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In vitro binding of bile acids and triglycerides by selected chitosan preparations and their physico-chemical properties

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Abstract

The selected 11 chitosan samples were evaluated for their fat- and bile acid-binding capacities, physico-chemical properties, and the correlations between each binding capacity and individual physico-chemical properties. The bile acid- and fat-binding capacities were estimated using in vitro assays, whereas the measured physico-chemical properties were deacetylation degree, swelling capacity, and solution viscosity. Chitosan samples might differ in their binding capacities against fat and/or individual bile acids. The bile acid-binding capacities were 0.20–0.61, 0.43–1.63, and 0.61–1.61 μmol/g chitosan for cholic, deoxycholic, and chenodeoxycholic acids, respectively. Stronger binding capacity of chitosan against a selected bile acid does not warrant greater binding capacity for other bile acid. The fat-binding capacity ranged from 1077–1239 g oil/g for the chitosan samples under the experimental conditions. No correlation was observed between any binding ability and individual physico-chemical properties, although the swelling capacity of chitosan was correlated to the solution viscosity ($r = 0.82$, $P = 0.02$) and deacetylation degree ($r = -0.62$, $P = 0.04$), indicating that none of the measured physico-chemical properties can be used to predict the fat- or bile acid-binding ability of chitosan.

Keywords: Chitosan; Bile acid-binding; Fat-binding; Deacetylation degree; Viscosity; Swell volume

1. Introduction

Chitosan, a polymer of glucosamine, is a deacetylated product of chitin. Growing evidence indicates that chitosan preparations may exhibit hypolipidemic effects and reduce the risk of cardiovascular diseases (Ikeda et al., 1993; Nagyvary et al., 1979; Trautwein, Jürgensen, & Erbersdobler, 1997), along with a number of other potential benefits to humans such as antigenotoxic and antitumor activities (Koide, 1998). The proposed mechanisms for the hypolipidemic activity included interfering lymphatic absorption of cholesterol and fat (Ikeda, Tomari, & Sugano, 1990; Ikeda et al., 1993), increasing fecal excretion of neutral steroids (Ikeda et al., 1993; Sugano, Fujikawa, Hiratsuji, & Hasegawa, 1978), and altering liver functions (LeHoux & Grondin, 1993). The interference of lymphytic absorption of cholesterol and fatty acids is believed partially due to the capacity of chitosan to directly bind all lipid components of micelles including triglycerides, cholesterol, and bile acids (Kritchevsky, 1987). It is also well known that the dietary fibers that are able to swell or gel in an aqueous system may reduce the gastrointestinal transition time and reduce the absorption of fat and cholesterol (Allen, Bristow, & Yu, 2004; Gallaher, Munion, Hesslink, Wise, & Gallaher, 2000).

It is well accepted that the hypolipidemic effects of dietary fibers are related to their physico-chemical properties such as their solution viscosity and average molecular weight (Ikeda et al., 1993; Sugano, Watanabe,

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Kishi, Izume, & Ohtakara, 1988). The chitosan derivative with a smaller molecular weight of 10,000–20,000 has been found to be as effective as the chitosan with a molecular weight of 50,000 in cholesterol-lowering capacity (Ikeda et al., 1993). These previous data suggested that the physico-chemical properties of dietary fibers including chitosan might influence their hypolipidemic capacity. Little information is known about the relationship of the physico-chemical properties of chitosan preparations and their potential interaction with bile acids and triglycerides, although the information is important for production and utilization of chitosan preparations with improved hypolipidemic potency. Thus, the aim of this research was to examine and compare the 11 chitosan preparations for their in vitro fat-binding capacity, potency to bind individual bile acids, deacetylation degree, solution viscosity, and swell volume. The correlation between fat-binding or bile acid-binding capacity and each of the tested physico-chemical properties was also determined. The information obtained from this research will be used to promote the production of chitosan preparations with improved fat-binding and/or bile acid-binding capacities.

2. Materials and methods

2.1. Materials

Eleven chitosan preparations were used in this study, including six prepared in Dr. Xia's laboratory at Southern Yangtze University (Wuxi, Jiangsu, PR China) and five purchased from commercial. Cholestyramine, individual bile acids (cholic, deoxycholic, and chenodeoxycholic acid), diphorase, 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate (MBTH), and serum bile acid testing kit were purchased from Sigma-Aldrich (St. Louis, MO), while olive oil was purchased from local supermarket. All other chemicals and solvents were of the highest commercial grade and used without further purification.

2.2. Preparation of chitosan samples

2.2.1. Preparation of chitin

Chitin was prepared according to a previously described procedure (Xia & Wang, 1991, 1992). Briefly, fresh shrimp shell (2.3 kg) was homogenized with equal parts water (w/w) and digested by proteinase (1 mg/l) for 3 h at 50 °C with stirring. The mixture was then filtered and the solid was collected. The solid was then boiled with 10% NaOH for 2 h, washed with pure water to neutral pH, and treated with 5% HCl at ambient temperature until no gas formation. The resulting solid was washed with pure water to neutral pH and oven dried at 60 °C to obtain 100 g chitin.

2.2.2. Preparation of chitosan with different degree of deacetylation

Five hundred grams of NaOH solution (50 g in 100 g water) was added to a reaction flask containing 100 g chitin (Xia & Wang, 1991, 1992). The reaction mixture was stirred for 3, 6, and 9 h in a 95 °C water bath to obtain chitosan samples with 80%, 87% and 92% degree of deacetylation, respectively. After cooling, the reaction mixture was filtered. The solid was collected, washed with pure water to neutral pH, and dried.

2.2.3. Preparation of chitosan with different molecular weight

Chitosan sample was dissolved in 2% acetic acid to obtain the reaction mixture with a final concentration of 2 g chitosan/100 g solution (Xia & Wang, 1992). The reaction solution was heated with stirring for 2, 4, or 8 h. The resulting mixture was neutralized with diluted NaOH. Absolute ethanol was added to the neutralized solution until the final ethanol concentration reaches 70 ml ethanol in 1 l solution to completely precipitate the chitosan. The chitosan samples with different molecular weights, but with the same degree of deacetylation, were collected by filtration, washed with pure water, and dried at low temperature.

2.3. Bile acid-binding capacity

In vitro bile acid-binding capacity of chitosan was measured according to a previously described procedure (Camire & Dougherty, 2003; Kahlon & Woodruff, 2003). Briefly, 200 mg of each chitosan sample was treated with 2 ml of 0.01 mol/l HCl, which simulated gastric condition, and incubated at 37 °C for 1 h. The sample pH was brought to 7.0 with 0.1 mol/l NaOH, and mixed with 5 ml of porcine pancreatin and 5 ml of 400 μmol/l bile acid stock solution prepared in 0.01 mol/l phosphate buffer (pH 7.4). The resulting mixture was vortexed and incubated for 1 h at 37 °C, which simulated intestinal conditions. After incubation, the mixture was centrifuged at 20,000*g* for 10 min at ambient temperature, and the supernatant was collected for bile acid quantification.

Bile acids in the supernatant were determined using commercial kit from Sigma-Aldrich (St. Louis, MO). The final assay mixture contained 200 μl of supernatant or bile acid standards, 0.25 ml of 1.22 mmol/l nicotinamide adenine dinucleotide, 0.25 ml of 5 mmol/l nitro blue tetrazolium salt, 0.2 ml of 625 units/l 3-α hydroxysterol dehydrogenase and 0.2 ml of 625 units/l diphorase. The mixture was incubated for 1 h at ambient temperature. After incubation, 200 μl of phosphoric acid was added to stop the reaction and the absorbance of

each reaction mixture was measured at 530 nm. The phosphate buffer without bile acid was used for a reagent blank and cholestyramine used as a positive control to verify the enzyme. The levels of unbound bile acids were obtained using a standard curve prepared with each of the three pure bile acids including cholic, deoxycholic, and chenodeoxycholic acids. The bile acid-binding capacity (μg/g sample) was calculated against a reagent blank. Triplicate assays were conducted for each chitosan against each bile acid.

2.4. Fat-binding capacity

Fat-binding capacity of chitosan was examined according to the previously described procedure with slight modification to simulate the gastrointestinal conditions (Babish, 2002; Lin & Humbert, 1974). Briefly, 20 mg of each chitosan samples was mixed with 1.25 ml of 0.6 mol/l HCl. After addition of 25 g olive oil, the mixture was vortexed thoroughly and incubated at 37 °C with shaking. After 2 h of incubation, 8 ml of 10 mmol/l phosphate buffer (pH 7.4) was added to the mixture and its pH was brought to 7.0 with 1 mol/l NaOH. The resulting mixture was incubated for 30 min at 37 °C, and centrifuged at 675*g* for 10 min. The supernatant oil was collected and measured as unbound oil. The fat-binding capacity of chitosan samples was calculated as gram of bound oil per gram of chitosan. The test was conducted in triplicate.

2.5. Deacetylation degree

The deacetylation degree of chitosan samples was obtained from glucosamine measurement according to the colorimetric method using MBTH (Chen & Chiou, 1999; Tsuji, Kinoshita, & Hoshino, 1969). Briefly, 50 μl of hydrolysed, nonhydrolysed chitosan solution, or glucosamine standard was mixed with 0.95 ml of water, 1 ml of sodium nitrite (5 g/100 ml), 1 ml of potassium hydrogen sulfate (5 g/100 ml). After 15 min of reaction, 1 ml of ammonium sulfamate (12.5 g/100 ml) was added and the mixture shaken for another 5 min, followed by addition of 1 ml of 3-methyl-2-benzothiazolone hydrazone (0.5 g/100 ml). The resulting mixture was heated in a boiling water bath for 3 min. After cooling to the ambient temperature, 1.5 ml $FeCl_3$ solution (0.25 g/100 ml) was mixed into the solution, and the mixture was kept for 50 min at ambient temperature before subjected to measure the absorbance at 650 nm. Chitosan sample was hydrolysed in 2.5 mol/l HCl at 121 °C for 3 h to liberate the acetylated amino groups for quantification of the total glucosamine residues of the chitosan. The glucosamine residues of chitosan samples were determined using a glucosamin standard curve. The deacetylation degree of chitosan samples was calculated and expressed as the percent deacetylated glucosamine residues against total glucosamine residues.

2.6. Swelling capacity

Swell volume of chitosan samples was measured according the procedure reported by Crosbie (1991). Approximately 0.4 g of chitosan sample was placed in a 10 ml cylinder and suspended in 10 ml simulated intestinal fluid without enzyme. The suspension was covered with parafilm and kept at 37 °C for 8 h with occasional shaking. After the incubation, the mixture was remained at ambient temperature for the sediment to settle. The volume of sediment was recorded. The swelling capacity was calculated as volume of sediment/weight of sample (ml/g).

2.7. Viscosity assay

Viscosity was measured with a Brookfield viscometer, Model DV-+Pro (Brookfield Engineering Laboratories, Inc., Middleboro, MA). One gram of chitosan was dissolved in 100 ml of acetic acid solution (1 ml in 100 ml solution), and used for viscosity measurements at 22 °C. The results were reported in the unit of centipoises.

2.8. Statistical analysis

Data were reported as mean ± SD for triplicate determinations. Analysis of variance and least significant difference tests (SPSS for Windows, Version Rel. 10.0.5., 1999, SPSS Inc., Chicago, IL) were conducted to identify differences among means.

3. Results and discussion

3.1. Bile acid-binding capacity

The hypocholesterolemic activity of chitosan preparations and other digestion-resistant polysaccharides were linked to their binding capacities against bile acids (Gallaher et al., 2000; Kim & Chun, 1999). In vitro assay has been widely accepted to estimate the potential bile acid-binding capacity of polysaccharides including chitosan, ready-to-eat breakfast cereals, and other dietary fibers (Camire & Dougherty, 2003; Kahlon & Woodruff, 2003; Kim & Chun, 1999). This research examined the selected chitosan preparations using the in intro assay for their abilities to directly bind individual bile acids, as well as the potential links between bile acid-binding capacity and the individual physico-chemical properties. This information is important for further improving the bile acid-binding capacity and physico-chemical properties of chitosan to promote their

potential utilization in functional food and supplemental products.

All chitosan samples were examined for their direct binding capacities against the selected bile acids including cholic, deoxycholic, and chenodeoxycholic acids. These bile acids were selected because of their solubility in the experimental solvents. The chitosan samples differed in their capacity to bind the selected bile acids (Fig. 1). The strongest binding capacity against both deoxycholic and chenodeoxycholic acids was observed for chitosan sample 9, which had the greatest deacetylation degree among all tested samples except chitosan sample 10 (Fig. 1). Interestingly, chitosan sample 10, which had the same deacetylation degree as that of sample 9, exhibited significantly weaker binding capacity against both deoxycholic and chenodeoxycholic acids than that observed in sample 9. The highest binding capacity against cholic acid was detected in chitosan sample 3 at a level of 0.59 μmol/g chitosan, which was significantly higher than most of the tested chitosan samples except chitosan sample number 5 (Fig. 1). Also noted was that the chitosan sample having the strongest binding capacity against a selected bile acid did not necessarily exhibit strongest binding capacity against other bile acids. This observation was supported by a previous study of the bile acid-binding capacity of raisin fibers and other materials against cholic and deoxycholic acids (Camire & Dougherty, 2003). It was found that the fiber having a stronger binding capacity against cholic acid might not show stronger deoxycholic acid-binding activity (Camire & Dougherty, 2003). Furthermore, no correlation was detected between individual bile acid-binding capacity and any other tested physico-chemical properties of chitosan under the experimental conditions. These data suggested that molecular weight as reflected by solution viscosity, deacetylation degree, or swelling capacity might not be used to predict the bile acid-binding capacity of chitosan.

In agreement to the observation by Kim and Chun (1999), an individual chitosan sample had greater or similar absorbing capacity against deoxycholic and chenodeoxycholic acids than that against cholic acid under the experimental conditions in this study. Kim and Chun (1999) compared chitosam and *o*-diethylaminoethyl chitosan (DEAE-chitosan) for their abilities to bind cholic and deoxycholic acids, and found that both absorbed greater amount of deoxycholic acid. These may be explained by the presence of two additional hydroxyl groups in the deoxycholic acid molecule, which provide stronger interaction with the hydroxyl and amino groups in the chitosan. The cholic acid-binding capacity was correlated to binding capacities against both deoxycholic and chenodeoxycholic acids under the experimental conditions ($r = 0.77$, $P = 0.06$). These data suggest the possibility to rapidly screen chitosan samples for their bile acid-binding capacities using cholic acid.

3.2. Fat-binding capacity

Chitosan and chitosan-based materials have been shown to effectively bind fat (Babish, 2002; Furda, 1998). It is believed that the bound fat has less absorption in vivo, and is excreted in feces (Furda, 1998). The ability of chitosan to directly bind fat may contribute to its overall hypolipidemic activity. This study estimated the fat-binding potential of the selected chitosan samples, and investigated the possible

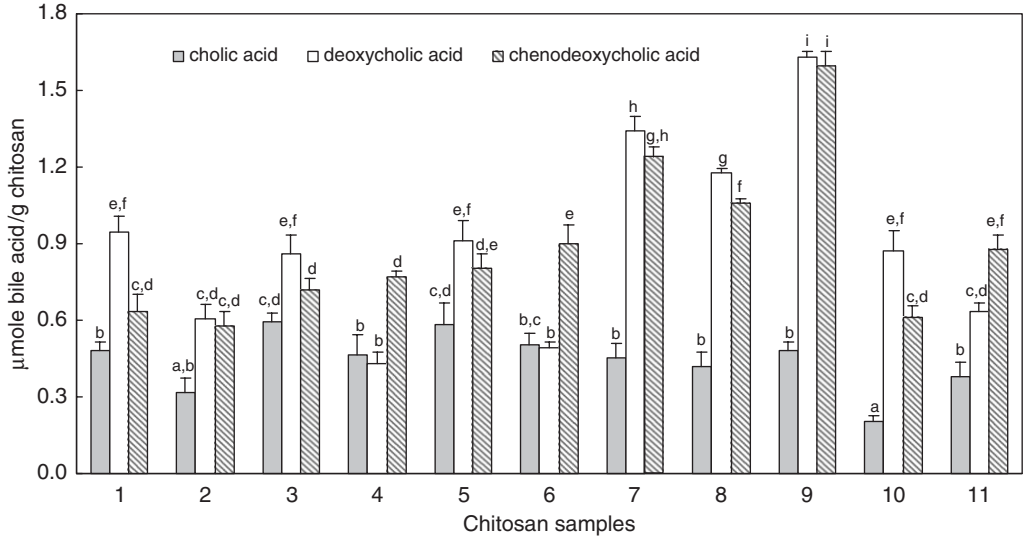

Fig. 1. Bile acid-binding capacity of chitosan samples. Numbers 1–6 represent chitosan samples prepared in our laboratory, whereas 7–11 represent chitosan samples from commercial. All tests were conducted in triplicate. The vertical bars represent the standard deviation of each data point. Values marked by the same latter are not significantly different ($P<0.05$).

correlation between the fat-binding ability and the measured physico-chemical properties.

The tested chitosan samples exhibited a fat-binding capacity of 1077–1239 g oil/g chitosan under the experimental conditions (Fig. 2). Chitosan samples 4 and 6, which had much higher molecular weight, did not have stronger fat-binding capacity than the rest samples, indicating that a greater molecular weight does not necessarily warrant a stronger capacity for chitosan to directly bind fat. Furthermore, the fat-binding capacity of chitosan is not correlated to any other measured physico-chemical properties.

3.3. Deacetylation degree

Deacetylation degree determines the anion-exchange capacity of chitosan, and may be related to its solution behavior and functionalities. The deacetylation degree of the tested chitosan samples ranged 86.5–95.7% under the experimental conditions (Fig. 3). The deacetylation degree was negatively correlated with the welling volume ($r = -0.62$, $P = 0.04$).

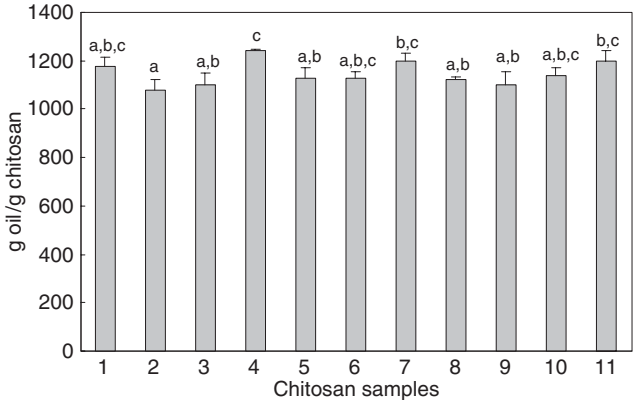

Fig. 2. Fat-binding capacity of chitosan samples. Numbers 1–6 represent chitosan samples prepared in our laboratory, whereas 7–11 represent chitosan samples from commercial. All tests were conducted in triplicate. The vertical bars represent the standard deviation of each data point. Values marked by the same latter are not significantly different ($P<0.05$).

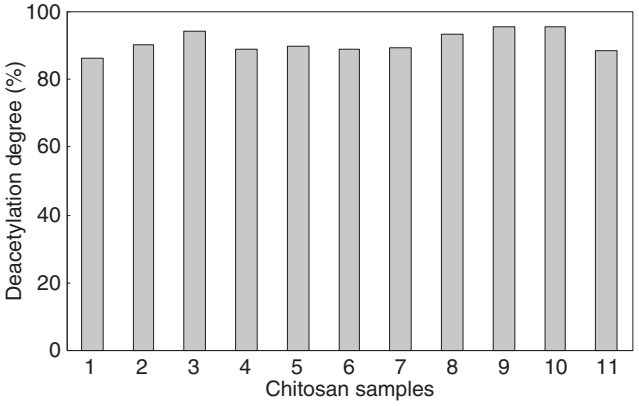

Fig. 3. Deacetylation degree of chitosan samples. Numbers 1–6 represent chitosan samples prepared in our laboratory, whereas 7–11 represent chitosan samples from commercial.

3.4. Swelling capacity

Swelling capacity measured as the swell volume was included in this research because it has served as a measurement to predict the cholesterol-lowering efficacy of dietary fibers (Allen et al., 2004). The swelling capacity of chitosan was measured as the swell volume. The tested chitosan samples had a swelling volume of 6.3–9.1 ml/g (Fig. 4). Statistical analysis showed that the swelling capacity of chitosan was positively correlated to the solution viscosity ($r = 0.82$, $P<0.01$), but was negatively correlated to the degree of deacetylation ($r = -0.62$, $P = 0.04$).

3.5. Viscosity

It is suggested that the ability of chitosan to increase the viscosity of intestinal contents may contribute to their cholesterol-lowering effect (Gallaher et al., 2000). In the present study, all the tested chitosan samples differed in their solution viscosity at ambient temperature (Table 1). The solution viscosity was not correlated to either bile acid or fat-binding capacity of chitosan under the experimental conditions. The two chitosan samples, numbers 4 and 6, exhibited more than ten-fold higher solution viscosity than the rest of the chitosan samples, but had relatively lower fat- and bile acid-binding capacities among the tested samples (Table 1, Figs. 1 and 2), suggesting that the hypolipidemic activity of chitosan may involve multi-mechanisms.

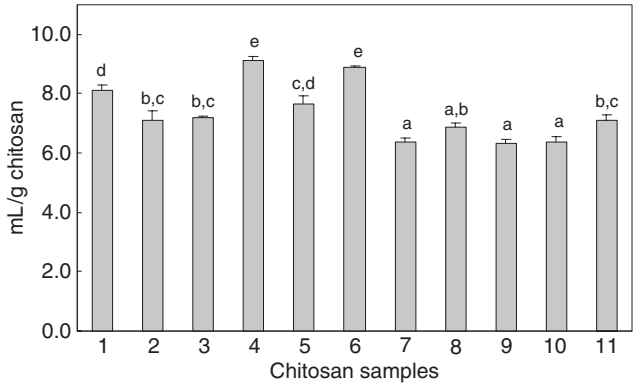

Fig. 4. Swell capacity of chitosan samples. Numbers 1–6 represent chitosan samples prepared in our laboratory, whereas 7–11 represent chitosan samples from commercial. All tests were conducted in triplicate. The vertical bars represent the standard deviation of each data point. Values marked by the same latter are not significantly different ($P<0.05$).

Table 1
Viscosity of chitosan solution at room temperature[a]

Sample no.	1	2	3	4	5	6	7	8	9	10	11
Viscosity (*cP*)	24.3a,b	14.6a,b	20.5a,b	535.3d	11.5a	532.3d	18.1a,b	22.3a,b	27.4b	22.8a,b	95.8c
Spindle speed (rpm)	50	50	50	3	50	3	50	50	50	50	50

[a]1 g/100 ml chitosan solution was prepared for each sample with acetic acid–water (1 ml acetic acid in 99 ml water). Numbers 1–6 represent chitosan samples prepared in our laboratory, whereas 7–11 represent chitosan samples from commercial. Viscosity of chitosan solution was tested using a small sample adaptor. All tests were conducted in duplicate and the means are used. Values marked by the same letter are not significantly different ($P<0.05$, $n=3$).

In summary, this study reported the bile acid-binding capacity, fat-binding ability, swelling capacity, deacetylation degree, and solution viscosity of the selected 11 chitosan preparations. No correlation was detected between the binding capacity and the measured physico-chemical properties. Additional research is required to determine the critical physico-chemical properties that may be associated to the binding capacities of chitosan against fat and/or bile acids.

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