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Preparation of 1,3,5-thiadiazine-2-thione derivatives of chitosan and their potential antioxidant activity in vitro

Xia Ji,[a,b] Zhimei Zhong,[a,b] Xiaolin Chen,[a,b] Ronge Xing,[a,b] Song Liu,[a,b] Lin Wang[a,b] and Pengcheng Li[a,*]

[a]*Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China*
[b]*Graduate University of the Chinese Academy of Sciences, Beijing 100039, China*

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Abstract—Three new kinds of 1,3,5-thiadiazine-2-thione derivatives of chitosan with two different molecular weight (SATTCS1, SATTCS2, TITTCS1, TITTCS2, CITTCS1 and CITTCS2) have been prepared. Their structures were characterized by IR spectroscopy. The substitution degree of derivatives calculated by elemental analyses was 0.47, 0.42, 0.41, 0.38, 0.41 and 0.36, respectively. The result shows that substitution degree of derivatives was higher with lower molecular weight. The antioxidant activity was studied using an established system, such as hydroxyl radical scavenging, superoxide radical scavenging and reducing power. Antioxidant activity of the 1,3,5-thiadiazine-2-thione derivatives of chitosan were stronger than that of chitosans and antioxidant activity of low molecular weight derivatives were stronger than that of high molecular weight derivatives. It is a potential antioxidant in vitro.

Chitosan is a linear homopolysaccharide consisting of D-glucosamine monomers linked through β-(1 → 4) glycosidic linkages. It is a cationic polysaccharide made from alkaline N-deacetylation of chitin. Chitosan has several reactive groups such as –OH, $–NH_2$ which can react with many compounds and new derivatives of chitosan were obtained. In order to enlarging the utilization of chitosan, in resent years, chemical modification to introduce a variety of functional group is a key point. Much work has been reported on the chemical modification of chitin and chitosan.[1,2] For example, carboxymethyl chitosan can easily dissolve in water which enlarges the scope of the use of chitosan[3,4]; quaternized diethylmethyl chitosan chloride showed higher antibacterial activity than chitosan.[5] Up to now, there are no studies on 1,3,5-thiadiazine-2-thione derivatives of chitosan. In this paper, we introduce 1,3,5-thiadiazine-2-thione on chitosan, synthesize three new kinds of 1,3,5-thiadiazine-2-thione derivatives of chitosan (TDTC) with two different molecular weight (SATTCS1, SATTCS2, TITTCS1, TITTCS2, CITTCS1 and CITTCS2).

Keywords: 1,3,5-Thiadiazine-2-thione derivatives of chitosan; Radical scavenging effect; Reducing power.
* Corresponding author. Tel.: +86 532 82898707; fax: +86 532 82968951; e-mail addresses: jixia04@126.com; pcli@ms.qdio.ac.cn

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Antioxidant activity is one of the essential natural of substance. Antioxidant can reduce oxidative damage such as that caused by ROS (reactive oxygen species). ROS in the forms of superoxide anion ($\cdot O_2^-$), hydroxyl radical (·OH) and hydrogen peroxide (H_2O_2) are produced by sunlight, ultraviolet light, ionizing radiation, chemical reactions and metabolic processes which have a wide variety of pathological effects, such as cancer, cardiovascular diseases, diabetes, and atherosclerosis.[6,7] Antioxidants exert their effects by scavenging ROS or preventing the generation of ROS.[8] In recent years, there has been increasing interest in finding natural antioxidants, since they can protect the human body from free radicals and retard the chronic progress of many diseases.[9] As a natural product, antioxidant activity of chitosan and its derivatives have attracted much attention.[10] Xing et al. had found that all kinds of sulfated chitosans possessed antioxidant activities and free radical scavenging activities.[11] Xue et al. reported that the chitosan derivatives prepared by graft copolymerization of maleic acid sodium onto hydroxypropyl chitosan and carboxymethyl chitosan sodium had scavenging activities against hydroxyl radicals.[12] In this paper, TDTC was studied as new antioxidant. We found that it could scavenge superoxide/hydroxyl radicals and had obviously reducing power. As a natural product, TDTC might have little toxicity than

chemical antioxidant agents. It was a potential antioxidant.

Chitosan was purchased from Qingdao Baicheng Biochemical Corp. (China), which has a deacetylation degree of 97% and viscosity average-molecular weight of 700 kDa. Phenazine methosulfate (PMS), ethylene diamine tetra-acetic acid (EDTA) were purchased from Sigma Chemicals Co. Nitro blue tetrazolium (NBT), nicotinamide adenine dinucleotidereduced (NADH) were purchased from Fluka Chemical Co. The IR spectrum was measured by a Nicolet Mangne-Avatar 360 instrument using KBr disks. The elemental analyses (C, H, N, S) were performed on a Heraeus vatioO-NCSH elemental analyzer. The other reagents were of analytical grade and used without further purification.

Chitosan oligomers were prepared according to previously reported method,[13] and the viscosity average-molecular weights (Mw) of chitosan oligomers were measured referring to the method of Jia.[14] Two different molecular chitosan oligomers were obtained: CTS1 (M_w = 2400), CTS2 (M_w = 3500).

TDTC were synthesized as shown in Scheme 1. CTS1/CTS2 (0.02 mol) was dissolved in H_2O (40 ml), then 20% KOH (5.6 ml) and CS_2 (1.2 ml) were added to the solution with stirring at 20 °C. After 2 h, 37% formaldehyde (0.04 mol) was added with stirring at 20 °C for 2 h. Then brown solution was obtained.

Different primary amines were suspended in potassium phosphate buffer (pH 7.8), mixed with above brown solution and stirred at 20 °C for 4 h. The reaction mixture was adjusted to pH 4 with 10% HCl, and filtrated. The obtained product was washed with distilled water and alcohol, and finally dried at 50 °C for 24 h.

According to the reference,[15] the reaction mixture, total volume 4.5 ml, containing all kinds of TDTC (0.025–0.8 mg/ml), was incubated with EDTA–Fe^{2+} (220 μM), safranine O (0.23 μM), and H_2O_2 (60 μM) in potassium phosphate buffer (150 mM, pH 7.4) for 30 min at 37 °C. The absorbance of the mixture was measured at 520 nm. Hydroxyl radical bleached the safranine O, so decreased absorbance of the reaction mixture indicated decreased hydroxyl radical scavenging ability.

The superoxide scavenging ability of TDTC was assessed by the method of Nishikimi.[16] All kinds of TDTC were mixed separately with PMS (30 μM), NADH (338 μM), and NBT (72 μM) in phosphate buffer (0.1 M, pH 7.4) and then incubated at room temperature for 5 min. The absorbance was read at 560 nm against a blank and the capability of scavenging superoxide radical was calculated using the following equation:

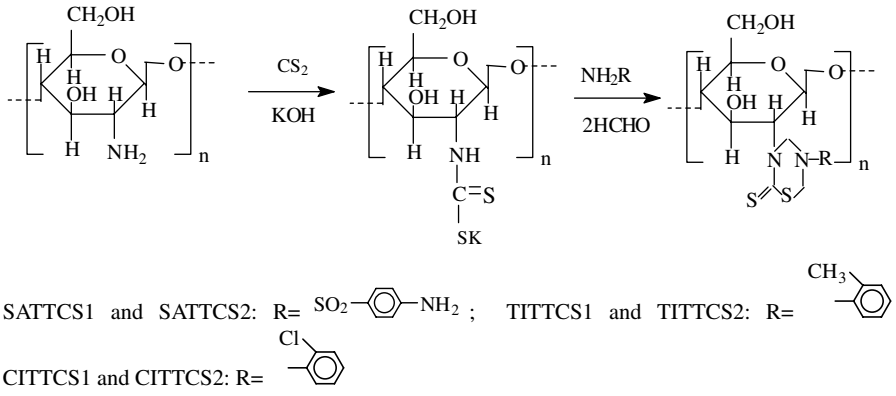
$$\text{Scavenging effect } (\%) = (1 - A_{\text{sample 560 nm}}/A_{\text{control 560 nm}}) \times 100$$

where $A_{\text{control 560 nm}}$ is the absorbance of the control (phosphate buffer, instead of NADH).

The reducing power of the all kinds of TDTC was quantified by the method described earlier by Yen and Chen[17] with minor modifications. Briefly, 1 ml of reaction mixture, containing different concentration of TDTC (0.075, 0.15, 0.225, 0.30 and 0.375 mg/ml) in phosphate buffer (0.2 M, pH 6.6), was incubated with potassium ferricyanide (1% W/V) at 50 °C for 20 min. The reaction was terminated by trichloroacetic acid solution (10% W/V) and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant was mixed with distilled water and ferric chloride (0.1% W/V) solution and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

All data are expressed as means ± SD. Data were analyzed by an analysis of variance ($P < 0.05$) and the means separated by Duncan's multiple range test. The results were processed by computer programmes: Excel and Statistical software (1999).

The result of the elemental analyses and the color of the TDTC are listed in Table 1, and the IR spectra of chitosan oligomers and the TDTC are shown in Figure 1. As shown in Figure 1, the IR spectra of chitosan shows peaks assigned to the saccharide structure at 897.85 and 1154.98 cm^{-1} and characteristic amino peak at



Scheme 1. Synthetic pathway for all TDTC.

Table 1. The elemental analysis result and the color of chitosan and TDTC

Compound	Elemental analyses (%)				Colors
	C	N	S	H	
Chitosan	44.82	8.63	0	6.82	White
SATTCS1	42.56	9.36	15.55	5.11	Yellow
SATTCS2	42.70	9.32	14.58	5.21	Yellow
TITTCS1	49.30	7.99	10.62	5.97	Brown
TITTCS2	49.08	8.02	10.09	6.01	Brown
CITTCS1	45.78	7.73	10.27	5.30	Hoariness
CITTCS2	45.71	7.80	9.44	5.41	Hoariness

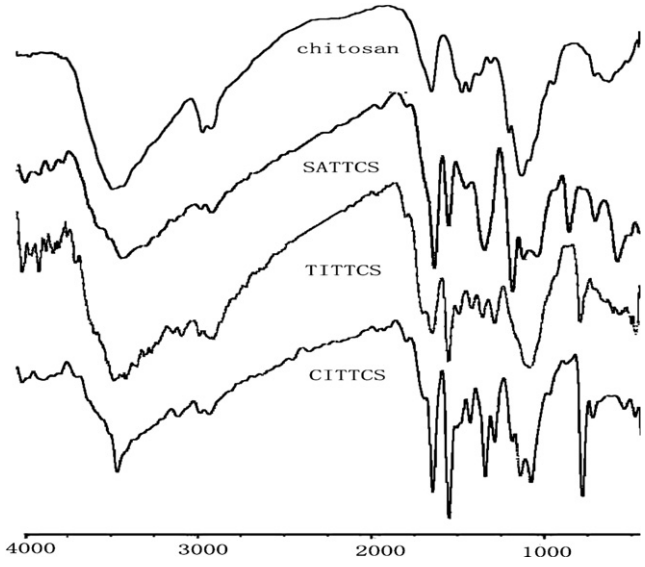


Figure 1. IR spectral data for chitosan and TDTC.

1600.03 cm^{-1}. Compared to the IR spectra of chitosan, the TDTC have strong peaks at 1508–1516 cm^{-1} which are assigned to the characteristic absorbance of thio-ketone groups [C═S],[18] and peaks at 1298–1310 cm^{-1} which are assigned to the characteristic of [N–C═S]. Moreover, there are strong peaks at about 1600 and 750 cm^{-1} corresponding to the phenyl groups.[19] The peaks at 939–941 cm^{-1}, due to the pyranose units in the polysaccharide, proved that the cyclic pyranosyl rings were not destroyed. These results indicated that the TDTC was obtained, and the substitution degree of SATTCS1, SATTCS2, TITTCS1, TITTCS2, CITTCS1 and CITTCS2 was calculated as 0.47, 0.42, 0.41, 0.38, 0.41 and 0.36, respectively, per glucosamine unit from the result of the elemental analyses.

The effect of TDTC on oxidative damage, induced by Fe^{3+}/H_2O_2 on deoxyribose, as measured by the thiobarbituric acid method, is plotted in Figure 2. As shown in Figure 2, apart from CTS1, CTS2 and chitosan, others had obvious scavenging activity. Furthermore the scavenging rate increased with increasing concentration. Scavenging activity of low molecular weight chitosan oligomer on hydroxyl radical was slightly stronger than that of high molecular weight chitosan oligomer. Moreover, scavenging activities of low molecular weight derivatives of chitosan were much stronger than that of high molecular derivatives of chitosan. In addition, concerning chitosan and TDTC of the same molecular weight, scavenging activity of TDTC on hydroxyl radical was more pronounced than that of chitosans. As show in Figure 2, IC_{50} of SACTCS1, SACTCS2, TITTCS1, TITCS2, CITTCS1, CITTCS2 was 6.27, 14.71, 42.29, 55.47, 94.46, 74.42 μM, respectively. However, IC_{50} of chitosan and chitosan oligomers could not be read. Polysaccharides with a scavenging effect on the hydroxyl radical have the same structural feature in that all of them have one or more alcohol or phenolic hydroxyl groups. And the scavenging ability was related to the number of active hydroxyl groups in the molecule.[20] 1,3,5-Disubstituted-tetrahydro-2H-1,3,5-thiadiazine-2-thione derivatives can hydrolyze in water solutions,[21] so TDTC have SH groups after hydrolyzing. Because SH group and hydroxyl groups have so many same properties, more SH groups in the molecule may induce stronger scavenging ability on hydroxyl radical. This maybe the reason that the scavenging ability on hydroxyl radical of TDTC was stronger than that of the chitosans. Furthermore, the derivatives of chitosan with higher substitution degree have stronger scavenging ability of hydroxyl radical, which also illuminate that the scavenging ability was related to the number of active SH groups.

The scavenging ability of the chitosan, chitosan oligomers and TDTC is shown in Figure 3. The inhibitory effect of TDTC on superoxide radical was related to concentration, and at a concentration from 0.025 to 0.4 mg/ml, the percentage scavenging effect valued from about 20.0% to not more than 46%. However, the scavenging rate of chitosan and chitosan oligomers increased with increasing concentration, which was not obvious. Scavenging activities of chitosan oligomers were stronger than that of chitosan. Moreover, scavenging activities of low molecular weight of chitosan and their derivatives was slightly higher than that of high molecular weight and their derivatives. Superoxide radical is a zwitterionic radical. It could react with free hydroxyl and amino groups in chitosan and its derivatives. Then superoxide radical was eliminated by this reaction. Unlike high molecular weight chitosan, low molecular weight chitosan own incompact structure and have more free hydroxyl and amino groups, the slighter change of molecular weight of chitosan will not bring much change of the number of free hydroxyl and amino groups in chitosan and its derivatives, it might be the reason that in our experiment the scavenging activity of superoxide radical of low molecular weight chitosan oligomer and its derivative was slightly stronger than that of high molecular weight chitosan oligomer and its derivatives. Furthermore, scavenging activity of TDTC on superoxide radical was more pronounced than that of chitosan oligomers. This might be caused by the hydrolyzation of TDTC, and more free SH groups were obtained after hydrolyzing compared to chitosan. So the derivatives have stronger scavenging activities.

Figure 4 depicted the reducing power of chitosan, chitosan oligomers and TDTC. The reducing power of chitosan, chitosan oligomers and TDTC correlated well with

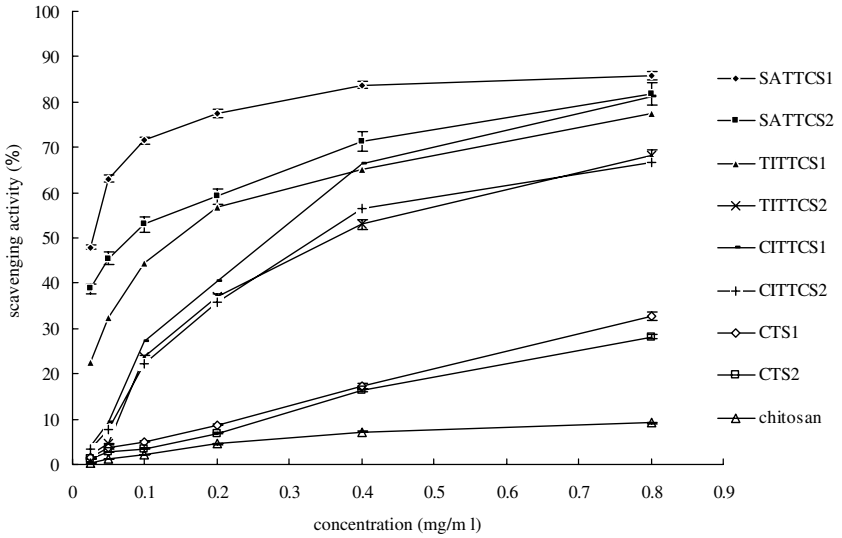


Figure 2. Scavenging effect of chitosan, chitosan oligomers and TDTC on Hydroxyl radicals. Valves are mean ± SD of three determinations.

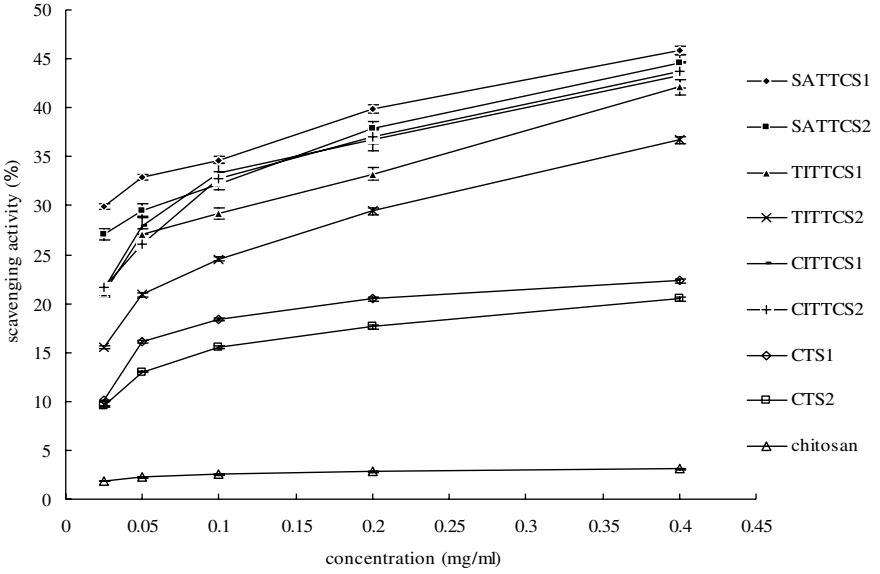


Figure 3. Scavenging effect of chitosan, chitosan oligomers and TDTC on superoxide radicals. Each values is expressed as mean ± SD ($n = 3$).

increasing concentrations. Figure 4 showed the reducing power increased with increasing chitosan, chitosan oligomers and TDTC concentration. Moreover, the reducing power of TDTC was relating more pronounced than that of chitosans and that of TITTCS1 was the most pronounced. Furthermore, the reducing power of low molecular weight chitosan oligomer and their derivatives was little more pronounced than that of high molecular weight chitosan oligomer and their derivatives. Mau et al.[22] reported reducing powers were 0.80, 0.89 and 0.92 at 1.0 mg/ml for ascorbic acid, α-tocopherol and BHA, respectively. The reducing power of TITTCS1, TITTCS2 and CITTCS1 (2.646, 1.636 and 0.797 at 0.4 mg/ml, respectively) was higher than that of ascorbic acid, α-tocopherol and BHA. Earlier authors[23] have observed a direct correlation between antioxidant activities and reducing power of certain plant extracts. The reducing power was generally associated with the presence of reductones[24] which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom.[25] Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. Our data on the reducing power of all kinds of TDTC suggested that it was likely to contribute significantly toward the observed antioxidant affect.

IR spectral analyses and elemental analyses were employed to demonstrate that three kinds of TDTC with two different molecular weights prepared. Moreover, the substitution degree of derivatives was higher with low molecular weight. Furthermore, the result of the present work indicated that all kinds of TDTC possessed antioxidant activities and antioxidant activities of low molecular weight derivatives were stronger than that of high molecular weight derivatives. This might be caused by the hydrolyzation of TDTC and the different compact structure of different molecular weight

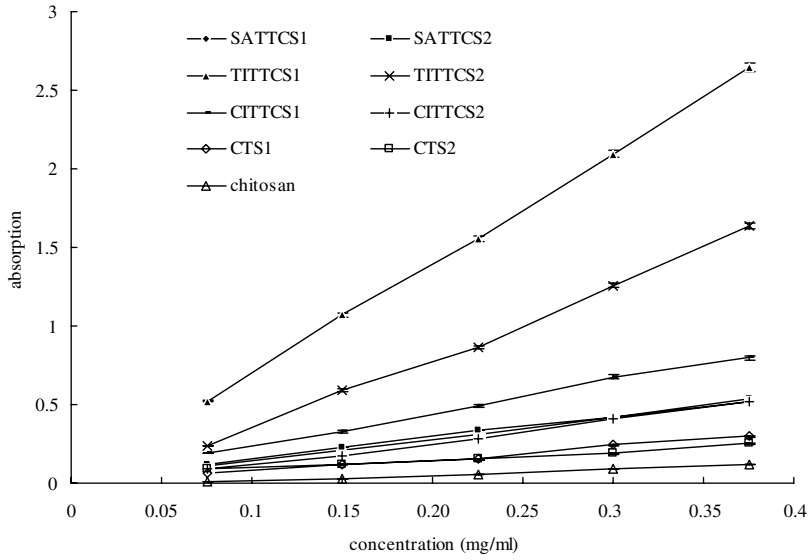


Figure 4. Reducing power of chitosan, chitosan oligomers and TDTC. Each values is expressed as mean ± SD ($n = 3$).

chitosan. However, their antioxidant activity in vitro and the different antioxidant mechanisms need to be further researched.

Acknowledgments

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