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Antioxidant and antimicrobial properties of chitosan-sugar complex

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Abstract: Antioxidant and antimicrobial properties of chitosan-sugar complex from six types of sugar (glucose, fructose, lactose, arabinose, maltose, and galactose) were investigated. Antioxidant properties were measured by the DPPH test and a measurement of reducing power. These two methods showed the same profile of antioxidant activity. All chitosan-sugar complexes demonstrated better antioxidant activity than chitosan. Chitosan-arabinose complex and chitosan-galactose complex seemed the most effective antioxidant activity. The IC_{50} values of chitosan-arabinose complex and chitosan-galactose complex were 26.38 g/ml and 28.50 g/ml, respectively. While antiradical efficiencies were 0.03791 and 0.03509, respectively. The reducing power of chitosan-galactose complex was similar to reducing power of BHA (Butylated hydroxyanisole) ($p > 0.5$). High correlation between antioxidant activity and browning product (A_{420}) was observed ($r=0.959$ for antiradical efficiency). It is indicated that browning product exhibited antioxidant activity. For antimicrobial activity, most chitosan-sugar complexes were more effective than chitosan. Chitosan-sugar complex could be potential alternative natural product for synthetic food additive replacement and also meet consumer safety requirement.

Keywords: Chitosan, Maillard reaction, antioxidant agent, antimicrobial agent

Introduction

Research on alternative natural products for synthetic food additive replacement has increased because of the need to meet safety standards. Natural food additives from many biological materials have attracted much interest because of their supposed safety. Potent sources of natural food additive compounds have been found in several types of natural material. One of these is chitosan. Chitosan, a deacetylated derivative of chitin, is a linear copolymer composed of mainly D-glucosamine and some proportion of N-acetyl-D-glucosamine with β-1,4- linkage (Rinaudo, 2006; Shahidi, 2007). Chitosan is biocompatible, nonantigenic, nontoxic and biofunctional (Hirano *et al*., 1990; Li *et al*., 1992; Tharanathan and Kittur, 2003). It has received much attention as a new excipient and/or functional material of high potential in the pharmaceutical and food industries (Illum, 1998). In a subacute toxicity study, Kim *et al*. (2001) showed that in rats no observed adverse effect level for chitosan oligosaccharide was considered to be over 2,000 mg kg^{-1}. Chitosan derived from shrimp has been recognized as a GRAS (Generally Recognized as Safe) for generally use in foods, including meat and poultry, and for various technique effects by the US food and Drug Administration (2005). In Korea and Japan, chitosan has been approved as a food additive since 1995 and 1983, respectively (Weiner, 1992; KFDA, 1995).

The Maillard reaction, or nonenzymic browning, occurs when carbonyl groups, usually from reducing sugar, condense with free amino groups, most commonly from peptides and proteins. It is largely responsible for the color and flavor of many processed foods (Ames, 1988). The antioxidant activity of browning products has been supported by many systems. The examples are low molecular carbonyl compound and amino acid model system (Kawashima *et al*., 1977), porcine plasma protein-sugar model system (Benjakul *et al*., 2005) and casein-glucose model system (Gu *et al*., 2009). The Maillard reaction products from these systems show antioxidant activity.

The applications of chitosan to use as antimicrobial material for food have been widely reported in literatures. For example, in fruit and vetgetables (Chien *et al*., 2007; Badawy and Rabea 2009), bread (Lee *et al*., 2002; Ahn *et al*., 2003), seafood (Tsai *et al*., 2002; López-Caballero *et al*., 2005), meat (Sagoo *et al*., 2002; Rao *et al*., 2005) and sausage (Lin and Chao, 2001; Soultos *et al*., 2008). The possible mechanisms for chitosan's antibacterial activity has

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been described by many scientists (Hadwiger *et al*., 1986; Papineau *et al*.,1991; Sudarshan *et al*., 1992). For example, the reaction of positive charged chitosan with negative charged molecules at the bacterial cell surface may show an effect on cell permeability and a mechanism related to the binding of chitosan with bacterial DNA to inhibit RNA synthesis. The antibacterial and/or antifungal characteristics of chitosan and its derivatives have been effective in commercial disinfectants. Chitosan has several advantages over other types of disinfectants in that it possesses a higher antibacterial activity, a broader spectrum of activity and a lower toxicity for mammalian cells (Liu *et al*., 2001).

The purpose of this study was to investigate antioxidant and antimicrobial properties of the chitosan-sugar complex. The Maillard reaction product or chitosan-sugar complex was prepared from the reaction of chitosan and six types of reducing sugar. This complex should serve the needs of recent consumer trends that tend towards an interest in natural products and also requires natural food additives to replace synthetic food additive.

Materials and methods

Chemicals

We purchased 2,2-Diphenyl-1-picrylhydrazyl (DPPH), chitosan (chi), glucose (glu), maltose (mal), fructose (fru), galactose (gal), lactose (lac) and arabinose (ara) from Sigma-Aldrich, Inc (St.Louis, MO, USA). We obtained acetic acid, methanol, potassium ferricyanide, trichloroacetic acid, ferric chloride, disodium hydrogen phosphate and sodium dihydrogen phosphate from Merck (Darmstadt, Germany). Butylated hydroxyanisole was purchased from Fluka (Switzerland).

Bacterial culture

Escherichia coli (TISTR 780), *Pseudomonas aeruginosa* (TISTR 781), *Staphylococcus aureus* (TISTR 1466) and *Bacillus cereus* (TISTR 687) obtained from The Thailand Institute of Scientific and Technological Research (TISTR) were used during this study. The bacterial cultures were grown on nutrient agar. The isolates were subcultured twice before inoculation.

Preparation of chitosan-sugar complex

Chitosan, in powder form, was dissolved in 1% (v/v) acetic acid at 1 % (w/v) on dry basis. This chitosan solution was used to prepare the mixed solution between chitosan and sugar. The mixed solution was prepared by adding 1% (w/v) sugar to chitosan solution. Then the mixture was autoclaved for 15 min. Six types of sugar (glucose, fructose, lactose, arabinose, maltose and galactose) were used to prepare the complex for this study.

UV-absorbance and browning color measurements

UV-absorbance and browning color measurements were used to indicate the intermediate stage and final stage of Maillard reaction, as described by Ajandouz *et al*. (2001). The aqueous solution of chitosan, sugars and chitosan-sugar complexes were measured at room temperature at 294 nm (for intermediate stages) and 420 nm (for final stage), respectively. The experiments were carried out in triplicate.

Antioxidant activity measurement

DPPH Assay

The DPPH assay was determined using the method of Singh *et al*. (2002). Samples were diluted to various concentrations with methanol. One hundred –microliter aliquots of various concentrations of the samples were added to 5 mL of 0.1 millimolar methanol solution of DPPH. Then the solution was shaken strongly. After a 20 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. The purple color bleaching of the DPPH reagent showed as positive antioxidant activity. Inhibition of free radicals by DPPH in percent (I %) was calculated as in equation 1. For control, methanol solution was used. The solution concentration with 50% inhibition (IC_{50}) was calculated from the plot of the inhibition percentage against the extraction concentration. Antiradical Efficiency, AE) was calculated as in equation 2. The assay was carried out in triplicate.

$$\%\ radical\ scavenging\ activity = \frac{(control\ OD - sample\ OD)}{control\ OD} \times 100 \quad \text{--------- 1}$$

Where control OD = absorbance value of control after 30 min

sample OD = absorbance value of sample after 30 min

$$AE = \frac{1}{IC_{50}} \quad \text{--------- 2}$$

Where AE = Antiradical Efficiency

IC_{50} = Concentration that had 50% inhibition

Reducing power determination

The reducing power of the solution was carried out as described by Oyaizu (1986). The various concentrations of the samples in distilled water

(2.5 ml) were mixed with sodium phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50 ºC for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 1000 rpm for 8 min. The upper layer of solution (5 ml) was mixed with distilled water (5 ml) and $FeCl_3$ (1.0 ml, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated reducing power.

Antimicrobial activity measurement

Antimicrobial activity of chitosan and chitosan-sugar complex was analyzed against four types of food spoilage and pathogenic bacterial (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus cereus*). The test cultures were inoculated into a 25 ml nutrient broth and incubated overnight at 37ºC. Different volumes of chitosan and chitosan-sugar complex were added to the nutrient broth tubes in order to obtain a final concentration of 5, 10,15 % (v/v). The nutrient broth tubes containing chitosan and chitosan-sugar complex were then inoculated with 0.1% bacterial suspension. At the initial time (0 h), each sample was serial diluted (10-fold dilution) in nutrient broth, 0.1 ml aliquot of each dilution was plated in plate count agar by the spread plate method, and counted after incubating at 37 °C for 18 h. This gave the initial bacterial amount and was expressed as log cfu/ml. After that, all the sample tubes were incubated for 6 and 24 h at 37 °C. The aliquots were again taken, serially diluted in nutrient broth, plate on plate count agar and count (expressed as log cfu/ml). Antibacterial activity of samples was evaluated from the decrease in log cfu/ml of test sample.

Statistical analysis

The data were subjected to analysis of variance (ANOVA). The significance of the difference between means was determined by the Duncan's Multiple Range Test ($p \leq 0.05$) using SPSS. The correlations between IC_{50}, Antiradical Efficiency (AE), $Absorbance_{294}$ and $Absorbance_{420}$ were performed using Pearson's correlation coefficient. The experiments were performed in triplicate.

Results and Discussion

Determination of browning reaction

Browning reaction of chitosan-sugar complex was studied via UV-absorbance and browning intensity. For intermediate stages, UV-absorbance of Maillard reaction was measured at 294 nm. While the absorbance at 420 nm was measured for the final stage of Maillard reaction. When the browning reactions of chitosan-sugar complex from 6 sugars were compared (Figure1), chitosan-arabinose complex seemed the most effective. The next orders of browning reactions were the complex of chitosan with galactose, glucose, lactose, maltose and fructose, respectively. During the early studies of Maillard reaction, several results showed that the browning of fructose solution in the presence of amino acids in the model system took place more rapidly than that of glucose (Hodge, 1953; Reynolds, 1965). Nevertheless, we find conflicting reports (Ellingson *et al*., 1954; Bobbio *et al*., 1981; Baxter, 1995). Moreover, it has also been reported that browning of fructose solution was either more or less extensive than that of glucose, depending on the heating conditions (Kato *et al*., 1969; Buera *et al*., 1987; Wijewickreme *et al*., 1997). For this study, glucose shows more reaction than fructose.

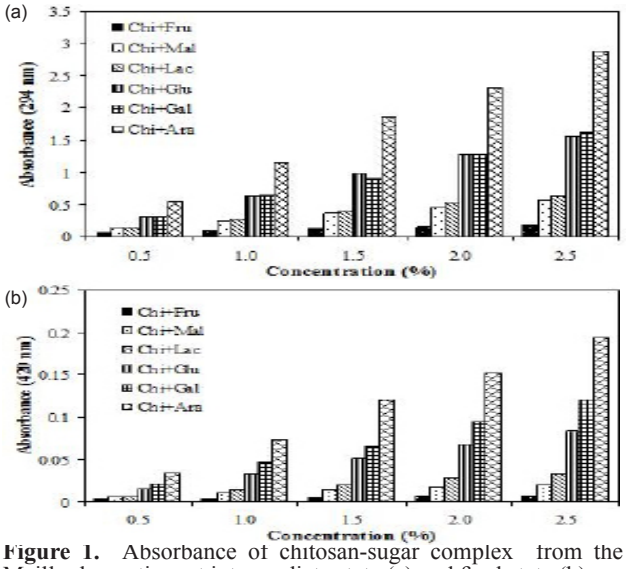

Figure 1. Absorbance of chitosan-sugar complex from the Maillard reaction at intermediate state (a) and final state (b)

Antioxidant activity measurement

DPPH radical scavenging activity

DPPH has been used extensively as a free radical to evaluate reducing substances (Cotelle *et al*., 1996). Percent DPPH scavenging activity of all chitosan-sugar complexes were concentration dependent (Figure 2). The activity of all chitosan-sugar complexes was so high though chitosan or sugar alone was low. These confirm the evidence that DPPH radical scavenging activity of chitosan and sugar complex was better than chitosan or sugar alone.

The IC_{50} and Antiradical Efficiency (AE) of chitosan and sugar are shown in Table 1. As IC_{50} and Antiradical Efficiency of chitosan were 2102.12 g/ml and 0.00048, IC_{50} and Antiradical Efficiency of sugars were vary from 19571.56 - 2531.34 g/ml and

0.00005 - 0.00040, respectively. The low IC_{50} and the high AE is the more effective. Then chitosan had better DPPH radical scavenging activity than sugar.

Table 2 shows IC_{50} and Antiradical Efficiency of chitosan-sugar complexes. Chotosan-arabinose complex appears the most effective. The result was in agreement with Benjakul *et al*. (2005) who report that antioxidant activity of the Maillard reaction product from galactose was better than from glucose and fructose, when studied in porcine plasma protein–sugar model system. The IC_{50} and antiradical efficiency of this complex are 26.38 g/ml and 0.03791, respectively. When the IC_{50} of chitosan-arabinose complex and chotosan-galactose complex is compared, it is not significantly different ($p > 0.5$). The antioxidant activity of chitosan-arabinose complex and chitosan-agalactose complex is quite similar, though antiradical efficiency is significantly different ($p \leq 0.5$). The correlation between antioxidant activity (IC_{50} and AE) and the intensity of nonenzymatic browning reaction was shown in Table 3. The intensity of nonenzymatic browning reaction was measured at 294 nm for intermediate stages and 420 nm for final stage. A high correlation between antioxidant activity and intensity of nonenzymatic browning reaction was observed. This indicated the effect of browning reaction on antiradical efficiency.

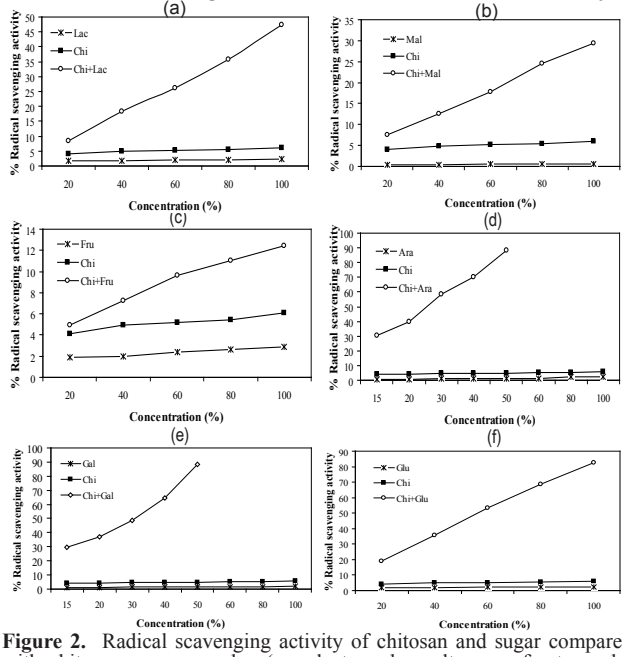

Figure 2. Radical scavenging activity of chitosan and sugar compare with chitosan-sugar complex (a : lactose, b: maltose, c: fructose, d: arabinose, e: galactose and f: glucose) by DPPH method at different concentration.

Table 1. IC_{50} and antiradical efficiency of chitosan solution and sugar solution

Solution	IC_{50}(g/ml)	Antiradical Efficiency
Maltose	19571.56[a]	0.00005[a]
Glucose	13487.11[b]	0.00007[a]
Lactose	7604.60[c]	0.00013[ab]
Glactose	4844.83[cd]	0.00021[ab]
Fructose	3710.59[cd]	0.00027[b]
Arabinose	2531.34[d]	0.00040[c]
Chitosan	2102.12[d]	0.00048[d]

Remark: - IC_{50}: The solution concentration (g of solution/ml) that had 50% inhibition.
- The different letters within the same column indicate significant differences between treatments after one-way ANOVA ($p \leq 0.05$).

Table 2. IC_{50} and antiradical efficiency of chitosan-sugar complex solution

Solution	IC_{50} (g/ml)	Antiradical Efficiency
Chotosan-fructose complex (Chi+Fru)	488.45[a]	0.00205[a]
Chotosan-maltose complex (Chi+Mal)	172.56[b]	0.00579[b]
Chotosan-lactose complex (Chi+Lac)	106.35[c]	0.00940[c]
Chotosan-glucose complex (Chi+Glu)	57.52[d]	0.01738[d]
Chotosan-galactose complex (Chi+Gal)	28.50[e]	0.03509[e]
Chotosan-arabinose complex (Chi+Ara)	26.38[e]	0.03791[f]

Remark: - IC_{50}: The solution concentration (g of complex solution/ml) that had 50% inhibition.
- The different letters within the same column indicate significant differences between treatments after one-way ANOVA ($p \leq 0.05$).

Table 3. Correlation between IC_{50} and antiradical efficiency and browning color of non enzymatic reaction ($Absorbance_{294}$ for intermediate stages and $Absorbance_{420}$ for final stage)

	IC_{50}	Antiradical Efficiency (AE)
$Absorbance_{294}$	-0.680**	0.918**
$Absorbance_{420}$	-0.713**	0.959**

** Correlation is significant at the 0.01 level (2-tailed).

The antioxidant efficiency was considerably improved when reacted chitosan with sugar, compared with chitosan or sugar alone. An increase in the antioxidant activity of chitosan-sugar complex may be the same reason as described by Guérara and Sumaya-Martinez (2003). They showed that the study of the chromatographic profiles obtained before and after the Maillard reaction of glucose and protein hydrolysates found changes in absorbance at 280 nm, indicating molecular rearrangements with phenolic structure that could be involved in the improvement of the antioxidant activities. In addition, Hayase *et al*. (1990) showed that melanoidins were strongly active in scavenging active oxygen species, may be another reason.

The antioxidant properties of the Maillard browning products (MRP) are widely documented. For example: glucose and lysine system (Yoshimura *et al*., 1997) ovalbumin and D-aldohexoses system (Sun *et al*., 2006) and coffee brews system (Cämmerer and Kroh, 2006). While the applications of MRP product were as an inhibitory agent towards black tiger shrimp polyphenoloxidase (PPO) (Matmaroh *et al*., 2006), an antioxidative agent for sardine products (Tanaka *et al*., 1988), an antioxidative agent for butter cookie (Bressa *et al*., 1996) and etc.

Reducing power determination

The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain through donating a hydrogen atom (Gordon, 1990). When compared with chitosan and sugar alone, chitosan-sugar complex of all sugar possessed the best ability to reduce iron (III) and also in a linear concentration dependent pattern (data not show). Chitosan-galactose complex exhibited the most effective and was similar to reducing power of BHA (Figure 3).

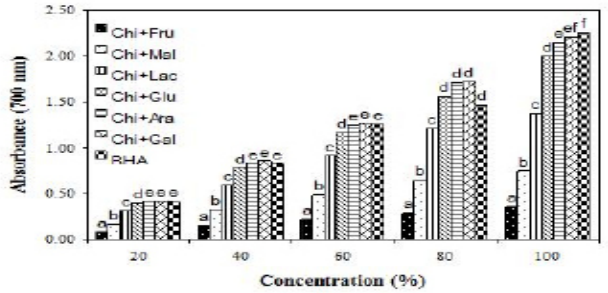

Figure 3. Reducing power of chitosan-sugar complex at various concentrations compared with BHA (100% complex solution: 1 g chitosan and 1 g sugar in 100 ml of 1% acetic acid solution and autoclaved, 100% BHA :1 g BHA in 100 ml distilled water.

Antimicrobial effect of chitosan-sugar complex

The antimicrobial properties of chitosan-sugar complex from six types of sugar (glucose, fructose, lactose, arabinose, maltose, and galactose) was analyzed against four types of food spoilage and pathogenic bacteria (*Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*). The obtained complex was the product of the Maillard reaction. The results were shown in figure 4-7.

The antimicrobial activity of chitosan was proposed by many mechanisms. Such as, the availability of an amino group on chitosan can absorb the essential nutrient that is necessary for bacterial growth (Cuero *et al*., 1991; Tsai *et al*., 2002). The interaction between the positive charge of the chitosan molecule and the negative charge of the microbial cell membrane, results in changes to the membrane permeability (Sudarshan *et al*., 1992; Helander, 2001). Film formation of chitosan over the surface of microbial cell membrane that prevent the nutrient from entering the cell (Zheng and Zhu, 2003). The mechanism of chitosan complex for antimicrobial activity from this study may be from more than one mechanism.

Effect of chitosan and chitosan-sugar complex on Bacillus cereus

In this study, we reported on antimicrobial activity of chitosan-sugar complex by mean of the Maillard reaction product. Figure 4 shows antimicrobial activity of chitosan and chitosan-sugar complex against *Bacillus cereus* at various concentrations (5%, 10% and 15%). After 6 hours, chitosan-fructose complex exhibited the best effect at all concentration. However, the growth of *Bacillus cereus* was completely suppressed and no viable cell could be detected at 15% concentration of all compounds after 24 hours. This event did not appear at low concentration, thus the bactericidal effect of chitosan and chitosan-sugar complex is concentration dependent. *Bacillus cereus* is a gram positive bacterial, then the mechanism of microbial inhibition may be the formation of chitosan and chitosan-sugar complex film over the surface of microbial cell membrane that prevent the nutrient from entering the cell (Zheng and Zhu, 2003). The other possible mechanism was the availability of the amino group on chitosan can absorb the essential nutrient that necessary for bacterial growth (Cuero *et al*., 1991; Tsai *et al*., 2002).

Effect of chitosan and chitosan-sugar complex on Staphylococcus aureus

The lethal effect of chitosan and chitosan-sugar complex on *Staphylococcus aureus* is shown in Figure 5. After 6 hours, the evidence showed that chitosan-lactose complex and chitosan-arabinose complex was the most effective at all concentrations. However, chitosan-galactose complex was the best antimicrobial agent after 24 hours of incubation at all concentrations. The inhibition effect of chitosan or chitosan-sugar complex for *Staphylococcus aureus* may be bacteriostatic effect, because number of bacteria increase when incubated bacteria culture with chitosan or chitosan-sugar complex unit 24 hours. *Staphylococcus aureus* is a gram positive bacterial, thus the mechanism of microbial inhibition may be the formation of chitosan and chitosan-sugar complex film over the surface of microbial cell membrane that prevent the nutrient from entering the cell (Zheng and Zhu, 2003). The other possible mechanism was the same as *Bacillus cereus*.

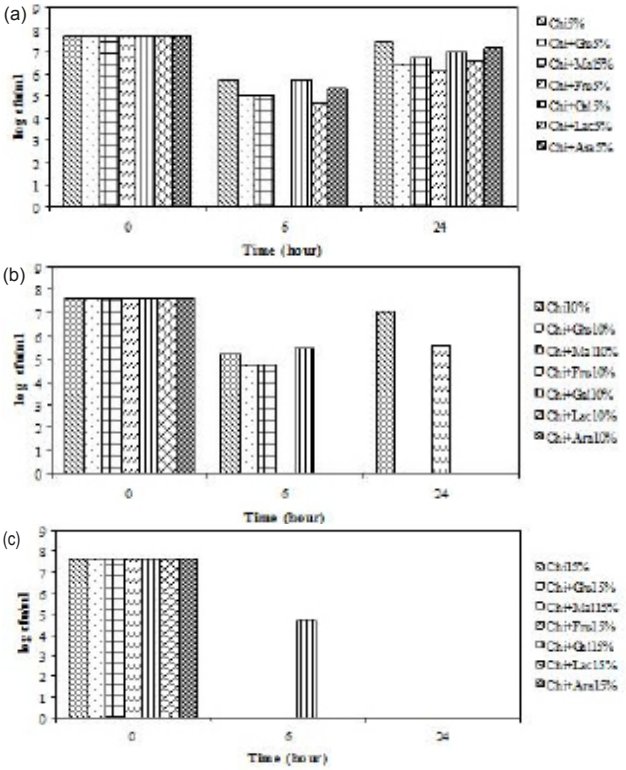

Figure 4. Microbial inhibition of chitosan and chitosan-sugar complex against *Bacillus cereus* at various concentration (a: 5%, b: 10% and c:15%).

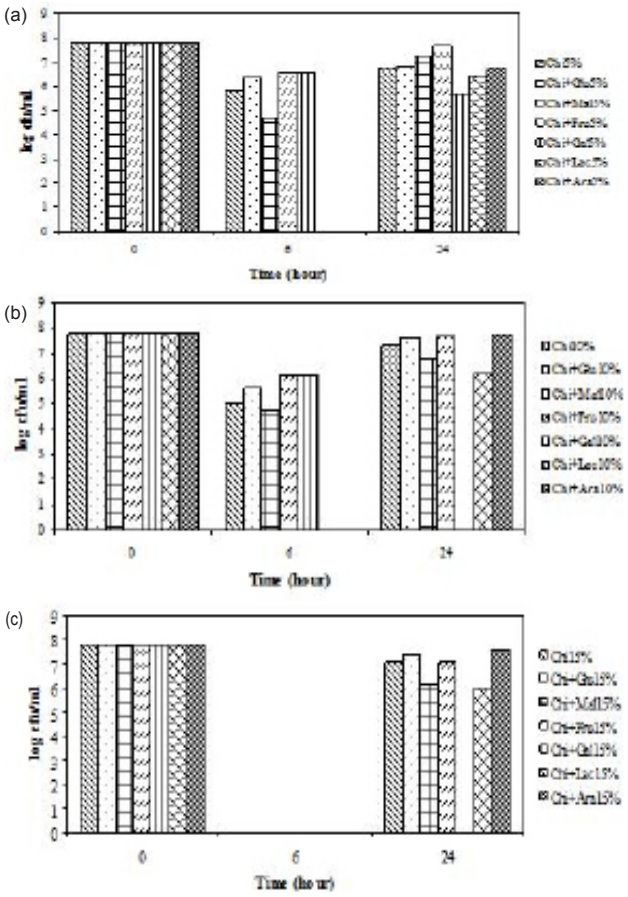

Figure 5. Microbial inhibition of chitosan and chitosan-sugar complex against *Staphylococcus aureus* at various concentration (a: 5%, b: 10% and c: 15%).

Effect of chitosan and chitosan-sugar complex on Pseudomonas aeruginosa

The antibacterial activity of chitosan and chitosan-sugar complex against *Pseudomonas aeruginosa* is shown in Figure 6. After 6 hours, chitosan-lactose complex present stronger antibacterial properties against *Pseudomonas aeruginosa* than the other complexes. After 24 hours, the variable count of bacterial cells was increased. The inhibitory effect of chitosan toward gram negative bacterial has been report to be due to the interaction between the positive charge of chitosan molecule and the negative charge of microbial cell membrane, resulting in changes in membrane permeability (Sudarshan *et al*., 1992; Helander, 2001; Babiker, 2002). The inhibitory activity of chitosan and chiotsan-sugar complex towards a gram negative bacterial like *Pseudomonas aeruginosa* should be as described by the mechanism proposed. The other possible mechanism was the availability of the amino group on chitosan that can absorb the essential nutrient which was necessary for bacterial growth (Cuero *et al*., 1991; Tsai *et al*., 2002).

Effect of chitosan and chitosan-sugar complex on Escherichia coli

The antimicrobial capacity of chitosan and chitosan-sugar complex on *Escherichia coli* was determined as described in the method. As can be seen from Figure 7, the result indicated that the antimicrobial activity of the inhibition agent was concentration dependent (except chitosan and chitosan-lactose complex). After 24 hours, as the concentration increased to 10% and 15% bacteria was completely eliminated. This better than inhibition against *Bacillus cereus*, that show no viable cell at 15% concentration of all compounds after 24 hours. Then chitosan and chitosan-sugar complex were more effective against Gram-negative bacteria. While the other studies showed that chitosan and chitosan derivative were more effective against Gram-positive bacteria (Jeon *et al*., 2001; Xie *et al*., 2002). As *Escherichia coli* is a gram negative bacteria, then the inhibition effect should be due to the interaction between the positive charge of chitosan molecule and the negative charge of microbial cell membrane, resulting in changes in membrane permeability (Sudarshan *et al*., 1992; Helander, 2001; Babiker, 2002) and the availability of the amino group on chitosan can absorb the essential nutrient that necessary for bacterial growth (Cuero *et al*., 1991; Tsai *et al*., 2002). The antibacterial activity of chitosan against *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus* was already reported (Darmadji and Izumimoto, 1994).

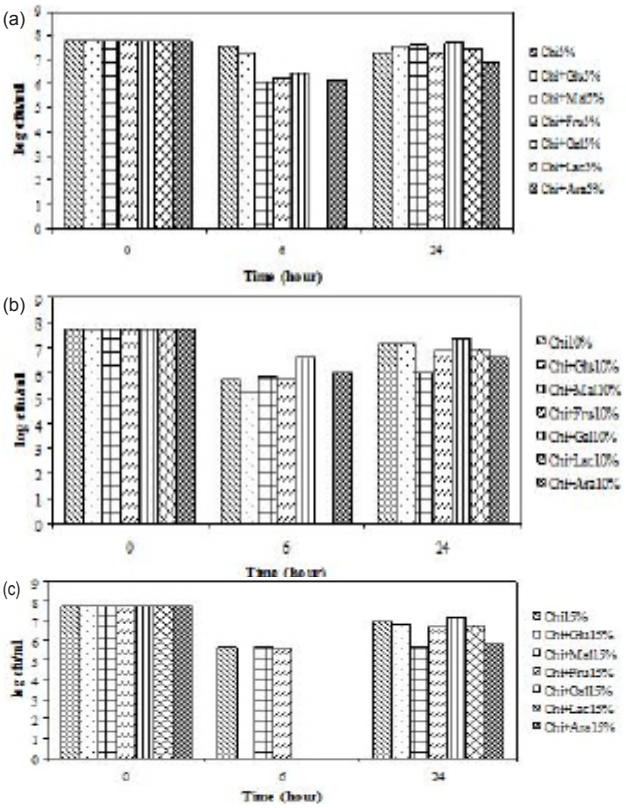

Figure 6. Microbial inhibition of chitosan and chitosan-sugar complex against *Pseudomonas aeruginosa* at various concentration (a: 5%, b: 10% and c: 15%).

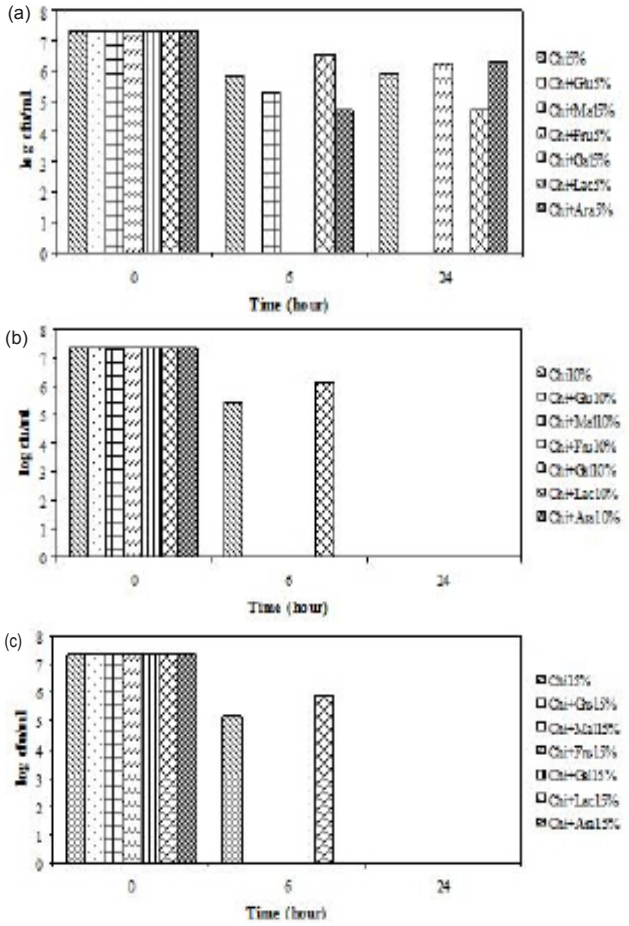

Figure 7. Microbial inhibition of chitosan and chitosan-sugar complex against *Escherichia coli* at various concentration (a: 5%, b: 10% and c: 15%).

Conclusions

Chitosan-sugar complex from this study show the potential to act as a better antimicrobial and antioxidant agent than chitosan alone. Compared with other chitosan-sugar complexes, chitosan-galactose seems to be the best antioxidant. However, antimicrobial activity depended on microbial type. Chitosan and chitosan-sugar complex were more effective against Gram-negative bacteria. This study demonstrates the potential of chitosan-sugar complex, an alternative natural product, to use for synthetic food additive replacement.

Acknowledgements

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