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In Vitro Toxicity and ZO-1 Gene Expression Analysis Of GliSOD_P61 Treatment In Caco-2 Cell

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ABSTRACT

Superoxide dismutase (SOD) is a natural antioxidant protein and considered as a potential therapeutic compound. To mediate permeability, SOD *Citrus limon* (SOD_Cl) was fused with gliadin peptide QQPYPQPQPF (GliSOD_P61).. The aims of the present work were to determine its toxicity and permeability mechanism by measuring gene expression of ZO-1 in Caco2 cells. In vitro toxicity and permeability testing was evaluated using Caco-2 cells. Cytotoxicity was determined using Alamar Blue and Trypan Blue Assay. For permeability testing, 6 days Caco-2 monolayers were grown in Transwell chambers and proteins (100 µg/mL) were added to the donor chamber. SDS-PAGE analysis was performed to determine the presence of proteins at the apical and basolateral compartments. ZO-1 gene expression analysis was determined using quantitative polymerase chain reaction (qPCR) using cDNA as template. To obtain cDNA, total RNA isolated from Caco-2 cell culture used in permeability testing was reverse transcripted.. In vitro cytotoxicity test results indicated in all concentrations range under study, none of the tested proteins showed any significant cytotoxicity. SDS-PAGE analysis revealed that the presence of GliSOD_P61 in basolateral compartments while SOD_Cl was not. The gene expression of ZO-1 gene caused by SOD_Cl and GliSOD_P61 was not significantly different. Gliadin peptides (QQPYPQPQPF) mediates permeability with the mechanism of the tight junctions opening without affecting the ZO-1 gene expression.
Keywords: SOD Cl, GliSOD_P61, ZO-1 , Gliadin, qPCR

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INTRODUCTION

Superoxide dismutase (SOD) is ROS-metabolizing enzymes. It can metabolize superoxide anionic radicals form hydrogen peroxide and molecular oxygen. One of the reports is SOD from lemon (*Citrus limon),* an antioxidant enzyme that stable over a wide range of pH and stable to digestive enzyme [1]. In the previous study, SOD *Citrus limon* was fused with gliadin peptide to mediate its membrane permeability. Results showed it have high activity on antioxidant properties and can permeate into Caco-2 cells monolayer. Evaluation of protein toxicity is important since cell deaths lead to high permeability. This increase in permeability can be caused by loss of cell membrane integrity [2]. Gene expression analysis to examine the mechanism of permeability also important to increase confidence in the results of permeability tests.

Currently, there is no data reported on the toxicity and permeability mechanism of the SOD *Citrus limon* and its fusion to gliadin peptides in Caco-2 cells. This study was done to evaluate the cytotoxicity and permeability mechanism of SOD_Cl and GliSOD_P61 in Caco-2 cells. In vitro human adenocarcinoma cell lines Caco-2 were used to represent characteristics of adult colon cells (mimicking the GI epithelium). It is the most extensively characterized and useful cell model in the field of drug permeability and in vitro toxicology [3][4]. Caco-2 cells, derived from a human colorectal carcinoma, undergo spontaneous enterocytic differentiation in culture. When they reach confluency on a semipermeable porous filter, the cell polarity, and tight junctions are well established [5].

The most common parameter used to evaluate toxicity is cell viability [6] which can be assessed by a variety of assays such as LDH leakage assay, a protein assay, the neutral red, the MTT assay and Alamar Blue Assay [7] [8] [9]. In this study, the effects of various concentrations of SOD_Cl and GliSOD_P61 on the viability of Caco-2 cells monolayer were assessed by measuring cell loss and metabolic activity using Alamar Blue assay (cytochrome oxidase activity) and Trypan Blue dye exclusion assay. Gene expression analysis of ZO-1 was determined using quantitative polymerase chain reaction (qPCR) since ZO-1 are considered as key elements of the TJ complex in paracellular permeability [10]. It is known that ZO-1 can be detached from the complex TJ due Zonulin, causing the opening of paracellular [11].

MATERIALS AND METHODS

Chemical materials:

Reagents for Sodium Dodecyl SulfatePolyacrilamide Gel Electrophoresis (SDS-PAGE) ; Tris base (Promega), glisin (Fisher Scientific), SDS (Promega)}, Pierce™ Unstained Protein MW Marker (Thermo Scientific), β- mercapthoethanol (Promega), bromophenol blue (Sigma-Aldrich), bis-acrylamide (Merck), amonium persulfat (Bio-Rad), Tetramethylethylenediamine (Thermo Scientific), coomassie blue (Merck), methanol, glacial acetic acid, Dulbecco's Modified Eagle Medium (DMEM) with 4 mM L-Glutamine (Lonza); Fetal Bovine Serum (FBS) (Life Technologies), 1% Non Essential Amino Acids (NEAA) (Lonza), puromycine 100 µg/mL, trypsin–EDTA solution (Gibco), Hank's Balanced Salt Solution (HBSS) (Gibco), trypan blue dye (0.45 w/v) (Gibco) , alamar blue (Invitrogrn), Trizol (Life Technologies), chloroform (Merck) , ethanol (Merck), DNAse I Recombinant Enzyme (Roche), RevertAid First Strand cDNA synthesis Kit (Thermo Scientific), ZO-1_CACO2_FOR (5'-ATCCCTCAAGGAGCCATTC-3'), ZO-1_CACO2_REV (5'-CACTTGTTTTGCCAGGTTTTA-3'), hGAPDH_f (5'-GGTATCGTGGAAGGACTCATGAC-3'), hGAPDH_r (5'-ATGCCAGT GAGCTTCCCGTTCAG-3') (IDT DNA), KAPA SYBR FAST qPCR Master Mix (2X) Bio-Rad iCycler (KAPA Biosystems), Water, diethylpyrocarbonate (DEPC) treated (1st Base), agarose (Thermo Scientific), DNA ladder 100 pb (Vivantis).

Instruments:

SDS-PAGE electrophoresis equipment (Bio-Rad), DNA electrophoresis equipment (Bio-Rad), Microplate Spectrophotometer (Multiskan™ GO, Thermo Fisher Scientific), µDrop™Plate (Thermo Scientific), CFX96 Real-Time PCR (Bio-Rad), Biosafety Cabinet (ESCO), CelCulture CO2 Incubator (ESCO), microscope (Olympus IX51).

Software : ImageJ V.1.43u, Primer BLAST, OligoAnalyzer (IDT DNA), UMelt™ dan Bio-Rad CFX Manager 3.1.

Methods:

Cell thawing

Caco2 cells frozen at passage 8-12 were thawed suspending in 10 ml of DMEM medium in a 50 ml falcon tube. Thawed cells were centrifuged at 1000 rpm for 10 min at 4 °C. Supernatants were removed, and cell pellets were re-suspended in culture medium, and seeded at 10^6/T-75 flask. Flasks were incubated at 37 °C and 5% CO_2 for 2–3 days.

In vitro cytotoxicity assays

In vitro cytotoxicity assays were performed according to the protocol described in the ISO 10993-5 guide [12]. Confluent cells (80-90%) in T-75 flasks were detached using a trypsin–EDTA solution followed by the addition of culture medium to neutralize trypsin, then the cells were pelleted by centrifugation at 1000 rpm for 10 min at 4 °C. Supernatants were removed, and cell pellets were re-suspended in culture medium and counted using a hemacytometer. Cells were seeded into 24-well plates at a density of 1×10^5 cells·cm^{-2}. Plates were placed in an incubator overnight to allow for attachment and recovery. After culturing for 24h, the cells were used for the cytotoxicity tests described below.

Alamar Blue Assay. Cells were treated with a sample of protein (SOD Cl and GliSOD_P61) for 1 h and 24 h of preincubation (10, 50 & 100 µg/ml) and then gently rinsed three times with HBSS (Hanks' Balanced Salt Solution), and 200 µL of fresh HBSS containing Alamar Blue was added to each well. The assay was performed according to the manufacturer's protocol. After incubating for 4h at 37ºC, the colorimetric change in the Alamar Blue solution during incubation was assessed from the absorbance values at 570 nm and 600 nm. Absorbance was measured with a Microplate Spectrophotometer (Multiskan™ GO, Thermo Fisher Scientific). Wells containing medium and Alamar Blue without cells were used as negative control.

Trypan Blue Exclusion Assay. The effect of protein (SOD Cl and GliSOD_P61) on membrane integrity was evaluated using trypan blue exclusion method as previously described [8]. Caco2 cells were plated in the 24-well plates (1×10^5 cells per well) and incubated for 24 h. Then, samples were introduced to cells with different concentrations (10, 50 & 100 µg/ml) in the culture medium. One and twenty-four hours later, the supernatant was collected and the cells were detached with 300 µL trypsin–EDTA solution. The mixture of the supernatant and detached cells was centrifugated at 1000 rpm for 10 min at 4 °C. Then, the residue was added with 800 µL Trypan blue solution and dispersed. After 5 min staining, cells were counted using cytometer. The dead cells were stained with blue color. Cell mortality (%) is expressed as a percentage of the dead cell number/the total cell number. The cells were stained with trypan blue dye (0.4% w/v) and the number of viable and dead cells were scored under the light microscope with Neubauer' hemocytometer.

Permeability Testing 6 Days Old Caco-2 Cell

Caco-2 cell monolayers (The human intestinal Caco-2 cell line was originally obtained Dexa Laboratory of Biomolecular Sciences) were prepared by cultivating Caco-2 cells culture medium (the antimicrobial penicillin and streptomycin solution were replaced by 730 nM (around 0.4 µg/ml) of the antibacterial puromycin. Cells were split after reaching 80-90% confluence. In the passage operation, the cells were detached from the culturing flask by trypsinization. Cells were seeded at 600.000 cells/cm^2 onto polycarbonate membrane filters cell culture inserts (Transwell, mean pore size 0.45 µm). The cells were allowed to grow and differentiate for 6 days. The medium was changed every second day [3]. Cells were incubated at 37ºC and samples SOD_Cl and GliSOD_P61 (100 µg/mL) were prepared in Hank's Balanced Salt Solution (HBSS) added to the donor chamber, and the receptor compartment also was filled with HBSS. After 2 h samples were taken and analyze using SDS-PAGE.

ZO-1 mRNA expression by real-time quantitative polymerase chain reaction

Total RNA was extracted from cells using Trizol (Life Technologies) then RNA was reversed transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit according to the manufacturer's instructions. Amplification conditions for GAPDH (housekeeping gene) and ZO-1 were performed with 1000 ng cDNA, 10 µL KAPA SYBR FAST qPCR Master Mix (2X), 1 µL (20 µM) of each primer and DEPC water. Cycling conditions were as follows denaturation for 3 minutes at 95 ºC, amplification for 40 cycles with denaturation at

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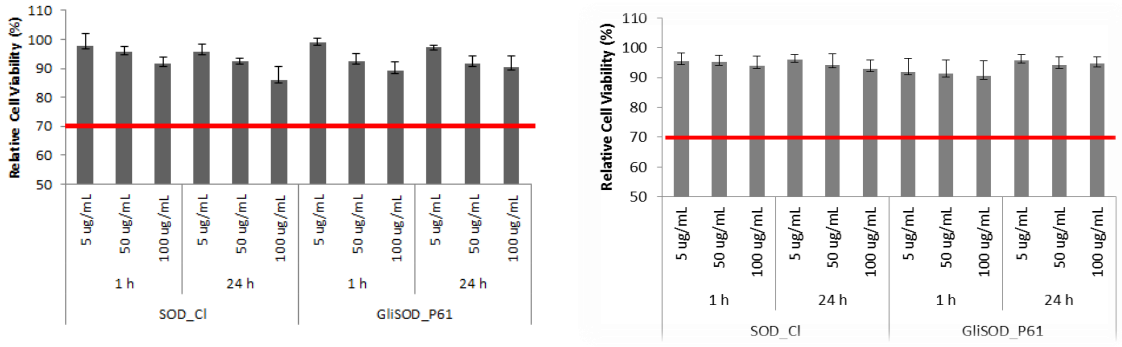
95 °C for 3s, annealing, and extension for 20 s at 60°C. PCR products were sequenced to validate the specificity of the amplification. The Livak method for relative gene expression analysis was used.

RESULTS & DISCUSSION

While viability shows the activity of cell mitochondria, the mortality indicates death of the cell. In this study, the cell mortality is monitored by Trypan blue exclusion assay [13]. Failure to exclude Trypan blue reflects disruption of membrane integrity [14] and the mortality is expressed by the ratio of dead cells in all cell. For viability assays, we performed Alamar Blue assays. Viability assay is a basic steps in toxicology that show metabolic activities explain the cellular response to a toxicant [15]. Alamar Blue reagent, which contains a specific oxidation-reduction indicator has been used to assess the effect on mitochondrial dehydrogenase activity based on a colorimetric reaction.

The cytotoxicity of the recombinant SOD Cl and GliSOD_P61 in Caco-2 cells in vitro are presented as a percentage of cell viability relative to the negative (100%) and positive controls, as determined using the Alamar Blue assay. The standard deviation was calculated for the 3 replicates. Quantification of cytotoxicity was determined by measuring cell death according to protocols described in ISO 10993-5, this international guide states that a reduction in cell viability by more than 30% is considered a cytotoxic effect. [12].



(A) (B)

Figure 1. In vitro toxicity experiment. Alamar Blue Assay (A) and Trypan Blue Assay (B) Relative viabilities of Caco-2 cells after being incubated with samples protein SOD_Cl and GliSOD_P61 with series of concentrations. Error bars depict the primary results based on standard deviation. The red line separates the cell viability values above and below 70%.

An incubation time used are 1 h was used to minimize instability or degradation of protein and 24 h was used for mimicking the GI transit time. Samples of protein (SOD Cl, GliSOD_P51, GliSOD_P61) were tested for 1 h and 24 h of preincubation (10, 50 & 100 µg/ml). According to the results of Alamar Blue Assay and Trypan Blue Assay, all concentrations range under study were not significantly different from that of control monolayers (Fig.1) indicating both of protein are non-toxic to human intestinal Caco-2 cell line. Based on microscopic observation there is no cell detachment from the monolayer, which would reflect cell death or damage to the cell-matrix contact (data was not shown).

Based on in vitro toxicity results, samples protein with concentration 100 µg/ml was used for permeability experiment since its non toxic and for easy protein visualization in SDS-PAGE. The permeation study was conducted for 2 h. SOD *Citrus limon* without gliadin peptide addition (SOD_CL) used as a control. Permeated protein across Caco-2 cell monolayer is presented (Fig.2). After a 2-h incubation with control (no protein), protein SOD_Cl (without gliadin P61) and GliSOD_P61 (SOD_Cl with gliadin P61), the protein electrophoresis showed the existence of GliSOD_P61 in basolateral compartment compared to SOD_Cl, there is no existence of SOD_Cl in basolateral compartment. The results from this study showed that GliSOD_P61 transported across the Caco-2 cell monolayer while SOD_Cl was not. These preliminary studies suggested that these gliadin P61 (QQPYPQPQPF) may have activity as absorption enhancers.

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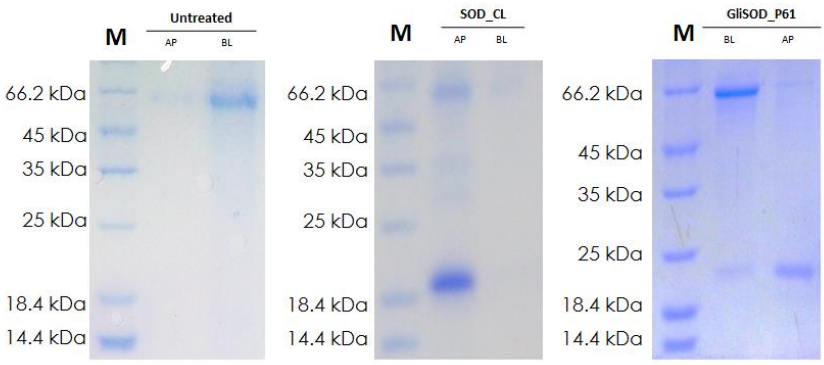

Figure 2. Analysis of SDS-PAGE of permeability study. (A) Untreated (B) SOD_Cl (C) GliSOD_P61, AP=Apical , BL=Basolateral

Analysis permeability mechanism by measuring gene expression of ZO-1 was done through RNA isolation. Caco-2 cells that have been used in the permeability test was then trypsinized for total RNA isolation. The total RNA was reverse transcribed into cDNA and it will be used as a template for gene expression analysis using qPCR method. In present study, ZO-1 gene expression was measured. To test primer specificity, dissociation curve analysis and agarose gel electrophoresis were done.

The result of ZO-1 primer specificity was shown in Figure 3. Dissociation curve shows a double peak, confirmed by the results of agarose gel electrophoresis of qPCR products also showed the presence of two bands with 209 and269 bp size. In silico analysis using NCBI BLAST software. Primer of ZO-1 is known to produce three different amplicons with size 209, 230 and 269 bp. The three types of amplicons are the amplification products of different mRNA variants. But amplicon is still a gene amplification products of ZO-1 mRNA.

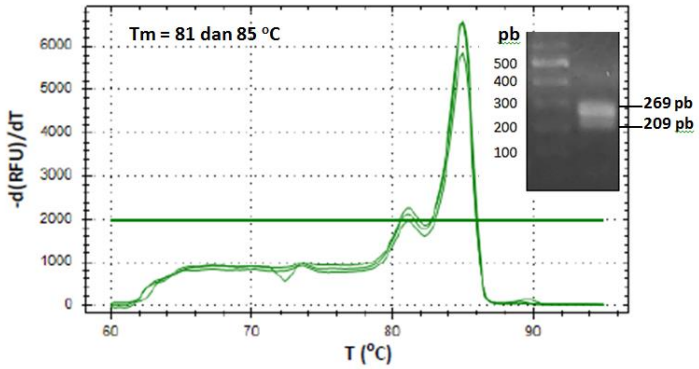

Figure 3. Melting curve (dissociation curve) and DNA electropherogram of ZO-1 amplicon using the primers ZO-1. A melting curve is the result of three different samples.

In this study, data interpretation was conducted using relative quantification Livak method [16]. Relative quantification method is to measure changes in the level of mRNA expression relative to other RNA expression levels. Furthermore, the relative parameters can be added, for example by comparing the expression level of target genes in the sample relative to the untreated control [16]. In relative quantification method Livak, the primer is assumed to have 100% efficiency, which means that in each cycle to form amplicons by 2-fold [17].

Results of ZO-1 gene expression analysis was shown in Figure 4. It shows the gene expression levels of ZO-1 in untreated samples was not significantly different with Caco-2 cells were treated with SOD_Cl and GliSOD_P61. Based on these results we can conclude that the mechanism of gliadin peptides on GliSOD_P61 in increasing the permeability of SOD does not involve ZO-1. A related study was found, the study showed that the increased permeability of the intestinal epithelium paracellular can occur without changes in the expression of ZO-1 [18].

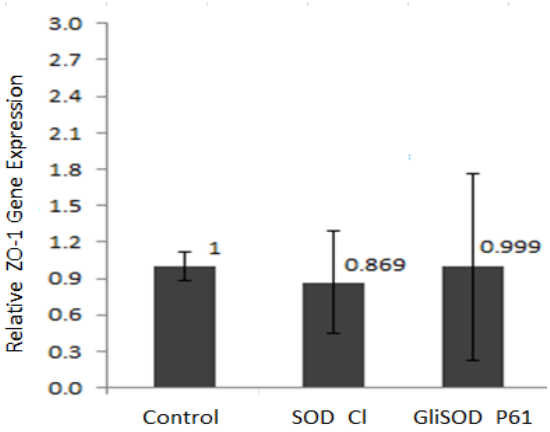

Figure 4 Effect of SOD_ Cl and GliSOD_P61 on the mRNA expression of junctional proteins ZO-1 in Caco-2 cells. mRNA levels of ZO-1 were expressed relative to each mRNA level of the control monolayers (untreated), which were assigned a value of 1. Data (means ± standard errors of the means) were compiled from three independent experiments.

CONCLUSION

GliSOD_P61 was found in basolateral compartments while SOD_Cl was not, indicating gliadin peptide (QQPYPQPQPF) enhanced Caco-2 cell permeability. SOD_Cl and GliSOD_P61 also in all concentrations range under study (10, 50 & 100 µg/ml) shown to be nontoxic to human intestinal Caco-2 cell line. The gene expression of ZO-1 gene caused by SOD_Cl and GliSOD_P61 was not significantly different. These data clearly indicate that GliSOD_P61 as a candidate for permeation enhancer to mediates permeability with the mechanism of the tight junctions opening without affecting the ZO-1 gene expression.

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