



# Production of chitin and conversion into chitosan from crab (*Scylla tranquebarica*) shells and evaluation of its antioxidant activities

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## Abstract

The present study was undertaken to extract chitin and chitosan from crab shell waste and investigate the chemical structure and antioxidant abilities by in vitro assays. Chitin was extracted, chitosan conversion was performed by chemical methods, and the yield was found to be 21%. Several active functional groups, NH primary and secondary amines, amides, and acetyl groups with different bond stretching were conformed in FT-IR spectral analysis. In the proton NMR, N-acetyl glucosamine and H-2 proton of glucosamine (GlcN) residues were identified in chitosan. The antioxidant activities were realized by dose dependent and the maximum activity was found 62, 71, and 58% in higher concentrations of DPPH, hydroxyl, and ferrous ion chelating effects. The derived chitosan is a nontoxic polymer with high chelating capability a good source for functional foods from sustainable source of marine waste.

**Keywords** Chitosan · <sup>1</sup>H NMR · IR spectra · *S. tranquebarica* · Scavengers

## 1 Introduction

Due to the wealth of knowledge accumulated on the applications of chitin, a natural biopolymer, and chitosan, the N-deacetylated form of chitin, over the past 7 decades, it may be considered a “golden era” of research in these fields [1]. A substantial number of research publications on chitin and chitosan demonstrate the growing importance of this polycationic linear amino-polysaccharide molecule [2]. Chitin is the second most common biopolymer on the planet after cellulose and is found as a structural component in the

exoskeletons of a variety of eukaryotic creatures such as crustaceans, insects, and fungal cell walls [3]. The chitosan biocompatibility, nontoxicity, and adaptability make it ideal for use in a variety of industries [4, 5]. The worldwide market for chitin- and chitosan-derived products, which were valued at US\$ 2900 million in 2017, is predicted to increase at a CAGR (compounded annual growth rate) of 14.8 percent to reach US\$ 63 billion by 2024 [6]. Chitin has similar chemical structure to cellulose, with 2-acetamido-2-deoxy-D-glucose (NAG) monomers linked through (1→4) links [7]. Chitin is insoluble in common solvents due to the stiff crystalline structure produced by binary hydrogen bonds between the amide group and either the primary hydroxyl group at position C-6 or the secondary hydroxyl group at position C-3 of the N-acetyl glucosamine residue. Chitin has two separate crystalline structures: one is the  $\beta$ -structure, which is found in crab and shrimp shells, and the other is the structure found in squid pens [8]. The crystalline structure of the  $\alpha$ -type structure is made up of an anti-parallel chitin molecule arrangement and a parallel chitin molecule arrangement for the  $\beta$ -structure. Depending on the origin, a looser molecular packing is hypothesized for the  $\alpha$ -type crystalline structure, whereas a tighter packing is predicted for the  $\beta$ -type structure. Chitin's insolubility prevents it from being chemically altered for use in animal bodies despite the fact that the hard crystalline structure is

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essential for fungus, insects, and crustaceans to keep their life form through exoskeleton cuticles [9, 10].

Chitin comes in three distinct allomorphs, and it is classified into 3 types, namely,  $\alpha$ -chitin,  $\beta$ -chitin, and  $\gamma$ -chitin. The main differences between the three are the level of hydration, the size of the unit cell, and the number of chitin chains per unit cell [11, 12]. Chitin's compact shape restricts its reactivity and solubility in most solvents, limiting its applicability. This has resulted in a number of chemical changes to chitin in order to develop more soluble and reactive derivatives, one of which being chitosan [13]. The demand for seafood has increased, as has the global seafood processing sector, over the past few decades [14]. As a result, the output of marine goods made from crabs, prawns, shrimps, and krills has increased exponentially [15]. Every year, 6–8 million tonnes of discarded seafood are produced. Shrimp heads, tails, and shells are undesirable byproducts of the food industry [16]. Due to the increase in shrimp waste, chitin extraction has received a lot of attention [17]. Chitin is produced by living things in the water on an annual basis in amounts of 1012–1014 tonnes [18].

Chitosan is a partially/poor soluble water soluble derivative of chitin, and through deacetylation process in the presence of strong alkali solutions at high temperatures, chitin is deacetylated. Chitosan is made up of cycles of pyranoses (1,4)-2-amino-2-deoxy- $\beta$ -D-glucose glycosidic links on N-glucosamine [19]. Along chitosan chains, acetylated or deacetylated units are randomly dispersed as a result of the deacetylation process [20, 21]. The present study was undertaken to explore the extraction of chitin from crab shells and conversion of chitosan by chemical methods and examined the antioxidant potential by in vitro screening.

## 2 Materials and methods

### 2.1 Collection and processing of samples

The crab *S. tranquebarica* shell from the dead animals was collected from the Nagapattinam (Tamil Nadu, India) fish processing unit near fishing harbor in sterile plastic bags, and the shell was washed with distilled water for the removal of adherent particles. The cleaned shells were air dried for 2 days, and then they were fine powdered by mortar and pestle.

### 2.2 Extraction of chitin

By demineralizing and deproteinizing the material after it had been ground up, chitin was obtained. Cuttlebone powder was first treated for 24 h with 2 N HCl to remove the mineral content and then for 24 h with 1N NaOH at 80 °C to remove the protein [22].

### 2.3 Conversion to chitosan

Takiguchi's [22] method was used to deacetylate the created chitin, turning it into chitosan. Chitin was warmed at 110 °C in a reflux oven for 6 h, cooled to room temperature, and then deacetylated in 40% aqueous NaOH. Precipitate was cleaned with distilled water. By dissolving chitosan in a 10% acetic acid solution and stirring it constantly for 12 h at room temperature, chitosan was purified. The pH was lowered to 10 with a 40% NaOH solution. For 24 h, the solution was dialyzed against deionized water, and to extract pure chitosan, the product was centrifuged at 10,000 rpm for 5 min.

### 2.4 FTIR spectral analysis of chitosan

An Agilent, Cary 630 FTIR spectrometer was used for the FT-IR spectroscopy of solid samples of chitosan. The 500  $\mu$ g of chitosan sample was placed on the sample holder of FTIR spectroscopy, and scan the functional groups at 300–3500  $\text{cm}^{-1}$  wave numbers.

### 2.5 NMR analysis of chitosan

In this analysis, using BRUKER Avance II 400 MHz NMR spectrometer, the proton NMR analysis of chitosan was explored. The sample was produced and examined following Arasukumar et al. [23]. The evaluation of the chemical changes was done in parts per million (ppm).

### 2.6 In vitro antioxidant assays

#### 2.6.1 DPPH radical scavenging effect

The Arasukumar et al. [23] method was used to determine the gelatin hydrolysate's ability to scavenge DPPH radicals. The 0.1 mM DPPH (Sigma-Aldrich, India) was dissolved in 100% MeOH (Merck, India) and combined with various amounts of chitosan tubes (1, 1.5, 2, 2.5, and 3 mg/ml). Through a drop in the wavelength of 517 nm, the continuous reduction of DPPH free radicals was investigated. In this experiment, BHT-butylated hydroxytoluene (Sigma-Aldrich, India) was used as the standard, and the scavenging potency was determined using standard formula.

#### 2.6.2 Hydroxyl radical scavenging effect

The chitosan's ability to scavenge hydroxyl radicals was tested using the previously described method of Arasukumar et al. [23]. The Fenton reaction was used to assess the sample's capacity to scavenge hydroxyl radicals. Smirnoff and Cumbes procedure, sodium phosphate buffer (150 mM,

pH 7.4), 10 mM FeSO<sub>4</sub> (Himedia, India), 10 mM EDTA (Himedia, India), 2 mM sodium salicylate, and 30% hydrogen peroxide were used to create the hydroxyl radicals. Chitosan was synthesized at various concentrations ranging from 0.5 to 2.5 mg/ml. The assay's standard was BHA-butylated hydroxyanisole (Sigma-Aldrich, India). After the combination was incubated for 1 h at 37 degrees, it was read using a spectrophotometer (Shimadzu UV-1800, Japan) at 510 nm, and the hydroxyl scavenging effect was calculated using a standard equation.

### 2.6.3 Chelating ability on ferrous ions

The ferrous ions chelating effect of chitosan was determined by following the method of Dinis et al. [24]. In this analysis, 1–5 mg/ml of sample concentration was used for the evaluation of chelating effects, and EDTA was used as standard chelating agent.

## 3 Results and discussion

### 3.1 Extraction and yield of chitosan from crab shells

Chitosan was nontoxic and biodegradable biopolymer, and it is a desirable bioactive polymer, making it a good candidate for further research due to its nontoxic nature. The use of chitosan is relatively restricted because it is insoluble in water at neutral or high pH regions. To create new biomaterials, it is therefore of utmost importance to chemically alter chitosan to produce molecules that are water soluble [25]. In this study, chitin was taken from the crabs' exoskeletons using chemical procedures including deproteinization and demineralization, and chitosan was produced from chitin using a deacetylation method, which removes acetyl groups to break big molecules down into smaller ones. Chitosan was extracted in 1859 by Rouget by treating chitinous material with very concentrated potassium hydroxide at boiling temperatures. Hoppe–Seyley gave it the name chitosan in 1894. Von Furth and Russo identified the fundamental characteristics of chitosan in 1906: it is soluble in aqueous acid solutions and crystallizable from aqueous alkaline solution [10]. Yield is an important factor for the commercialization and industrialization of the chitosan production for large scale [23]. The yield percentage of *Scylla tranquebarica* crab shell-derived chitosan was found to be 21% by chemical method extraction. Arasukumar et al. [23] have reported the highest chitin yield of 35% but showed lower chitosan yield compared with *Scylla tranquebarica*. Zentz et al. [26] isolated and measured the amount of chitosan in the nacre of the pearl oyster *Pinctada maxima* and the abalone *Haliotis tuberculata*. The mangrove gastropod *Nerita* (*Dostia*) crepidularia's operculum and shell were harvested by

Palpandi et al. [27]. The N-deacetylation of shrimp chitin by alkali treatment was studied by Harish Prashanth et al. [28] in controlled environments (under N<sub>2</sub> atmosphere and in the presence of thiophenol is compared to the conventional N-deacetylated chitosan as an oxygen scavenger) and compared with the traditionally N-deacetylated chitosan. Yield of the chitin and chitosan depends on the animals and chemical diversity as well as mainly on the extraction methods.

### 3.2 IR spectral analysis

One of the most effective and widely used analytical techniques for studying the qualitative identification of functional groups with bond types that are displayed in samples based on the vibrations of atoms in a molecule is FT-IR spectroscopy [29, 30]. The functional groups were discovered in the isolated chitosan through FT-IR spectroscopy technique, and results are depicted in Fig. 1 and Table 1. In these analytical results, totally, 10 peaks were detected, 459–3427 cm<sup>-1</sup>, in chemically converted chitosan from crab shell extracted chitin. The peak intensity of 459 and 623 cm<sup>-1</sup> revealed that NH was primary and secondary amines, and the wave numbers of 1008 and 1145 cm<sup>-1</sup> confirmed the presence of C–O–C bridge and skeletal vibrations involving C–O–C stretching (Fig. 1 and Table 1) [23]. The signals of chitosan revealed that the β-chitosan depends on the bond stretching [27]. The current analysis agrees with Dimzon and Knepper's [31] work, which found amide bands at 1660 and 1550 cm<sup>-1</sup> and an amine band at 1600 cm<sup>-1</sup> in training sets chitosan and unidentified chitosan. The bands between 14389, 1523, and 1645 cm<sup>-1</sup> reflects the presence of OH and CH deformation ring and NH<sub>3</sub><sup>+</sup> bending vibrations and amide-I [30]. The bands 2094, 2937, and 3427 showed the C–H asymmetrical stretching, N–H<sub>2</sub> asymmetrical stretching, and primary amines were functional groups [32]. Chitosan was isolated from the mussel shell using the FT-IR technique, and Abdulkarim et al. [33] reported the functional groups' distinctive –NH<sub>2</sub> band of 3447 cm<sup>-1</sup> and a carbonyl group band of 1477 cm<sup>-1</sup>. Similar to this, Rumengan et al. [34] discovered various active functional groups in chitosan from rotifers, including amide band, hydroxyl, and amino bands at ranging spectra up to 3500 cm<sup>-1</sup> (*Brachionus rotundiformis*).

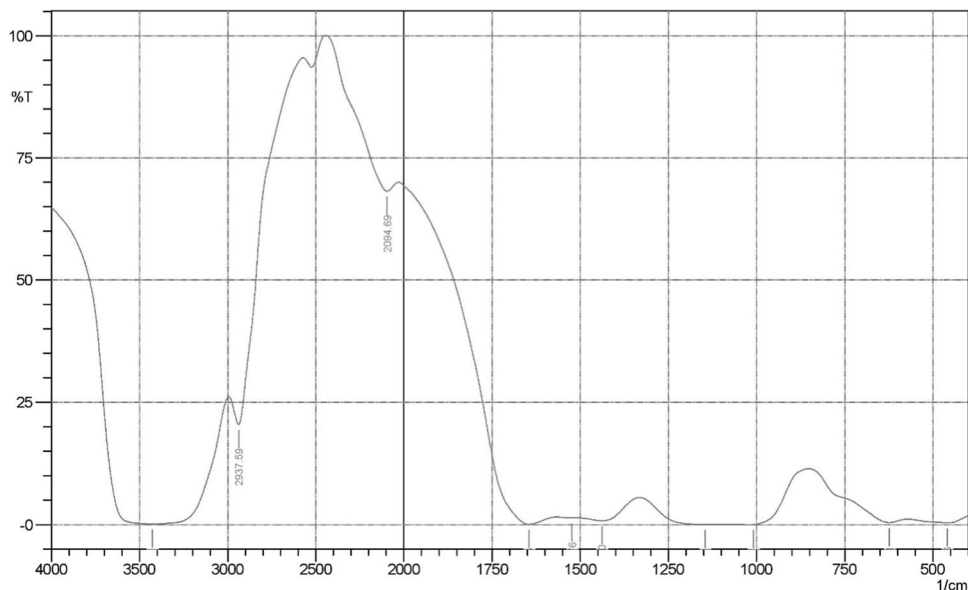
### 3.3 <sup>1</sup>HNMR spectra of chitosan

NMR spectroscopy is a very useful instrument in the arsenal of a chemist, especially an organic chemist, and is nuclear magnetic resonance spectroscopy. It is the study of how magnetic nuclei with nuclear spin interact with radiofrequency electromagnetic radiations while being affected by the right magnetic field [35, 36]. In the results of the present study, the proton NMR technique was used to identify the possible chemical structures and types of proton, which was

presented in the chitosan sample, and the results are displayed in Fig. 2. From the results, 7 major chemical shifts ( $\delta$ ) were identified between 1.07 and 3.88 ( $\delta$ ) ppm. The peaks 1.95 and 2.33 ( $\delta$ ) ppm revealed the occurrence for sugar signals of N-acetyl glucosamine (GlcNAc), and chemical shifts at 3.88 ( $\delta$ ) ppm revealed H-2 proton of glucosamine (GlcN)

residues [37]. In several types of chitosan generated from crustacean shells, Sagheer et al. [38] observed the acetyl protons, H2-6, H-2D deacetylation units, and methyl proton chemical shifts at different ppm. In partially water-soluble, partially N-acetylated chitosan, Martinou et al. [39] used  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy to trace the action pathway of

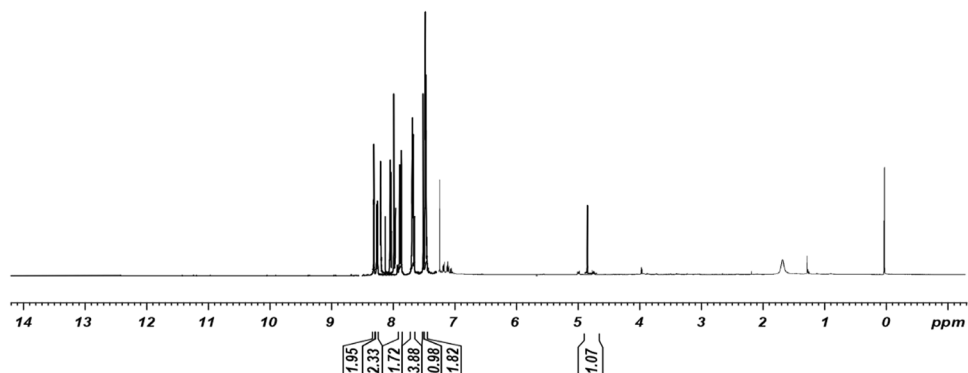
**Fig. 1** FTIR analysis of chitosan extracted from crab shells



**Table 1** IR spectra of chitosan

Sl. no.	Peak	Intensity	Corr. intensity	Base (H)	Base (L)	Area	Corr. area
1	459.06	0.376	1.214	569	399.26	364.743	52.9
2	623.01	0.439	2.627	850.61	570.93	433.034	34.163
3	1008.77	0.014	0.139	1010.7	852.54	255.604	0
4	1145.72	0.039	0.805	1330.88	1114.86	508.03	34.496
5	1438.9	0.814	2.193	1506.41	1332.81	303.52	34.446
6	1523.76	1.377	0.059	1566.2	1508.33	106.681	0.669
7	1645.28	0.071	12.953	2029.11	1568.13	434.139	80.587
8	2094.69	68.175	6.479	2443.81	2031.04	39.081	7.388
9	2937.59	20.473	14.598	2991.59	2573.04	108.061	11.025
10	3427.51	0.157	42.619	4000.36	2993.52	1379.178	991.541

**Fig. 2** Proton NMR spectral analysis of chitosan from crab shell



*Mucor rouxi*'s chitin deacetylase. The sugar signals of the chitosan was clearly identified by the  $^1\text{H}$  NMR spectroscopy.

### 3.4 Antioxidant activities of chitosan

Antioxidants are chemical or natural compounds that can stop the free radical chain reaction of oxidation from beginning or spreading by halting it and supplying hydrogen molecules. Because of this, stable free radicals are unable to initiate or grow supplemental oxidation of lipids and interact with biomolecules including amino acids, proteins, and DNA [40]. The *Scylla tranquebarica* chitosan's in vitro antioxidant activities, specifically its capacity to scavenge DPPH, hydroxyl radicals, and ferrous ions radicals, were investigated, and the results are shown in Figs. 3, 4, and 5.

From the findings of the antioxidant assays, the DPPH radical scavenging effects of chitosan have shown dose dependent scavenging effects, i.e., activity was increased with increasing of sample concentration. The maximum DPPH radical scavenging effect was noticed in 62% followed by 41 and 33% at the chitosan concentrations of 2.5, 2, and 1.5 mg/ml, respectively (Fig. 3). The lower effect was observed in 9% at 0.5 mg/ml, and the superior DPPH radical was scavenged in BHT (standard) for 91% at 2.5% concentration (Fig. 3). The maximum activity in the hydroxyl radical scavenging experiment was reported to be 71, followed by 56 and 27% at doses of 2.5, 2, and 1.5 mg/ml of chitosan, respectively (Fig. 4). The poor scavenging effects were observed in 0.5% for 14%; at the same time, the high effects were noticed in BHA (standard), and it showed 92% of scavenging effect at 2.5 mg/ml (Fig. 4). The ferrous ion chelating effects of the chitosan showed dose dependency, and the maximum effects were found to be 58% followed by 45 and 30% at 4, 3, and 2 mg/ml, and standard EDTA has shown better chelating effect of 92% at 4 mg/ml (Fig. 5). The results of the current investigation are comparable to those of Subhpradha et al. [41], who revealed that

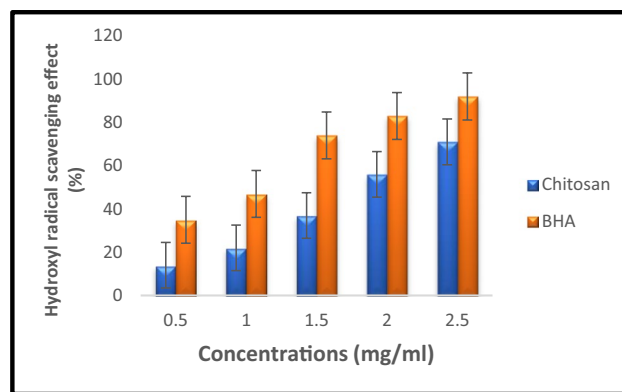


Fig. 4 Hydroxyl radical scavenging ability

phosphorylated (chemically modified) chitosan from squid pen had the highest DPPH, ABTS, superoxide, and hydroxyl radical scavenging effect. Similar to this, Seedeve et al. [42] investigated the antioxidant capacity of chemically modified (sulfated) chitosan from *Sepia prashadi* and discovered that superoxide, iron, and hydroxyl radicals showed a modest ability to scavenge antioxidant activity. Xing et al. [43] found that superoxide, hydroxyl, and ferrous ion radicals had superior scavenging abilities at concentrations of 3.269 and 0.05 mg/ml, respectively. According to Yen et al. [44], chitosan has a 28.4% scavenging efficiency for DPPH radicals, a 62.3–77.6% scavenging efficiency for hydroxyl radicals, and an 82.9–96.5% chelating efficiency for ferrous ions at a concentration of 1 mg/ml. According to Lin and Chou [45], the N-alkylated disaccharide chitosan derivatives with DS of 20–30% had the highest DPPH radical scavenging capacities of 80–95% at 0.1 mg/ml. The derivatives with DS of 40–50% and 60–70% were next in line. It appears that following sulfation or after N-alkylation of the disaccharide, chitosan's ability to scavenge might be diminished or might be increased.

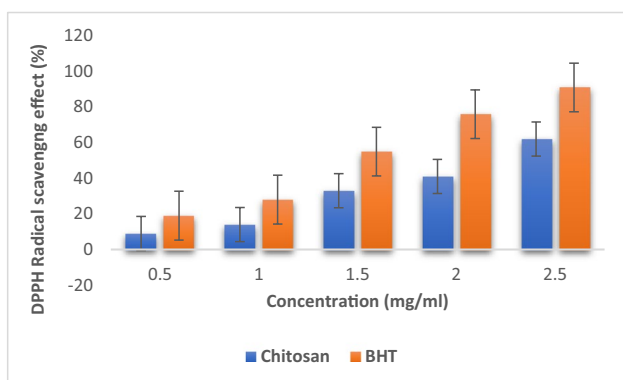


Fig. 3 DPPH radical scavenging ability

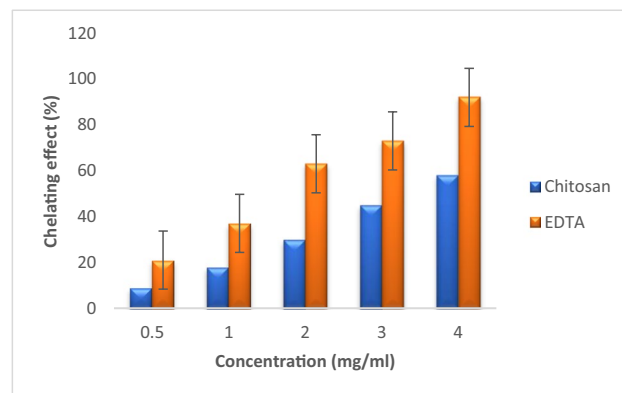


Fig. 5 Effect on the chelating ability of ferrous ions

## 4 Conclusion

In the present investigation, the chitin was extracted from the shells of crab (*Scylla tranquebarica*) waste, and the chitin was converted into chitosan by chemical conversion of deacetylation. The chemically converted chitosan has several active functional groups such as primary and secondary amines and amides, and is conformed as sugar signals. The proton NMR signals of the chitosan also revealed the sugar molecules presence of N-acetyl glucosamine (GlcNAc) and glucosamine (GlcN) residues, and it played a vital role in bioactivity of radical scavenging effects. Chitosan has demonstrated positive antioxidant scavenging activities on the main free radicals at low concentrations, although its precise mode of action is yet unknown. The present study's findings suggest that chitosan derivatives could be exploited in tissue engineering as a potential natural antioxidant and as biomedical and pharmaceutical industrial agents due to their nontoxic properties.

**Author contributions** SJS: conceptualization, methodology, formal analysis, investigation, writing (original draft and review and editing), and visualization. KT, AT, KPS, KJJ, and MMM: formal analysis, writing (review and editing), and project administration.

**Data availability** The data are available upon request from the authors.

## Declarations

**Ethical approval** In this study, animal experiment was not applicable.

**Consent to participate** In this study, animals and human trials not applicable.

**Consent to publication** Not applicable.

**Competing interests** The authors declare no competing interests.

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