_P51 and GliSOD_P61. (A) Effect of pH on the activity of SOD_Cl ( ), GliSOD_P51 ( ) and GliSOD_P61 ( ). The purified SOD was incubated in 0.05 M buffers pH range from 2.0 to 11.0 at 37°C for 60 min. (B) Effect of temperature on the activity of SOD_Cl ( ), GliSOD_P51 ( ) and GliSOD_P61 ( ). SOD was preheated at different temperatures (from 37°C to 80°C) for 60 min, respectively at pH optimum. The residual enzyme activity was determined. Results are presented as mean ± SD. (n = 3-8). An asterisk (*) sign indicates means are significantly different ($p < 0.05$).

Thermal stability is important to the commercial application of enzymes in several ways. The results showed that faster degradation of the three SODs was observed as higher temperature, especially above 50°C (Figure 3B). SOD_Cl, GliSOD_P51 and GliSOD_P61 were found to have moderate thermostability since the three of SODs retained more than 84% of their activity after incubation at 50°C for 1h. Their thermal stability was similar to SOD from *C. moschata* which retained more than 50% of its activity after incubation at 55°C for 2h (Qin *et al*., 2012) and to SOD from *Glycine max cv* that was not affected under 50°C, and gradually inactivated as the temperature was raised (Wang *et al*., 2012). Compared to SOD_Cl, there are significantly decreased residual activity at 60°C for GliSOD_P51 while GliSOD_P61 at 40-80°C. GliSOD_P61 displayed lower thermal stability compared to SOD_Cl and GliSOD_P51. These data conclude that addition of gliadin peptide decreases thermal stability of SOD_Cl. Based on the results above, the effect of the gliadin peptide on SOD_Cl stability showed that the introduction of gliadin peptide influences pH stability and thermal stability. However, since proteins showed good stability at pH 7 and 37°C, further permeability studies will be performed at pH 7.4 and at 37°C. This condition will minimize protein instability or degradation.

Effect chemicals on SOD stability

Depending on the type and concentration, ions or metal ions may also stabilize or destabilize a protein. The common sources of ions are excipients used in formulations or reagents used during manufacturing process while common sources of metal ions are the metal impurities from manufacturing equipment. In the present study, the changes of the SOD activity under different concentrations of different metal or ionic solutions were studied. The results are depicted in Table 3. Generally, treatments with KCN, EDTA, $CaCl_2$, H_2O_2, and $ZnSO_4$ caused reduction of activity of the three SOD proteins with various degrees and the activity level decreased with the increase of the metal or ionic solution concentration except for GliSOD_P51 treatment with $CaCl_2$. In contrast, the treatments with KCl, $MgCl_2$, and $ZnSO_4$ showed strong inhibition activity as the concentration decreased.

Compare to SOD_Cl, the addition of gliadin peptide in GliSOD_P51 and GliSOD_P61 increased their resistances when incubated with metal or ionic solutions at all concentrations. It can conclude that addition gliadin peptide may have caused more tight folding thus less exposed active sites leading to higher resistances, respectively than that of SOD_Cl. KCN and H_2O_2 are known to be inhibitors for Cu/Zn-SODs (Kumar *et al*., 2016; Paital *et al*., 2016). H_2O_2 is a strong oxidant and it can block the reduction and oxidation of Cu ion (Wang *et al*., 2012) or involves in the destruction of a histidine residue associated with copper (Bray *et al*., 1974). Significant inhibition by EDTA proved that SOD is a metalloenzyme. EDTA is a common chelating agent, which affects the enzyme activity by chelating the metal factor of the enzyme, in

this case, Cu^{2+} ion. Although it has a conserved Zn^{2+} binding site, the dismutation activity of SOD_Cl, GliSOD_P51 and GliSOD_P61 were lost in the presence of Zn^{2+} ions and an addition of 100 mM $ZnSO_4$ leads to precipitation (data was not shown). $ZnSO_4$ decreases SODs activity since it can combine with the active site of the enzyme and replaces Cu^{2+} ion (Wang *et al*., 2012).

Research (Wang *et al*., 2012) showed that chemical agents such as EDTA, $MgCl_2$, H_2O_2 and $ZnSO_4$ had a similar effect when incubated with *black soybean* SOD which remarkably inhibited the enzyme activity. This observation indicated that the effect of chemicals on SOD activity was highly dependent on the type of metals and their concentrations. Generally, taken together from the stability data from this study, the addition of gliadin peptides influences unit activity and stability however three of SODs showed good stability for their potential use in the pharmaceutical field, formulation purposes, and storage of the recombinant SODs.

CONCLUSIONS

In conclusion, SOD_Cl, GliSOD_P51 and GliSOD_P61 from synthetic codon-optimized gene were overexpressed in *E. coli* at a high level relative to total proteins as a soluble protein. The purified protein was confirmed to be as SOD_Cl, GliSOD_P51 and GliSOD_P61 based on its molecular weight size in a SDS-PAGE analysis and their dismutase activity using NBT-riboflavin oxidation-reduction based assay. In this paper, we show that two gliadin peptides fused to SOD_Cl retain the antioxidant activity of the GliSOD_P51 and GliSOD_P61 protein but slightly alter their specific activity, kinetic parameter, pH and thermal stability and stability against chemicals. However, the two of proteins still display high specific activity, quite stable to thermal exposure and activity at a wide pH range indicating they are kinetically stable and industrially relevant enzymes. Moreover, their activity at acid pH showed potentiality for oral administration. The presented work is the first report on high level expression of the three SODs in *E. coli* after codon optimization and the properties of the three SODs.

LIST OF ABBREVIATIONS

CAI	Codon Adaptation Index
GliSOD_P51	Gliadin Peptide P51 (LGQQQPFPPQQPYPQPQPF) fused with SOD_Cl
GliSOD_P61	Gliadin Peptide P61 (QQPYPQPQPF) fused with SOD_Cl
NBT	Nitro blue tetrazolium
PAGE	Polyacrilamide Gel Elctrophoresis
SDS	Sodium Dodecyl Sulphate
SOD	Superoxide Dismutase
SOD_Cl	SOD Citrus limon,
TEMED	Tetramethylethylenediamine.

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