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Evaluation of α -Chitosan from Crab Shell and β -Chitosan from Squid Gladius Based on Biochemistry Performance

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Featured Application: New trends on marine biomaterials.

Abstract: The objective of this study is to innovatively evaluate the biochemistry performance of α -chitosan from *Portunus trituberculatus* shell and β -chitosan from *Illex argentinus* squid gladius by using the weighted composite index method, and provide a theoretical basis for better development and utilization of chitosan biomedical materials. To build a composite evaluation system, seven key indicators, including molecular weight (M_w), deacetylation degree (DD), water binding capacity (WBC), fat binding capacity (FBC), thermal stability (TS), primary structure and secondary structure, which significantly affect chitosan biochemical characteristics, were determined and analyzed. The viscosity average M_w of chitosan was in the range of 22.5–377.1 kDa, and the DD was 83.4–97.8%. Thermogravimetric (TG) and differential scanning calorimetry (DSC) analyses of commercial chitosan (CS), crab chitosan (CSC) and squid chitosan (CSS) showed a downward trend in TS, while WBC and FBC showed an obvious upward trend. FT-IR had a similar profile in peak shape, but the peak position slightly shifted. CD indicated that chitosan maintained the double helix structure and multiple secondary structural elements. The composite weighted index values of CS, CSC and CSS were 0.85, 0.94 and 1.31 respectively, which indicated that the CSS biochemistry performance was significantly better than CSC, and β -chitosan has great potential in biomedical materials.

Keywords: chitosan; biochemistry; weighted composite index

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1. Introduction

The biosynthesis of chitin on earth is billions of tons every year, of which marine organisms produce more than one billion tons. It is an inexhaustible biological resource and a natural macromolecular compound with an output second only to cellulose [1]. Chitosan (CS), consisting of β -(1,4)-2-amino-2-deoxy-D-glucose units, is a natural cationic linear amino polysaccharide polymer deacetylated from chitin [2]. Its molecular chain contains abundant functional amino, N-acetyl amino and hydroxyl groups, resulting in relatively active properties [3]. The physiochemical characteristics and biological properties of chitosan are closely influenced by the molecular weight (M_w), deacetylation degree (DD) and sources [4]. In general, according to the arrangement of carbohydrate chains, chitosan can be divided into three different structural forms: α -form, β -form, and γ -form, each of which has different biological functions and natural sources [5]. α -form, the most common, is isolated from the exoskeleton of crustaceans, the cell wall of yeast and the cuticle of arthropods, consisting of two reverse and parallel chains with high thermodynamic stability due to hydrogen interactions between the chains. The second common chitosan structure,

β -form, is usually found in squid cartilage and composed of two parallel chains, showing a certain degree of hardness, flexibility, and fluidity, possibly due to the weak intermolecular forces caused by parallel polymer chains. The β -form chitosan in squid cartilage has superior affinity and reactivity under various physical and chemical treatments compared to the α -form chitosan in crab and shrimp shells, due to the β -form's weaker intermolecular forces than the α -form [6]. Currently, chitosan is extensively applied in versatile fields, ranging from biomedical to cosmetics, textiles, food industry and agriculture, due to its outstanding properties, including solubility, bioactivity, biocompatibility, biodegradability, non-toxicity and non-immunogenicity [7–9], etc.

Conventional methods to extract chitosan from marine organisms are generally accomplished using three different methods: chemical (hot alkaline), microbial culture and enzyme degradation. The enzymatic degradation mainly uses chitin deacetylase to catalyze the deacetylation of chitin molecules, but the main principle of microbial fermentation is to remove the acetyl group of chitin molecules contained in the cell wall by catalysis of the enzymes produced by the microorganism itself (such as *Aspergillus niger* and *Rhizopus oryzae*). The yield of chitosan by the enzyme and microbiological methods is superior with no obvious problems of molecular chain degradation or alkaline pollution [10], but the operation is more complex and the cost is higher, so it is difficult to be popularized in the industry. At present, chitosan is produced industrially mainly by the hot alkali method, which has high reaction speed and high DD, but requires hazardous chemical compounds, and is energy intensive [11]. In order to solve this problem, chitosan was prepared by ultrasonic assisted EDTA combined with the high-pressure steam method, and the physiochemical properties and biological structure of chitosan were characterized and analyzed in this study. Under the action of ultrasonic cavitation, most of the minerals can be eliminated in a short time. The hydrogen bond in the molecule can be destroyed due to the thermal energy being converted into mechanical energy under saturated steam and high pressure, and the temperature condition, which can disintegrate the chitosan crystalline structure and make the reaction, protein removal rate and DD greatly improved [12,13]. Deproteinization and deacetylation react at the same time, eliminating the intermediate washing process. The alkali liquor in the reaction can be reused after the crystallization purification treatment after being utilized to a certain extent. The production process is simple, the production time is shortened, energy consumption and environmental pollution are reduced, and the purposes of economic, efficient and green environmental protection are truly achieved.

Chitosan biochemistry performance is closely related to its biomedical applications. Molecular structural, physiochemical, and bio-functional aspects were taken into account to study chitosan biochemistry performance according to the published literature [14,15]. Infrared spectroscopy and thermogravimetric analysis are commonly applied to study and analyze the quality of the stability of chitosan. The group composition and variation in its molecular structure can be observed through the former, while the thermal stability (TS) characteristics can be analyzed beside the latter [16,17]. Sandipan Chatterjee et al. [18] characterized the biochemical efficacy of fungi chitosan by circular dichroism, and the results showed that the isolated chitosan could be used for specific drug delivery applications. Shichao Bi et al. [19] determined chitosan M_w and DD and studied the structural stability and biocompatibility. Ya-Ling Huang et al. [20] reported that the β -chitosan extracted from the squid gladius after a high pressure (500 MPa) treatment could significantly improve the fat binding capacity (FBC) and water binding capacity (WBC), and had higher antioxidant capacity.

Recently, the industry community is mainly interested in α -chitosan extracted from shrimp and crab shells, but the content of minerals is high, while the chitosan is relatively low in shrimp and crab shells. The transparent sword-shaped skeleton of the squid is called the gladius or pen, and usually is a by-product in squid industrial processing. To improve the utilization rate of marine resources and reduce environmental pollution, the squid gladius, which is significant in yield and holds valuable bioactive substances

such as β -chitin, protein and fat, can be used as a good source of chitosan products with potential application prospects in the food and pharmaceutical trades [21]. Currently, some researchers have studied the extraction and structure characterization of chitosan from marine biological by-products, and some interesting new applications have emerged. With the continuous improvement of the understanding of chitosan and the further deepening of the research on its application, chitosan has attracted more and more attention. The quality standard of chitosan established recently is only limited to the physiochemical properties, but the related biological performance is not involved yet. The comprehensive evaluation method for chitosan biochemistry performance, which is helpful to control the biological application of chitosan from different marine sources, has not been reported. A weighted composite index method groups the same or different index values for statistical processing to standardize the index values of different measurement units and different properties, to give various weights according to the importance, and finally to transform them into a weighted composite index. In this paper, the estimation method of the chitosan biochemistry performance index was established based on the close relationship between chitosan properties and the biomaterials application, which can provide scientific guidance for chitosan biological application.

2. Materials and Methods

2.1. Materials

Squid gladius of *Illex argentinus* were provided by Shanghai Fisheries Group Co., Ltd., Shanghai, China, which were washed twice with water, dried, pulverized using a grinder and passed through a 30 mesh sieve and stored at $-20\text{ }^{\circ}\text{C}$. Dried crab shell powder of *Portunus trituberculatus* was supplied from Lianyungang. CS was purchased from China National Pharmaceutical Group Corporation. All chemicals and solvents used in this study were purchased at the available analytical level or at the highest purity level, and all solutions were freshly prepared in distilled water.

2.2. Preparation of Chitosan

According to a method proposed by Yang Jinfeng [22], with some modification, demineralization was performed with ultrasonic assisted EDTA, and deproteinization and deacetylation were executed simultaneously by “one pot cooking”. The screened squid gladius powders and crab shell powders were quantitatively weighed and treated with 0.5 M EDTA decalcification solution at the ratio of 1:15 (g/mL) under constant stirring, respectively. Thirty-seven percent of HCl was added to fully dissolve the inorganic salt, the pH value was adjusted to 4.0, ultrasonic water bath reaction was conducted at $25\text{ }^{\circ}\text{C}$ for 30 min, and the ultrasonic frequency was controlled at 24~71 kHz. After dropping 37% of the HCl solution, stirring and standing for 10 min until no bubbles were observed and the decalcified reaction solution was obtained after decalcification and after filtration, the filter residue was collected and washed to neutrality with deionized water. Forty percent (w/v) of NaOH was added to the demineralized filter residue (the dosage was 1:5 (g:mL)), placed in a high-pressure reactor (BIOBASE, BKQ-B50II, China) at $120\text{ }^{\circ}\text{C}$, 0.225 MPa for 4 h, cooled, filtered, and the reactants washed to neutral. The alkali liquor in the reaction was reused after the crystallization purification treatment after being used to a certain extent. For purification, chitosan was continuously dissolved in 10% acetic acid solution at room temperature for 10 h, and the pH value was adjusted to 10 with 40% NaOH solution. The solution was dialyzed with deionized water for 24 h, the product was centrifuged at 5550 g for 10 min in a centrifuge (HITACHI, CR21G, Tokyo, Japan), and then lyophilized to obtain purified chitosan.

2.3. Characterization of Chitosan

2.3.1. Determination of the Molecular Weight

The viscosity average M_w of chitosan was determined by Ubbelohde Viscometer dilution method. Chitosan was dissolved in 0.2 M sodium chloride/0.1 M acetic acid at

25 ± 0.1 °C and determined using Ubbelohde Capillary Viscometer (Loikaw, China, capillary diameter: 0.4–0.5 mm). The M_w was calculated using the Mark–Houwink parameters as follows:

$$[\eta] = K(M_w)^\alpha$$

where $[\eta]$ is the characteristic viscosity, K and α are the characteristic constants of a specific polymer solvent system (selected according to DD) [23].

2.3.2. Measurement of Deacetylation Degree

Chitosan DD was analyzed using UV 1st derivative spectrophotometry method [24]. The calibration curve was made by plotting the 1st derivative value at 203 nm as a function of N-acetyl-D-glucosamine and D-(+)-glucosamine hydrochloride. Dry chitosan (0.01 g) was dissolved in 10 mL of 0.1 M acetic acid solution and added to 100 mL distilled water. The 1st derivative spectrum of chitosan was detected, and the content of N-acetyl-D-glucosamine was calculated according to the calibration curve.

2.3.3. Water Binding Capacity

WBC of the chitosan samples, which was interpreted as grams of water bound per gram of dry chitosan, was determined using the method of Huang and Tsai [18]. 1 g of dried chitosan was mixed with 10 mL distilled water at room temperature for 24 h, after centrifugation under centrifugal force of 4025 g for 10 min, and then the supernatant was discarded. After drying for 4 h at 70 °C in vacuum oven (CIMO, DZF-6030, China), the residue weight was determined and WBC was calculated by the following equation:

$$\text{WBC (\%)} = A_1 / A_0 \times 100$$

where A_0 is the weight of dry chitosan and A_1 is the weight of water bound by chitosan sample.

2.3.4. Fat Binding Capacity

FBC of the chitosan samples, which was expressed as grams of oil retained per gram of dry chitosan, was detected by the method of Wang and Kinsella [25] with some modification. 1 g dried CS, CSC, CSS chitosans was separately mixed with 20 mL soybean oil in the 50 mL centrifuge tube and made the sample completely dispersed. Then, tubes were placed at room temperature for 1 h with interval shaken for 5 s every 10 min followed by centrifugation at 4025 g for 25 min at room temperature. After decanting the supernatant, the tube weight was measured and FBC was obtained by the following equation:

$$\text{FBC (\%)} = A_1 / A_0 \times 100$$

where A_0 is the weight of dry chitosan and A_1 is the weight of fat bound by chitosan sample.

2.3.5. Thermogravimetric Analysis (TGA)

The TG209 F3 thermogravimetric analyzer (Netzsch, Weimar, Germany) was used in the experiment. The temperature of chitosan samples was raised from 30 °C to 700 °C at a heating rate of 10 °C /min under N_2 (purity: 99.999%) atmosphere. Derivative thermogravimetric analysis (DTG) curve was obtained depending on the differential thermogravimetric curve.

2.3.6. Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry was performed using DSC823e differential scanning calorimeter (Mettler Toledo, Shanghai, China). The sample was heated from -30 °C to 220 °C at a heating rate of 10 °C /min under N_2 atmosphere.

2.3.7. Fourier Transform Infrared Spectroscopy Analysis (FT-IR)

All samples were previously dried in a vacuum oven at 40 °C for 24 h under reduced pressure. The characteristic infrared spectra of samples were obtained by 64 scans at resolution of 4 cm⁻¹ between the wavelength range of 400~4000 cm⁻¹ in Nicolet is 10 spectrometer (Thermal Fisher Scientific, Waltham, MA, USA).

2.3.8. Circular Dichroism Spectroscopy (CD)

The CD spectroscopy of the chitosan was recorded using J-815 circular dichroic spectrometer (JASCO, Tokyo, Japan). 0.7 mL chitosan solution with concentration of 50 mg·L⁻¹ prepared by 2% acetic acid was analyzed for CD scanning under scanning rate 100 nm·min⁻¹ between the wavelength range of 260~340 nm in the optical path 2.00 mm quartz cuvette. The data of HT (voltage of photomultiplier tube) greater than 800 in the range of test wavelength were omitted, and the rest data were analyzed for circular dichroism spectrum.

2.3.9. Scanning Electron Microscopy (SEM)

S3400N scanning electron microscope (Hitachi, Japan) was used to observe the surface and microstructure of the chitosan samples. The samples were placed on the double-sided carbon tape and mounted on an aluminum specimen metal disk for vacuum gold spraying. The images were photographed at 400× magnification 10 kV accelerating voltage.

2.3.10. Polarized Optical Microscopy (POM)

The chitosan powder was coated on the glass slide, and the crystal morphology of the chitosan samples was observed under the color background via DM4500p polarizing microscope (Leica, Wetzlar, Germany).

2.4. Weighted Composite Index Evaluation

2.4.1. Index Selection, Classification and Determination of Weight Coefficient

According to the principle of scientificity, practicability and sensitivity of statistical data, and referring to relevant literature, three dimensions of chitosan characteristic indexes measured by the above methods were selected, including seven indexes which have significant influence on the biochemistry performance of chitosan: M_w, DD, WBC, FBC, TS, primary structure (PS) and secondary structure (SS).

Analytic hierarchy process (AHP) and expert consultation were used to calculate the weight coefficient. Relevant literatures and reports were searched [14,18,19,26,27], experts and scholars in the field of chitosan were organized to discuss and design questionnaires. The relative importance of each layer of indicators was judged according to the hierarchical scaling method, and the weight coefficient of each layer of indicators were calculated.

2.4.2. Data Standard Normalization Processing

The original data table of n rows (n evaluation objects) and m columns (m evaluation indexes) were listed. To eliminate the unit limitation of the original data, all index data were standardized and converted into the values of 0~10 interval. For getting rid of the multi variable order of magnitude, root operation was performed on the data with order of magnitude difference.

X_i is the actual value of each index, and M is the standard value of the index. The purpose of evaluation is to evaluate the biochemistry performance of CS, crab chitosan (CSC) and squid chitosan (CSS), so the average level of three kinds of chitosan was selected as the standard value (M). Then, M was taken as the reference value, X_i was compared with M , in which the M_w is inverse indexes, and the rest are positive indexes. The positive index refers to the index that the larger X_i , the better the evaluation, which is reflected by X_i/M . Inverse index refers to the index that the smaller X_i , the better, which is affirmed in M/X_i .

2.4.3. Calculation and Evaluation of Weighted Composite Index

The purpose of weighted general quality index (GQI) is to integrate several quality indexes of chitosan into one index GQI reflecting the general chitosan biochemistry performance. The establishment of weighted composite index evaluation method refers to Kristiana Dolge [28] with slightly changed.

GQI was calculated followed by the formula:

$$GQI = \sum_{i=1}^n a_i Y_i$$

where a_i is the weight of the i -th quality index, which is distributed according to the importance, and $\sum a_i = 1$; Y_i is the dimensionless value of the i -th index, and the composite index of chitosan biochemistry performance is obtained by adding the three indexes; n is the number of indexes.

2.5. Statistical Analysis

All values were expressed as means $\pm$ standard deviation of three independent experiments. Experimental data were analyzed via one-way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) version 17.0 (IBM, New York, NY, USA). The differences were considered at $p < 0.05$ for statistical significance.

3. Results and Discussion

3.1. Composition of Experimental Raw Materials

The proximate components of the dried shell of *Portunus trituberculatus* shell and *Illex argentines* squid gladius are shown in Table 1. Protein content of squid gladius is significantly higher than that of crab shell, suggesting that gladius of *Illex argentines* can be used as an excellent protein source (59.34%). Previous studies showed that the protein content of gladius in various squid species ranged from 36.5% to 74.6% [29–31]. Ash content of *Portunus trituberculatus* shell (mainly calcium carbonate) was 58.68%, while *Illex argentines* gladius only 0.86%. Bianli Wang et al. [32] reported that the content of calcium carbonate in *Eriocheir sinensis* was around 55%. Therefore, demineralization is necessary when chitosan obtained from marine crustacean shell. Cortizo et al. [33] reported that gladius of *Illex argentines* squid contain 1% ash, 2.3% fat, 64% protein and 31% chitin. Hence, compared with marine crustacean shell, chitosan extracted from squid gladius the demineralization can be avoided.

Table 1. Proximate composition of the dried shell of *Portunus trituberculatus* and squid gladius of *Illex argentinus*.

Name	Protein (%)	Fat (%)	Ash (%)	Moisture (%)	Yield (%)
Shell	18.36	3.22	58.68	2.32	NA ^a
Gladius	59.34	2.06	0.86	1.13	NA ^a
CS	ND ^a	ND ^a	0.18	0.35	NA ^a
CSC	ND ^a	ND ^a	ND ^a	0.32	11.81 ^b
CSS	ND ^a	ND ^a	ND ^a	0.26	26.15 ^b

^a NA—Not applicable; ND—None detected (below detection limits). ^b calculated according to dry weight of crab shell and squid gladius respectively.

As shown in Table 1, protein and fat were not detected in prepared chitosan and CS, indicating high purity. Ash content of CSC and CSS was lower than the detection level, and the difference was statistically significant ($p = 0.03$) compared with CS, which may be related to the purity of samples. Chitosan samples have relatively low moisture content, which is linked to the drying process and the extent of exposure to the atmosphere during storage. Chitosan is hygroscopic, minimal water content chitosan often has good shelf stability.

3.2. Extraction of Chitosan

The dried *Portunus trituberculatus* shell and *Illex argentinus* squid gladius were used as raw materials respectively. After demineralization, chitosan was directly prepared by high pressure heating in alkali water mixture. Deproteinization and Deacetylation were carried out simultaneously by “one pot cooking”. The specific preparation scheme of chitosan is presented in Figure 1. Chitosan yields (Table 1) of *Portunus trituberculatus* shell and *Illex argentinus* squid gladius were 11.8% and 26.1% respectively on the basis of dry weight calculation. S. Haiji et al. [34] reported that the yields of chitosan obtained from shrimp, crab and cuttlefish were 14.9%, 5.3% and 1.2%, respectively. B. E. Abdelmalek et al. [31] reported that the yield of β -chitosan from European squid (*Loligo vulgaris*) was 19.5% based on wet weight. Huang et al. [18] reported that the yield of chitosan extracted from gladius of *Illex argentinus* squid was 23.5%–34.5% under different pressure conditions.

Figure 1. Flow scheme for the isolation of marine-derived chitosan.

3.3. Analysis of Molecular Structural Characteristics

3.3.1. Molecular Weight

The time of chitosan solution flowing through capillary tube was measured by Ubbelohde viscometer at 25 ± 0.1 °C. According to the flow time, the specific viscosity (η_{sp}) of chitosan solution was calculated, and then the specific viscosity (η_{sp}/C) was obtained, the results were shown in Table 2. The relationship between the specific viscosity (η_{sp}) and the concentration (C) of three kinds of chitosan in 0.2 M sodium chloride/0.1 M acetic acid was shown in Figure 2. Under the same solvent and temperature, the intercept $[\eta]$, which can directly show the difference of M_W , was obtained by extrapolating the straight line to intersect with the ordinate. The viscosity average M_W obtained as a result of extrapolation according to the Mark–Houwink equation ($[\eta] = K(M_W)^\alpha$) were shown in Table 3, and the data was consistent with its apparent viscosity. The highest M_W of CS was 377.1 kDa, followed by CSC (249.8 kDa) and CSS (22.5 kDa). M_W has an important impact on chitosan physiochemical properties and biological function. Chitosan M_W can affect the viscosity of chitosan solution [35], the DD lower and the M_W higher, the chitosan solution viscosity stronger. Chitosan with M_W of 10 kDa has many excellent physiological functions, such as inhibiting the growth of tumor cells, reducing cholesterol, enhancing immunity [34]. The difference of M_W of the three tested chitosan samples is due to the different sources of raw materials and treatment methods. It is obvious that high temperature and high pressure flash explosion is a controllable method to obtain low M_W chitosan.

Table 2. Data of chitosan viscosity measurement.

C(g/100 mL)	CS		CSC		CSS	
	η_{sp}	η_{sp}/C	η_{sp}	η_{sp}/C	η_{sp}	η_{sp}/C
0.20	0.93	463.31	1.43	713.03	0.41	204.58
0.13	0.55	413.22	0.82	616.22	0.21	153.86
0.10	0.39	389.10	0.55	546.07	0.13	130.44
0.07	0.24	367.25	0.33	490.28	0.07	105.21
0.05	0.18	359.34	0.22	449.31	0.05	94.01

Figure 2. η_{sp}/C -C diagram of chitosan.

Table 3. Raw data of chitosan chemistry performance indexes.

	Molecular Structural		Physiochemical		Bio-Functional		
	M _w (kDa)	DD (%)	WBC (g/g)	FBC (g/g)	TS	PS	SS
CS	377.1	83.4	3.3	2.7	5.25	0.28	1.03
CSC	249.8	92.7	5.2	2.9	4.70	0.30	1.07
CSS	22.5	97.8	10.8	3.9	4.56	0.31	1.46

3.3.2. Deacetylation Degree

The DD can significantly influence chitosan physiochemical properties and biological activity, which is attributed to with DD raised, the -NH₂ group of chitosan molecules protonated in acidic environment to form NH₃⁺ ions, and the charge density on the carbon chain increased [36]. The variation of chitosan DD could help to define the different properties of the following chitosan samples. It was found that the M_w of CSS (3122.3 kDa) is higher than that of CSC (728.1 kDa) (not listed) when the reaction time is shortened to make their DD approximate to CS, which was similar to the result of Wang Aiqin et al. [37]. Therefore, under high temperature and pressure can reduce the effective breaking degree of β-1,4-glycosidic bond and obtain high M_w chitosan when the DD of CSC and CSS near commercial chitosan(CS). With the reaction time was prolonged, the DD of CSS and CSC were 97.8% and 92.7% respectively, which were significantly ($p = 0.03$) higher than that of CS (83.4%) (Table 3). The DD of prepared chitosan was similar to that reported by Zhu et al. [38] And Chen et al. [39]. M_w of CSC decreased and CSS decreased sharply, indicating the molecular chain of chitosan broken with the DD deepening, and CSS was more degraded than CSC may be related to their different molecular crystalline structure.

3.4. Analysis of Physiochemical Characteristics

3.4.1. Water Binding Capacity

The WBCs of three chitosan samples are shown in Figure 3. It was observed that the WBC of CSS (10.8 g water/g chitosan) was significantly higher than that of CSC (5.2) and CS (3.3). This difference may possibly be attributed to the high temperature and pressure treatment degrading β-chitosan into smaller molecules more easily and increasing the chitosan surface area, and a higher DD, exposing more hydrophilic amino groups in the chitosan molecular chain, resulting in the formation of hydrogen bonds with water molecules and showing better hygroscopicity and moisture retention. Tian Ye et al. [40] observed that for hydrophilic polymers, polar groups can strongly interact with water molecules. Therefore, the more polar groups, the greater the water absorption, indicating that the water absorption of chitosan increases with the increase in DD.

3.4.2. Fat Binding Capacity

Chitosan cannot be digested and absorbed in the human body for a short time; it can absorb multiple times its own weight in oil, effectively prevent the digestive system from absorbing cholesterol and triglycerides, and prevent the accumulation of cholesterol and fatty acids in the body. As shown in Figure 3, the FBC of CSS (3.9 g oil/g chitosan) was the highest among the three chitosan samples, followed by CSC (2.9) and CS (2.7), which was similar to its WBC. This was explained by the higher binding ability of low M_w chitosan with oil, which was consistent with the results reported by Ocloo et al. [41]. With the increase in DD, the exposed, positively charged NH₃⁺ rise, and the oil is adsorbed via the charge action. Additionally, the decrease in M_w makes the surface area of oil molecules larger, so it has greater fat binding ability. Jin Qiu et al. [42] observed that for the FBC of the average M_w of α-chitosan (1–1800 KDa) and β-chitosan (1–2340 KDa), the latter had higher DD, more amino groups and stronger charge attraction. The results showed that the maximum FBC of β-chitosan was higher than that of α-chitosan, but the regularity was distinct, indicating that chitosan FBC may be related to M_w, DD, and different molecular conformations also.

Figure 3. Water-binding capacity (WBC) and fat-binding capacity (FBC) of chitosan. Different small letters under the data in the same group indicate significantly different ($p < 0.05$).

3.4.3. Thermal Stability Analysis

The TGA and DTG curves of three chitosan samples are presented in Figure 4. The thermal degradation process can be roughly divided into three stages: before 200 °C, between 200–450 °C and after 450 °C according to the curves, which are respectively attributed to the loss of bound water, main chain fracture and complete carbonization of chitosan [43]. All samples had a slight weight loss before 100 °C, which was caused by the precipitation of trace water from chitosan. The initial mass loss of CS was 8.3%, while the mass loss of CSC and CSS were 10.1% and 8.7% respectively, indicating that chitosan prepared by high temperature and a high-pressure flash explosion could absorb more water in the form of bound water and unbound water, and could remove a certain amount of water at high temperature. The thermal decomposition mainly occurs at 250–350 °C, during which the O-bridge of the chitosan main chain and acetyl group broke, and the thermal weight loss was very slow after 450 °C. There was a strong weight loss peak on the curve at around 300 °C, where the thermal decomposition rate of chitosan reached the maximum. The maximum weight loss of CS was 59.1% at 308.09 °C, and that of CSC and CSS was 53.1% and 51.2% at 293.31 °C and 293.55 °C, respectively. The different thermal decomposition behaviors of α -chitosan and β -chitosan may be attributed to the distinct intermolecular forces in their different crystalline structures, especially the loss of hydrogen bonds.

It can be observed from the endothermic peak in the DSC curve spectrum of the tested samples in Figure 4 that the exothermic peak temperature of CS (129.11 °C) is the highest, followed by CSC (126.28 °C) and CSS (119.60 °C), and the results show the same trend as TGA and DTG. The M_w , DD, source and extraction method are important factors affecting the TS of chitosan.

The quantitative calculation formula of TS value is as follows: maximum point temperature of weight loss rate $\times$ thermal degradation temperature range $\times 10^{-4}$. The chitosan TS values are shown in Table 3.

Figure 4. Thermogravimetric analysis (TGA) and derivative thermogravimetric analysis (DTG) curves of commercial chitosan (CS) (A), crab chitosan (CSC) (B), squid chitosan (CSS) (C) and differential scanning calorimetry (DSC) spectra of chitosan (D).

3.5. Analysis of Bio-Functional Characteristics

3.5.1. FT-IR Analysis

The infrared spectra of the CS, CSC and CSS are shown in Figure 5. CS was analyzed by FT-IR spectroscopy as a reference. The results showed that the FT-IR spectra of CS, CSC and CSS exhibited similar tendencies, but the peak positions were slightly shifted. Characteristic peaks located at 1664 cm^{-1} , 1597 cm^{-1} and 1325 cm^{-1} in CSC were assigned to the amides I, II and III band, but 1660 cm^{-1} , 1592 cm^{-1} and 1321 cm^{-1} in CSS were observed respectively [44]. The fundamental frequency of the alcohol hydroxyl group was around 3600 cm^{-1} , and the stretching vibration absorption peaks of hydrogen bond (O-H and N-H) moved to a low frequency direction due to the role of the hydrogen bond in the chitosan molecular chain. The broad peak around $3417\text{--}3442\text{ cm}^{-1}$ was attributed to O-H and N-H stretching vibration on the chitosan molecular chain. It can be observed that the O-H absorption strength of chitosan increased and the absorption peak of CSS became sharper by comparing the absorption peak of CSC and CSS at 3432 cm^{-1} and 3417 cm^{-1} , respectively. According to the M_W of chitosan as determined above, the M_W of CSS was much lower than that of CSC, which indicated that the decrease in M_W may lead to an increase in the amount of hydroxyl in the product. The intramolecular and intermolecular hydrogen bonds were enhanced, and the molecular structure was improved. This is because with the decrease in M_W , the entanglement between the molecules decreases, which is conducive to the orderly arrangement of molecules. The characteristic absorption peaks of C₆-OH primary alcohol hydroxyl and C₃-OH secondary alcohol hydroxyl were noticed at 1026 cm^{-1} and 1166 cm^{-1} in CSC, but 1023 cm^{-1} and 1160 cm^{-1} in CSS, respectively. The microscopic differences of the above infrared spectra may be related to the antiparallel and

parallel arrangement of the molecules, explaining the difference in molecular structures between α -chitosan and β -chitosan. β -chitosan has higher physiological activity than α -chitosan due to the simpler molecular structure and weaker intermolecular force.

Figure 5. FT-IR spectra of chitosan (a) and fitted FT-IR spectra of CS (b), CSC (c), CSS (d).

FT-IR is based on the composition of molecular groups and sensitive to polar groups of chitosan, which is closely related to the biochemical characteristics of chitosan. Hydroxyl and amino groups are the main active functional groups of chitosan. So the eigenvalue of its PS is defined as the ratio of hydroxyl and amino groups. The FT-IR spectra of chitosan were fitted with sub peaks, and the results in Table 4 show that the PS values of CS, CSC and CSS were 0.28, 0.30 and 0.31, respectively. PS values of the three kinds of chitosan showed an increasing trend, which was attributed to the exposure of more active amino groups with the increase in DD and the decrease in M_w .

Table 4. Primary structure value of CS, CSC and CSS.

	-OH&-NH ₂	All Groups	PS
CS	1225.89	4360.71	0.28
CSC	1291.36	4306.67	0.30
CSS	1306.55	4216.13	0.31

PS means primary structure value, which is the ratio of hydroxyl group to amino group in the molecular structure of chitosan.

The value in the figure is the peak area calculated by peak fitting, and the hydroxyl and amino values are the sum of the fitting peak areas at CS (3442 cm^{-1} , 1173 cm^{-1} , 1030 cm^{-1}), CSC (3432 cm^{-1} , 1166 cm^{-1} , 1026 cm^{-1}) and CSS (3417 cm^{-1} , 1160 cm^{-1} , 1023 cm^{-1}) respectively.

3.5.2. CD Analysis

Circular dichroism spectroscopy is an effective method to study the three-dimensional structure of an organic biomacromolecule, which can provide the absolute configuration,

conformation and other information of molecules. Domard [45] reported that the absorption peak height was related to the content of acetyl group. Farooqahamed SK [46] deemed that the absorption of CD is independent of the configuration of α and β heterocephalic carbon, the length of the molecular chain, the ionic strength and the pH.

The CD spectral of chitosan samples are shown in Figure 6. It can be observed that each sample has a wide negative absorption peak at around 210 nm, indicating that chitosan still maintains a double helix structure, which is caused by the $n-\pi^*$ electronic transition of NH-CO chromophore in chitosan molecule. The low CD absorption peak of CSS is attributed to high DD and less carbonyl chromophores, while the absorption peak of CSC and CS are obvious, indicating that the absorption peak decreases with the increase in DD. The positive Cotton effect appeared at 180–200 nm, which is due to the existence of C-O and O-H groups in the polysaccharide molecule, indicating that α -helix and β -sheet existed in the SS of chitosan. The content of conformation unit was calculated by CD according to Chen et al. [47]. The results in Table 5 showed that the content of the β -sheet in chitosan is higher than that of α -helix. The spectrum of CSC was similar to that of CS, but the shape and position of the peak changed, and a positive peak appeared at 190 nm and 184 nm, respectively, which indicated that not only the DD of the samples treated by high temperature and high pressure increased, but also the stereoscopic configuration changed. CSS formed a weak positive peak at 196 nm and 183 nm, and a strong negative peak at 190 nm, indicating that it contained β -turn structure. The reason for this difference may be that the α -helix and β -sheet of α -chitosan are interphase in space, and most of the folding chains are parallel, while the α -helix and β -sheet of β -chitosan are separated in space, and most of the folding chains are antiparallel.

Table 5. Different secondary structure contents of CS, CSC and CSS.

	α -Helix	β -Sheet	β -Turn	Random Coil
CS	8.5	35.1	7.1	49.3
CSC	8.3	36.6	6.9	48.2
CSS	7.7	39.5	11.5	40.3

Number in the table is the proportion of each secondary structure of chitosan.

Figure 6. Circular dichroism spectroscopy (CD) spectra of CS (A), CSC (B), CSS (C).

The SS conformation units α -helix, β -sheet and β -turn have great influence on the biological function of polysaccharides, so the eigenvalue of SS (Table 3) is expressed by the ratio of the total content of α -helix, β -fold and β -turn to random coil. The effect of the advanced structure on function is more important than that of PS, and the stereo structure of polysaccharide is one of the determinants of its physiological activity. The results laid a foundation for further study on the relationship between the structure–activity relationship and biological activity function of chitosan.

3.6. Morphological Observation

3.6.1. Scanning Electron Microscopy

As presented in Figure 7, the morphological structure of chitosan was studied by SEM; the surface morphologies of CS and CSS are obviously different. The surface of CS is clean and lamellar, which is the typical surface morphology of chitosan [16]. CSC has round pores and ultrafine fibers, showing irregular multilayer conformation. CSS consists of round pores, microfibers and microcrystals, with a large number of prominent microfibers, and the particles in the shape are uneven and fragmented. Kucukgulmez et al. [48] observed the fragmentation with fibrillar structure in the SEM of chitosan. The diverse morphology of chitosan may be due to the different arrangement of hydrogen bonds between the chitosan samples [49].

Figure 7. SEM micrographs of CS (a), CSC (b), and CSS (c).

3.6.2. Polarized Optical Microscopy

Different M_w and DD affect the crystal structure of chitosan, especially the loss of intramolecular and intermolecular hydrogen bonds [50,51]. The molecular chain of chitosan has regularity, and there are intramolecular and intermolecular hydrogen bonds, which are conducive to the formation of a crystalline state. Polymers generally form spherical crystals first, and then form flat rectangular acicular crystals after further crystallization. Figure 8 shows the POM photos of different types of chitosan. It can be observed that the crystallinities of CS, CSC and CSS tend to increase, which may be related to the molecular structure of chitosan. The molecular chain of CSC is composed of two reverse parallel chains, which may have high thermodynamic stability due to the strong hydrogen bond interaction and high degree of entanglement. However, the intermolecular force of CSS is weak due to the parallel polymer chains [52], representing high crystallinity.

Figure 8. Polarized optical microscopy (POM) microscopes of CS (a), CSC (b), CSS (c).

3.7. Weighted Comprehensive Index Evaluation Method

3.7.1. Original Data

As shown in Table 3, the raw data table of the chitosan biochemistry performance evaluation index composed of seven indexes is established.

3.7.2. Determine the Weight Coefficient

Weight coefficient refers to the number employed to represent the proportion of each factor in the whole when an entirety is decomposed into several factors. The index weight reflects the relative importance of the index overall. For important indexes, the weight is larger; to the contrary, it is smaller. The commonly used methods to determine the index weight are Delphi, principal component analysis, the entropy method and the mean square error method. These methods are cumbersome and need significant calculations. In the composite evaluation of chitosan biochemistry performance, the combination of an analytic hierarchy process (AHP) and expert consultation should be used, because an AHP can simply compare and judge the evaluation weight of each index, and the expert consultation can increase the credibility and authority of the evaluation weight. The final weight coefficient is shown in Table 6.

Table 6. Content and weight coefficient of chitosan chemistry performance indexes.

Dimension	Indicator	Impact on <i>GQI</i>	Weight
Molecular structural (0.4)	M_w	–	0.20
	DD	+	0.20
Physiochemical (0.3)	WBC	+	0.12
	FBC	+	0.12
	TS	+	0.06
Bio-functional (0.3)	PS	+	0.12
	SS	+	0.18

3.7.3. Standardization Results

Before the standardization, the whole set of index data (such as M_w and WBC) with an order of magnitude difference need to be performed with the same power root to make them all become values between 0 and 10, so as to get rid of the huge ratio difference caused by the different digital factor magnitude. If the data in the same column have no order of magnitude difference but are all greater than 10, which should be divided by the same multiple until all converted into values between 0 and 10. The result of the data processed by the root operation is shown in Table 7.

Table 7. Data of chitosan biochemistry performance indexes processed by root operation.

	Molecular Structural		Physiochemical			Bio-Functional	
	M_w	DD	WBC	FBC	TS	PS	SS
CS	7.2	0.83	1.8	2.7	5.25	0.28	1.03
CSC	6.2	0.93	2.3	2.9	4.70	0.30	1.07
CSS	2.8	0.98	3.3	3.9	4.56	0.31	1.46
M	5.4	0.91	2.5	3.2	4.84	0.30	1.19

The cubic root operation was carried out based on the raw data of chitosan M_w , and the quadratic root operation was performed according to the raw data of chitosan WBC.

All the original indicator data were standardized and converted to 0~10 interval values. Then the average value of each index of the evaluation object was taken as the standard value to index the raw data. The normalization results of each index standard are shown in Table 8. It can be observed that the M_w , DD, WBC, FBC and SS values of three kinds of chitosan after the standard normalization treatment are significantly different, while the TS and PS values are less different.

Table 8. The index value after standard normalization.

	Molecular Structural		Physiochemical			Bio-Functional	
	M_w	DD	WBC	FBC	TS	PS	SS
CS	0.75	0.91	0.72	0.84	1.09	0.94	0.87
CSC	0.87	1.02	0.92	0.91	0.97	1.01	0.93
CSS	1.93	1.08	1.32	1.22	0.94	1.04	1.23

3.7.4. Calculation of Composite Index

The indexes of molecular characterization, physiochemical properties and structural characterization were calculated according to the weight of each index followed by the formula: $GQI = \sum_{i=1}^n aiYi$ and the composite index of chitosan was obtained by adding the three indexes. The results are presented in Figure 9. From the overall results, the most significant feature is that the CSS composite index (1.31) is obviously higher than CSC (0.94) and CS's (0.85), which is consistent with the research results recently published [17,19]. In addition, molecular structure accounts for the largest proportion in the comprehensive index, and physiochemical and bio-functional also hold a large proportion. This is mainly due to the low viscosity average M_w and high DD of CSS, and the parallel structure between β -chitosan molecules and weaker molecular binding force, which is superior to α -chitosan (CSC and CS) in molecular structure, physical and chemical properties and biological functions.

Figure 9. Composite index of chitosan biochemistry performance. Different lowercase letters under the data in the same group indicate significant difference ($p < 0.05$).

The higher the weighted composite index value, the better the chitosan biochemistry performance. The advantage of the weighted composite index method for evaluating chitosan biochemistry performance is that it can make the proportion of each index in the overall performance analysis clear, and can analyze and evaluate more objectively and comprehensively. The rationality of the comprehensive evaluation results mainly depends on the selection of representative indicators, the setting of reasonable weight coefficients

and the use of scientific statistical methods. In order to make the evaluation results of constant quality indicators comparable, it is necessary to determine a consistent evaluation index system and weight coefficient. Reasonable evaluation method can better control biological applications of chitosan obtained from different marine resources.

4. Conclusions

In summary, the chitosan biochemistry performance from different sources and preparation technologies are distinctive. The yield of chitosan from different marine organisms was effectively enhanced though the flash explosion “one pot cooking” method of high temperature and pressure, and the physiochemical properties of DD, WBC and FBC were significantly improved; this appears to be a new potential way to prepare chitosan with high yield and DD. The biochemistry performance of CSS extracted from *I. argentinus* squid gladius is significantly better than that of CSC obtained from *P. trituberculatus* crab shell, suggesting that β -chitosan is a potential biomedical material. The selected seven key indicators, namely M_w , DD, WBC, FBC, TS, PS and SS, which have a major impact on the quality of chitosan from three levels of molecular structural and physiochemical properties and bio-functional characteristics, are appropriate. The chitosan biochemical characteristics can be reflected reasonably from the composite index calculated on the basis of the above seven indicators.

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