Comparison of Depolymerization of Chitosan into Chitooligosaccharides from the Shrimp Shell Waste of *Parapeneopsis Stylifera* by Non-Specific Enzymes

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Abstract

Two different non-specific proteolytic enzymes (papain and pepsin) and two carbohydrase enzymes (α -amylase and β -amylase) were used for the depolymerization of chitosan to produce chitooligosaccharides (COS). The COS produced using papain, pepsin, α -amylase and β -amylase were characterized for physico-chemical, structural, thermal and antioxidant activities. Chitooligosaccharides produced using pepsin enzyme had higher solubility compared to other three COS. Structural variation of chitosan and their oligosaccharides were studied by FTIR analysis and it revealed the existence of various structural difference among the oligosaccharides and chitosan. Thermal behavior of chitosan and oligosaccharides were examined using DSC analysis, and it exhibits difference in the glass transition temperature among the four oligosaccharides and native chitosan. Anti-oxidant analysis such as DPPH radical scavenging activity and reducing power revealed the antioxidant ability of chitooligosaccharides. Among the four COS, pepsin-COS had the highest DPPH radical scavenging and reducing power activity. According to the patterns of molecular weight reduction, antioxidant properties, thermal behavior properties, four enzymes α -amylase, β -amylase, pepsin and papain found suitable for hydrolyzing chitosan into chitooligosaccharide. As it is superior to chitosan, chitooligosaccharide can be utilized in food industry as bio-preservatives to enhance the quality, safety and shelf life of the products.

Statement Of Novelty

Kiddy Shrimp (*Parapeneopsis styliifera*) processing industrial waste is one of the major sources of raw material for chitin production. Chitosan, a major derivative of chitin, is proven to have an array of health benefits. However, their usage in food and other industries is hampered because of their poor solubility. Addressing this problem would maximize the utilization of shrimp industries waste. Enzymatic depolymerization of chitosan is an ideal technique to improve the solubility as it produces low molecular weight chitosan, also known as chitooligosaccharides. Studies on comparative depolymerization efficiency of chitosan of shrimp shell waste from non-specific protease and carbohydrase are scarce. The results of the present study provide in depth knowledge on the depolymerization of chitosan by non-specific enzymes.

Introduction

Indian shrimp industry has assumed a greater importance during the last couple of decades due to the increasing demand among the local and international consumers. Around 85 species are accounted for in Indian waters in which 55 species are economically significant. India has 256 Non-EU plants and 353 EU plants with the production capacity of 14,692.47 MT and 20,440.34 MT respectively. During the year 2019–2020, exports from India amounted to 12,89,651 MT of seafood and shrimp accounted 6,52,253 MT. Especially Gujarat is having 130 seafood processing plants with the capacity of 6632.94 MT [1]. The waste generation during shrimp processing accounts nearly 40–60 %. Disposing these wastes generated by the shrimp processing industries is a major challenge to developing countries like India. Shrimp head generally constitutes 34–45 % and cuticle (exoskeleton) constitutes 10–15 % of the whole shrimp. These

wastes contain protein (35–40 %), chitin (10–15 %), minerals (10–15 %) and traces of caroteno pigments on dry weight basis [2].

Chitin is the world's second most abundantly available natural polymer next to cellulose. The major source of chitin is from the marine resources mainly in shells of crustacean such as shrimp, crab and lobster. It is a linear polysaccharide chemically made up of β -1-4 N-acetyl D- glucosamine and they are not readily soluble in water and weak acids. Chitosan is a main derivative of chitin and it is prepared by partial deacetylation. It is the long chain hetero-polymer of long linear chain consisting of β -(1–4)-linked D-glucosamine (deacetylated units) (GlcN) and N-acetyl-D-glucosamine (acetylated units) (GlcNAc). Unlike chitin, chitosan dissolves under mild acidic conditions. Chitosan is known for its specific properties like bioactivity, biodegradability, chelation ability, absorption capacity and film forming ability. Though these two forms are having beneficial activities, their poor solubility hinders their usage in food and other industrial applications.

Now researchers are exploring chitin and chitosan derivatives which are having high biological activities and water solubility. In this respect, chitooligosaccharides (COS) has received a greater attention due to its array of health beneficial properties. These COS are the degraded products of chitosan and having the molecular weight of < 3900 Da and degree of polymerization of less than 20 [3]. Compared to chitin and chitosan, COS have low molecular weight, low viscosity, high solubility, good absorption capacity, biodegradability and biocompatibility. More over COS display several bioactivities such as anti-oxidative, anti-bacterial, anti-fungal [4], anti-tumour [5, 6] and anti-inflammatory activity [7, 8].

Physical, chemical and enzymatic methods are available to produce COS and all these methods are having their own merits and demerits. Enzymatic methods using specific chitinolytic enzymes are giving controlled hydrolysed products but high costs of Chitinolytic enzymes and lower yields hamper their practical application and feasibility compared to other methods [3]. Considering the demerits, researchers have reported the alternative low cost, high productivity and feasible commercial production process using non-specific enzymes which are available in plenty at low cost [9]. Proteolytic enzymes are most commonly used for depolymerization of chitosan. The position of N-acetyl Glucosamine, Glucosamine, distribution of these residues (pattern of acetylation) and β -1-4 glycosidic linkage plays an important role in the depolymerization of chitosan. Several authors have reported the use of commercial proteolytic, lipolytic as well as carbohydrases enzymes in chitooligosaccharides production [10–17]. Chitosan is also found susceptible to glycanases, lipases, proteases and a tannase derived from bacterial, fungal, mammalian and plant sources ([9]. Though several researchers studied the hydrolytic effectiveness of different enzymes for the production of COS, only very limited research works are reported on the performance comparison of proteolytic enzymes (papain and pepsin) and carbohydrase enzymes (α -amylase and β -amylase) on the depolymerization of chitosan to produce chitooligosaccharides. Hence, the present study was aimed to compare the functional activity of COS using four different enzymes viz., α -amylase, β -amylase, pepsin and papain. This study also provided a comprehend knowledge on cheap and commercial proteases and carbohydrase with reference to COS production and their properties.

Materials And Methods

Raw materials

Shell waste of *Parapeneopsis styliifera* was collected from local processing unit, Veraval, Gujarat, India. DPPH (2 –Diphenyl 1-picrylhydrazyl) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Papain, pepsin, and α -amylase were procured from Himedia, India and β -amylase from Infinita Biotech, India. All the other chemicals and reagents used were of analytical or guaranteed reagent grade.

Preparation Of Chitooligosaccharides

Preparation of chitin and chitosan

The chitin from shrimp shell waste of *Parapeneopsis styliifera* was prepared using acid and alkali treatment according to the method described by Benhabiles et al [18]. The chitosan was prepared as described by Renuka et al [19]. Briefly, the chitosan preparation was accomplished by boiling with alkali, repeated water washing and air drying. Chitosan with 76.43 % degree of deacetylation and intrinsic viscosity of 8496.4 dL/g was used further for the preparation of chitooligosaccharides as detailed below.

Preparation Of Chitooligosaccharides Using Different Enzymes

Chitosan was dissolved in 1% acetic acid and filtered through Buchner funnel to remove impurities. Four different enzymes viz., α -amylase, β -amylase, pepsin and papain were used to depolymerize the chitosan. Optimum conditions for each enzyme are given in Table 1. as reported by Wu [15]; Rokhati et al [17]; Laokuldilok et al [20]; and Roncal et al [10]. The reaction mixture was pre-equilibrated to attain the desired temperature before adding the enzymes. After enzymes addition, the reaction mixtures were incubated for the specified duration and at the optimum pH based on the nature of enzymes (Table 1). The enzyme activity was arrested by keeping the reaction mixture in a boiling water bath for 20 min. The chitooligosaccharides formed as a result of depolymerisation reaction was neutralized using 3M NaOH and filtered through Whatman No 4 filter paper. The filtrate was concentrated in a rotary evaporator (Heidolph Instruments, Germany) under reduced pressure. This concentrated chitooligosaccharides was precipitated by the addition of 70 % methanol and centrifuged using (Biofuge Stratos, Thermo fisher scientific, USA) at 5000 g for 10 min and repeated the process with 80 % and 90 % methanol to reduce the impurities. The final precipitate of 90 % methanol was freeze dried and named as α -amylase-COS, β -amylase-COS, Papain-COS and Pepsin-COS accordingly. The yield of four different chitooligosaccharides were calculated using the following formula:

$$\text{Yield}(\%) = \frac{\text{Weight of the freeze dried COS (g)}}{\text{Weight of the chitosan (g)}} \times 100$$

Table 1
Hydrolysis condition of chitosan for different enzymes

Enzyme	Time (h)	Temp (°C)	pH	E/S	Method
α-amylase	16	50	5	1:100	Wu, 2011
β-amylase	16	50	5	1:100	Rokhati et al., 2013
Papain	16	40	4	1:100	Laokuldilok et al., 2017
Pepsin	16	50	4.5	1:100	Roncal et al., 2007

Functional Properties Of Chitooligosaccharides

Determination of degree of deacetylation (DD)

Degree of acetylation (DA) of chitosan and COS was determined from FTIR spectral data according to the method described by Moore and Roberts [21]. DA was calculated using the following formula:

$$DD(\%) = 100 - [(A_{1655} / A_{3450}) \times 100] / 1.33$$

Where, A_{1655} is the intensity of the absorption band of amide I, used as the specific band for N-acetylation, A_{3450} is the intensity of the absorption of the hydroxyl group band, used as the reference band. 1.33 is the ratio of the absorbance at 1655 cm^{-1} to that of the absorbance at 3450 cm^{-1} for fully N-acetylated chitosan.

Average Molecular Weight

The average molecular weight of COS was determined using the viscometric method of Roberts & Domszy [22] with slight modifications. Chitosan and COS solution with various concentrations (0.5 mg/ml, 1.0 mg/ml and 1.5 mg/ml) were prepared using 0.3 M acetic acid. The viscosity was measured at 25 °C using an Ostwald glass viscometer (Borosil, India). The average molecular weight of chitosan was calculated using the following Mark–Houwink equation as described by Cabrera & Van Cutsem [23].

$$\text{Intrinsic viscosity } (\eta) = (MW)$$

Where $K = 3.5 \times 10^4$, MW - molecular weight and $a = 0.76$

Solubility

The solubility of COS was determined according to the method described by Lin et al [24] with slight modifications. The pre-weighed COS sample was dissolved in distilled water and 0.001% acetic acid in

the ratio of 1:1 and thoroughly mixed under 120 rpm of magnetic stirring (Tarsons Products Pvt. Ltd, India) for 10 min at room temperature. The mixture was centrifuged at 6000 x g at room temperature for 30 min and filtered through pre-weighed whatman filter paper No. 4. The filter paper was dried at $60 \pm 2^\circ\text{C}$ in a hot air oven (Bio techno lab, India) until a constant weight was obtained then re-weighed to determine the residual solid weight. The solubility of COS in water and 0.001 % acetic acid were calculated using the following formula:

$$\text{Solubility (\%)} = (1 - (\text{Residual COS (g)})/(\text{Initial weight of COS (g)})) \times 100$$

Fourier Transform Infrared (Ftir) Analysis Of Chitooligosaccharides

The FTIR spectra of the COS samples was obtained to elucidate the structural information using NICOLET spectrometer (iS5 NICOLET, Thermo Scientific, USA). The samples and KBr were mixed at the ratio of 1:100 and the pellets pressed to what pressure were used to read the spectra in the wave numbers region of $400\text{--}4000\text{ cm}^{-1}$. Spectrum signals were collected in 36 scans at a resolution of 4 cm^{-1} . Spectral data analysis was carried out using the OMNIC 9.0 software programme.

Differential Scanning Calorimetric (Dsc) Analysis Of Chitooligosaccharides

Calorimetric analysis of COS was measured using a differential scanning calorimeter (DSC 25, TA Instruments, New Castle, USA). COS samples (20 mg) were placed in DSC pans that were hermetically sealed. The thermal profile was analysed in a temperature range of $0\text{--}220^\circ\text{C}$. All tests were run in scan mode at a heating rate of $5^\circ\text{C}/\text{min}$. A hermetically sealed empty pan was used as a reference. The total denaturation energy, as well as the enthalpy change was determined by integrating the area under the curve using the Trios software supplied with the instrument.

Antioxidant Properties Of Chitooligosaccharides

DPPH radical scavenging activity

Chitooligosaccharides prepared were evaluated for their ability to scavenge the free radicals using 2,2-Diphenyl 1-picrylhydrazyl (DPPH) radical scavenging assay, according to the method described by Yen et al [25]. An aliquot of 1.5 ml of COS solution prepared in 0.001% acetic acid at different concentrations (1, 2, 3, 4 and 5mg/ml), was mixed with 1.5 ml of 0.1 mM DPPH prepared in 99.5 % ethanol. Similarly, to compare the activity, 1.5 ml of chitosan dissolved in 1 % acetic acid was tested. Acetic acid 1 % solution was used as control. The reaction mixtures were incubated under dark conditions for 30 min at room temperature. The absorbance was measured at 517 nm in a double beam UV–VIS spectrophotometer

((T90 + UV/ViS Spectrometer, PG Instruments Ltd, India). DPPH radical scavenging activity was calculated using the following formula:

$$\text{DPPH free radical scavenging activity (\%)} = 1 - \left(\frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \right) \times 100$$

Reducing Power Assay

Ferric reducing power of COS was determined according to the method of Oyaizu [26]. One ml of COS was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1 % potassium ferricyanide. The reaction mixture was incubated at 50 °C in an incubator (Ocean Life Science Corp, UP, India) for 30 min and then the reaction was terminated by adding 2.5 ml of 10 % trichloroacetic acid. From the reaction mixture, 2.5 ml was diluted with equal volume of distilled water. Finally, 0.5 ml of 0.1 % FeCl₃ was added and vortexed in a cyclo-mixer (Remi Laboratory Instruments, India). The mixture was incubated at room temperature for 10 min and the absorbance was measured at 700 nm in a spectrophotometer (T90 + UV/ViS Spectrometer, PG Instruments Ltd, India). Higher optical density of reaction mixture at 700 nm is the indication of higher reducing power.

Statistical analysis

All the experiments were performed in triplicate and data were presented as means ± standard deviation. Analysis of variance (ANOVA) was performed and means comparisons were carried by Duncan's multiple range tests using SPSS package (SPSS 20.0, SPSS Inc, Chicago, IL, USA). Significant differences were defined as $p < 0.05$.

Results And Discussion

Yield of chitooligosaccharides obtained using four different enzymes

Yield of chitosan and chitooligosaccharides derived from chitosan using four different enzymes are given in the Table 2. The average yield of chitosan was 4.41 % on the basis of wet weight of shrimp shell waste. Ahing & Wid [27] reported the chitosan yield of 4.09 % from shrimp shell wastes. Chitosan yield is subjected to variation depends on process parameters employed such as time, temperature, solvent to raw material ratio and stirring speed during chitin and chitosan preparation. In addition, the state of quality of raw material, type of species, residual mineral and proteins are also determining the yield of chitosan [28]. The yield of COS obtained in the present study was found to be remarkably different with reference to type of enzyme used. Pepsin derived COS showed the higher yield of 64.98% followed by β -amylase (59.33 %), papain (48.43 %) and α -amylase (39.59 %). The result obtained in the present study

for pepsin COS is higher than the reported value of 52.2 % by Roncal et al. [10]. Pepsin is more effective in hydrolysis of chitosan indicates that it can cleaves on four different types of glycosidic bonds (GlcNAc) - GlcNAc, GlcN - GlcN, GlcNAc - GlcN and GlcN – GlcNAc as similar to specific enzymes [29]. In a study, Kumar et al [30] revealed that pepsin yield higher amount of COS when compared to papain and pronase enzymes. Moreover, non-specific enzyme yielded more COS than the chitosinase and chitinase [31]. In this study, papain COS showed the yield of 48.43 % which is similar to the report of Lin et al [12], where he reported 49.55 % of COS from papain.

Table 2
Different parameters of chitosan and four different chitooligosaccharides

Samples	Yield (%)	Degree of deacetylation	Molecular weight (kDa)	Solubility (%)	
				Water	0.001% acetic acid
Chitosan	4.41*	76.43	111.18	9	66
α -amylase-COS	39.56	80.32	7.25	59	100
β -amylase-COS	59.33	81.10	6.90	61	100
Papain-COS	48.43	79.91	7.45	58	100
Pepsin-COS	64.98	83.91	5.10	69	100

In earlier study conducted by Wu [15] showed 91.2% COS yield when α -amylase was used for hydrolysing the chitosan. This is higher than the results obtained in the present study (39.56 %). Moreover, the chitosan hydrolysis is subjective to the molecular weight of chitosan, degree of deacetylation of chitosan, the enzyme to substrate ratio, hydrolysis duration, temperature and the type of enzyme, stirring speed [20]. Chitosan hydrolysis efficiency of enzymes studied is in the order of pepsin > β -amylase > papain > α -amylase with respect to COS production from chitosan of *P.stylofera*.

Degree Of Deacetylation (Dd) Of Chitooligosaccharides

The ratio of glucosamine to N-acetyl glucosamine in a polymer system is called degree of acetylation (DD). The alteration of chitin into chitosan is determined by the amount of formation of glucosamine residues in the polymer system upon removal of acetyl group. The DD of COS is the most vital factor determining its applications in food, medical, water treatment and pharmaceutical field [32]. It influences biological properties viz., adsorption enhancer, antioxidant and antimicrobial activity and other properties like biodegradability, bioavailability and biocompatibility depend on the process conditions [18, 33]. The DD values of different COS produced in the present study is given in Table 2. The four COS had DD ranged from 79.91 to 83.91 %. The higher DD found in pepsin-COS followed by β -amylase-COS, α -amylase-COS and papain-COS. Enzymatic degradation affects the degree of deacetylation of samples [34].

This study revealed that DD of the chitosan (76.43) was slightly lower compared COS. In the case of COS, the DD significantly differed ($p < 0.05$), When the degree of deacetylation reaches 50 %, chitosan is readily dissolved in weak acids. DD of chitosan determines the DD of chitooligosaccharides. The depolymerisation of chitosan affected the DD of chitooligosaccharides. The DD of COS has shown a significant difference agreed with the report of Kumar et al [11]. The change in the DD is evidenced by the change in solubility of individual COS and appearance of small band in the FTIR spectra of each COS. DD is mainly affected by the concentration of NaOH and temperature employed for deacetylation and the deproteination process of chitosan [35].

Functional Properties Of Chitooligosaccharides

Average molecular weight of chitooligosaccharides

The molecular weight of chitosan and four different COS determined using viscosity are presented in Table 2. The viscosity of the chitosan is proportional to the molecular weight of chitosan. As chitosan is a linear polysaccharide, the change in their viscosity reflects the corresponding changes in the molecular weight due to depolymerization and changes in the distribution of acetyl groups. The raw material and the chitin preparation methods influences the molecular weight of chitosan [28]. The molecular weight of commercial chitosan is ranging from 1.5 kDa to 2000 kDa. In this study, molecular weight of the prepared chitosan was 111.18 kDa. The average molecular weight of α -amylase, β -amylase, papain and pepsin COS were 7.25, 6.90, 7.45 and 5.10 kDa, respectively. The molecular weight of COS is the key in determining the biological activities of chitooligosaccharides [36]. Though there is no specific molecular weight to define the COS, there are different postulates about the molecular weight of COS. For instances, some authors have stated that chitosan with the average molecular weight of 3.9 kDa as COS [37]. While Jeon et al [38] suggested COS with > 10 kDa has good biological activities. Papain rapidly reduced the molecular weight of chitosan by the action of endoenzyme hydrolysis. Although the accurate mechanism of degradation of chitosan by papain is not clear, papain is usually used to break α (1–4) glycosidic bonds but not β (1–4) bonds, which mainly links monomers in chitosan [39]. In this study, molecular weight reductions also suggested that pepsin, β -amylase, papain and α -amylase possessed excellent chitosanolytic activities and reduced molecular weight of chitosan significantly. α amylase-COS showed the molecular reduction of 118.18 to 7.25 kDa which is lower to the earlier report on molecular weight of α -amylase-COS (730 Da) by Wu [15]. The higher reduction in molecular weight of chitosan by pepsin and papain could be due to high affinity towards molecules containing ring structures and eventually hydrolysis of more number of glycoside linkages of chitosan [40].

Water Solubility Of Chitosan And Different Chitooligosaccharides

Water solubility of chitosan and four different chitooligosaccharides are given in Table 2. The COS are not fully dissolved in water but attained 100% solvation in 0.001 % acetic acid. Among the four COS samples, Pepsin derived COS shown highest water solubility of 69 % followed by β -amylase-COS (61 %), α -amylase-COS (59 %) and papain-COS (58 %). Chitosan had only 9 % solubility in water. Degree of acetylation and pattern of acetyl group are fundamentally influence the solubility of chitosan. Water solubility increases with the lowered molecular weight. As noted earlier, the lower molecular weight pepsin-COS had high water solubility, while the high molecular weight papain-COS had low solubility. But all four COS were completely dissolved in 0.001% acetic acid, while the chitosan had only 66 % solubility. The lower solubility of a molecule is the major limitation for the usage in the food and pharmacological industry. More intra-molecular hydrogen bonds present in the low molecular weight chitooligosaccharides enhance the water solubility by readily interacting with available water molecule [24]. Apart from that, the breakdown of GlcN – GlcN units or GlcNAc – GlcNAc linkage reduced the molecular weight and intra viscosity, thereby it is readily soluble in the water [41]. The higher solubility of pepsin-COS indicating of both endo and exo action of enzyme reaction [30].

Fourier Transform Infrared (Ftir) Analysis Of Chitooligosaccharides

Fourier transform infrared (FTIR) spectra provide the structural information of functional groups of polysaccharides. FTIR spectra of native chitosan and their oligosaccharides are shown in Fig. 1–4 and Table 3. Though, the chemical structure of COS is similar to native chitosan, the FTIR spectra shows some difference in their banding pattern. Many fine absorption peaks are present in COS when compared to chitosan.

Table 3
Functional group profiling of chitosan and chitooligosaccharides

Spectra (cm ⁻¹)	Functional groups	Chitosan	α - amylase	β - amylase	Papain	Pepsin
895– 897	β (1–4) glucoside bonds	895	897	896	895	896
1100– 1200	C–O–C stretch, symmetric and asymmetric	1152	1152	1154	1152	1155
1315– 1320	amide III n-acetyl glucosamine residues	1324	1337	1320	1342	1323
1492	CH ₂ bending and orientation of primary hydroxyl group	1421	1413	1412	1411	1412
~ 1550	Amide II NH bending in polysaccharide	1595	1566	1568	1566	1570
1600– 1650	Amide I C = O bending	1652	1641	1656	1641	1641
2350– 2360	CO ₂ bonding	Absent	2362	2363	2362	2363
2850– 2950	C–H, stretch, symmetric and asymmetric	2921	2922	2923	2922	2922
3450	OH stretch	3438	3429	3424	3440	3423

FTIR spectra of chitosan had a single broad peak at around 3438 cm⁻¹. It indicates that chitosan has β -conformation. The β -conformation refers to the arrangement of individual chitosan polymer chain in a parallel manner. The hydrogen bonding network is weaker in β -conformation compared to α -conformation. Thus, it facilitates easy binding of the enzymes into the network of chitosan molecules [42].

The absence of a strong absorption band around 3000–3500 cm⁻¹ reveals the presence of inter and intra molecular hydrogen bonding in 6CH₂OH (primary hydroxyl group) and 3-OH groups in chitosan and COS [13]. A broad band above 3000 cm⁻¹ represented to the intermolecular crystal lattice of a molecule, which is related to the hydrogen bonding network of the molecules, and it reveals the incorporation of water molecule into molecular structure. In chitosan, this band appeared at 3438 cm⁻¹ and there was a slight shifting in the four COS like in α -amylase-COS at 3429 cm⁻¹, β -amylase-COS at 3424 cm⁻¹, papain-COS at 3423 cm⁻¹ and pepsin-COS at 3440 cm⁻¹. This slight difference in the band among the four COS, indicated a decrease in the ordered structure of chitooligosaccharides. This difference is probably due to the decrease in the degree of acetylation in COS, which results in lower degree of hydration of the

molecule affecting the crystallinity index and thus the orderliness. When compared to chitosan downward shift of the absorption band above 3000 cm^{-1} indicated the presence of more orderliness in COS [11]. Presence of a distinct band around 2922 cm^{-1} in both chitosan and different COS indicate the -CH vibration mode, which represented a good internal reference for comparison of band absorbance [30].

Amide I group which associated with C = O bending in polysaccharides is usually represented by an absorption band around 1650 cm^{-1} . Here the amide I peak was present at 1652 cm^{-1} for chitosan, 1641 cm^{-1} for α -amylase-COS, 1656 cm^{-1} for β -amylase-COS, 1655 for papain-COS and 1641 cm^{-1} for pepsin-COS. A peak around 1320 cm^{-1} represents the presence of n-acetyl glucosamine residues in the chitosan [43]. While compared to the native chitosan, the height of this peak decreased in all COS. This indicated in all COS the amount of n-acetyl glucosamine residues was reduced and it leads to the lower degree of acetylation in all COS. It was further evidenced by progressive weakening of the amide I band [44].

Amide II group represent the NH bending in polysaccharide and it generally present near 1550 cm^{-1} [44]. In this study, chitosan had the amide II region around 1595 cm^{-1} while α -amylase-COS and pepsin-COS had at 1566 cm^{-1} , 1570 cm^{-1} , β -amylase-COS at 1568 cm^{-1} , and papain-COS at 1566 cm^{-1} . In polysaccharides, amide III band is around 1320 cm^{-1} represents the n-acetyl glucosamine residues [14]. In chitosan it is at 1324 cm^{-1} while α -amylase-COS had at 1337 cm^{-1} , β -amylase-COS at 1320 cm^{-1} , papain-COS at 1342 cm^{-1} , pepsin-COS at 1323 cm^{-1} . The reduced absorption intensity at this region reveals the reduction of acetyl content and association of depolymerization. In polysaccharides, band around 1429 represents the CH_2 bending and orientation of primary hydroxyl group [44]. In chitosan it was around 1421 cm^{-1} , whereas α -amylase-COS had at 1413 cm^{-1} , β -amylase-COS at 1412 cm^{-1} , papain-COS at 1411 cm^{-1} , pepsin-COS at 1412 cm^{-1} . Compared to chitosan this peak for all the four chitooligosaccharides had lower wave numbers. The lower wave number in COS, probably due to the changes in hydrogen bonding pattern and environment of primary -OH groups which is affected by the reduction of acetyl content in the COS [30].

Among the four COS, α -amylase and pepsin-COS had a peak around 2362 cm^{-1} , at the same time β -amylase and papain had this peak at 2362 cm^{-1} . But in the case of chitosan this peak was absent at this region. A peak around 1380 cm^{-1} indicates the presence of CH bending in polysaccharides. This peak is present in chitosan while absent in all the four COS. The peak around $895\text{--}897\text{ cm}^{-1}$ in chitosan and their oligosaccharides represents the presence of β -(1-4) glucoside bonds [15].

Differential Scanning Calorimetric (Dsc) Analysis Of Chitooligosaccharide

Thermal behaviour of chitooligosaccharides was studied using differential scanning calorimetric analysis. DSC analysis gives the information about the temperature and enthalpies associated with

phase transition of COS. Glass transition and solid melting properties of chitosan and four different COS are given in Table 4.

Table 4
DSC thermogram of chitooligosaccharide prepared from chitosan using α -amylase, β -amylase, papain and pepsin

Sample	$T_{gi}(^{\circ}\text{C})$	$T_{gp}(^{\circ}\text{C})$	$\Delta H_g(\text{Jg}^{-1})$	$T_{mi}(^{\circ}\text{C})$	$T_{mp}(^{\circ}\text{C})$	$\Delta H_m(\text{Jg}^{-1})$
Chitosan	40.08	45.10	1.76	113.99	146.00	240.18
α -amylase-COS	55.23	56.85	16.084	63.76	92.03	119.04
β -amylase-COS	53.61	56.80	57.47	109.50	137.26	261.34
Papain-COS	48.88	49.12	1.19	113.08	136.89	158.58
Pepsin-COS	56.10	58.55	36.33	99.56	131.31	247.30
T _{gi} -Onset glass transition temperature; T _{gp} -Peak glass transition temperature; T _{mi} -Onset solid-melting temperature; T _{mp} - Peak solid-melting temperature; ΔH_g -Enthalpy change for glass transition; ΔH_m - Enthalpy change for solid-melting.						

The onset of glass transition temperature was 40.08 °C which is lower than any of the chitooligomers produced in this study. Among the chitooligomers produced pepsin-COS had the highest onset of glass transition temperature (56.10 °C) followed by α -amylase-COS (55.23 °C), β -amylase-COS (53.61 °C) and papain-COS (48.88 °C). Similar order was recorded for peak glass transition temperature (pepsin-COS > α -amylase-COS > β -amylase-COS > papain-COS > chitosan). Greater glass transition temperature is mainly due to the presence of high amount of cross linking [45]. Moreover, dried substances are hygroscopic in nature (tendency to absorb water from the environment) and the water molecules are partially crystallizable. Evaporation of this crystallizable water is also responsible for increase of glass transition temperature [46]. Glass transition temperature gives the information about the polymer rigidity. Here all COS had glass transition temperature near or above 50 °C. It means the COS is in rigid or glassy state when it is used at a temperature above the glass transition temperature. So, COS can be used in food packaging material to improve and give the strong physical properties such as strength, stress, shear and compression of the packaging material.

In the case of solid melting temperature chitosan had initial temperature of 113.99 °C and the peak temperature at 146.00 °C. Papain-COS also had more or less similar values as that of chitosan and the values were 113.08 and 136.89 °C respectively. The lowest solid melting temperature was noted in α -amylase-COS. It had 63.76 °C as onset solid-melting temperature and 92.03 °C as peak solid-melting temperature.

Higher enthalpy changes of 261.34 Jg⁻¹ was observed in β -amylase-COS, followed by pepsin-COS (247.30 Jg⁻¹), chitosan (240.18 Jg⁻¹), papain-COS (158.58 Jg⁻¹) and α -amylase-COS (119.04 Jg⁻¹).

This phenomenon could be due to the presence of high amount of bound water in the sample, because the moisture presents in the sample play a vital role in the solid melting phenomenon [46].

Antioxidant Properties Of Chitooligosaccharides

The antioxidant activity of chitosan and its derivatives depends on their ability to obstruct hydrogen or electrons or to donate the protons.

Dpph Free Radical Scavenging Activity

The hydrogen-donating ability of chitooligosaccharides is determined using DPPH free radical scavenging activity. The ability of COS to scavenge the long-lived purple coloured organic nitrogen radical of DPPH to pale yellow hydrazine was measured at 517 nm. The DPPH free radical scavenging activities of chitosan and four different COS increased with the increase in concentration (1–5 mg /ml) and are shown in supplementary file 1.

Compared to chitosan the DPPH free radical scavenging activities of COS were significantly high ($p \leq 0.05$). Chitosan had very low DPPH free radical scavenging activity of 27.85 %. Among the four different COS, pepsin-COS had the highest DPPH scavenging activity and it was 74.49 % at 5 mg/ml concentration followed by papain-COS (70.59 %), β -amylase-COS (62.32 %) and α -amylase-COS (41.85 %). The higher amount of freely exposed amino groups in the COS could be ascribed for the higher reducing ability [47]. Earlier studies have concluded that the amino group present in the COS is involved in DPPH free radical scavenging activity. The lower activity observed for chitosan compared to COS could be due to steric hindrance posed by larger molecules and also the solvent system modifies the radical and antioxidant reaction differently. Chitosan is soluble in higher strength of acetic acid (1 %), whereas, COS are soluble in low strength of acetic acid. These results indicate that the chitooligosaccharides formed by the enzyme hydrolysis had the ability to donate the electrons or protons and possess the bioactive property like free radical scavenging activity.

Many studies have suggested that low molecular weight COS had higher potential to scavenge the free radicals [48, 49]. In this study, among the four different COS, pepsin-COS had the lowest molecular weight of 5.1 kDa with highest radical scavenging activity. Similar results were reported for low molecular weight chitosan and chitooligosaccharides having the molecular weight of < 3 kDa and < 5 kDa [50, 8]. Though the exact mechanism of radical scavenging activity of COS is not fully elucidated, the amino groups and hydroxyl groups which present in the pyranose ring of COS can scavenge free radicals and convert it into stable macromolecule radicals. Likewise, COS scavenge the free radical DPPH compounds by donating a hydrogen ion and reduced the DPPH [51]. Many antioxidants that quickly react with peroxy radicals in vivo may slowly react or even may be inert with DPPH [52].

Effective concentration of COS needed to scavenge 50% of DPPH free radicals were calculated to know the EC_{50} values of different COS and are given in the Fig. 5. The EC_{50} values of different COS were significantly lower than that of chitosan ($p \leq 0.05$). Lower EC_{50} value indicates the higher potency of radical scavenging. The lower molecular weight pepsin-COS exhibited low EC_{50} value at 2.97 mg/ml followed by papain-COS (3.34 mg/ml), β -amylase-COS (3.86 mg/ml) and α -amylase-COS (5.64 mg/ml). Chitosan had the EC_{50} value of 9.98 mg/ml which indicates ineffective nature towards radical scavenging compared to the derived COS. Moreover, strong intra-molecular hydrogen bonding which is usually present in the high molecular weight chitosan forms a compact and rigid structure [41]. Apart from that, free amino groups (NH_2) which is present in the COS taking the hydrogen ion from the solution and forms the ammonium groups (NH_3^+) which react with free radicals by an addition reaction [20, 53, 54]. Additionally, the hetero-chitooligosaccharide radical scavenging activity depends on their degree of deacetylation and molecular weight [55].

In the present investigation, all COS from chitosan using different enzymes exhibited free radical scavenging activity as assessed by DPPH free radicals probe. The radical scavenging activity was found to vary with the enzymes used for deriving the COS. Results clearly demonstrate that COS produced by enzymatic hydrolysis has the ability to donate the electron or protons when reacts with the DPPH free radical and thus could act as antioxidant.

Reducing Power Assay

The compounds which could reduce iron (III) to iron (II) are detected using FRAP assay. Reducing reaction of iron (III) to iron (II) exhibit a standard reduction potential. So far, a wide range of compounds have been screened for their antioxidant potential using FRAP assay. The principle behind this assay is potassium ferricyanide (Fe^{3+}) reduced into potassium ferrocyanide (Fe^{2+}) by the reducing substances and this compound reacts with ferric chloride which forms ferric-ferrous complex and this complex can be absorbed at 700 nm.

The reducing power of bioactive compounds is linked with electron donating capacity [56]. Reducing powers of chitosan and different COS at different concentrations (0.5–5 mg /ml) are given in Fig. 6. The results explained the reducing power of chitosan and four COS increased with the increase in concentrations ($p < 0.05$). Chitosan had the lowest absorption at 700 nm (0.27 for 5 mg/ml). The highest activity was recorded for pepsin-COS in all the concentration when compared to other three COS. Reducing power of all the four COS and chitosan is significantly different between concentrations ($p < 0.05$).

Among the COS, pepsin-COS had the absorption of 0.68 followed by papain (0.45), α -amylase-COS (0.40) and β -amylase-COS (0.39) at 5 mg/ml concentration ($p > 0.05$). There was a slight difference in reducing power between α -amylase-COS and β -amylase-COS, when compared to other COS. The results indicate that the lower MW had the greater ability to reduce Fe^{3+} ions. The lower molecular weight pepsin-COS

showed the higher reducing absorption than other COS in all concentration. This may be because of the higher exposure of the readily available free amino groups in the smaller COS [47]. Several authors have also reported similar increase in reducing power with the increase in concentration. Li et al [48] observed that degree of polymerization lesser than 6 homo COS showed the higher reducing power with increasing concentration. Chitosan from crab shells with the DD in the range of 83.3–93.3 % was not effective for reducing power [25]. Laokuldilok et al [20] found that the lower the molecular weight of COS higher the reducing power.

Conclusions

Difficulties in the solubility of chitosan in water hinder their usage in foods and other applications. Conversion of high molecular weight chitosan into low molecular chitooligosaccharides paves the way to increase their utilization because of the water soluble nature. Recent researches were focusing on low cost, high productivity method which should be suitable for commercial production of COS. Among the different methods of COS production, using non-specific chitosanolytic enzymes such as proteases, amylases and lipases has geared-up due to their availability and low cost compared to the chitinolytic enzymes. In this study four different non-specific enzymes viz., α -amylase, β -amylase, papain and pepsin were used to produce the chitooligosaccharides and their yield, water solubility, average molecular weight and degree of deacetylation, structural, thermal behaviour and anti-oxidative properties were examined. Yield of chitooligosaccharides for all the four enzymes varied and factors like hydrolysis nature of enzyme, mode of action (endo or exo enzymatic action), and enzyme ability to attack the chains of substrate and so on could be attributed to the variation observed. In the case of degree of deacetylation only slight variation was noted in all oligosaccharides and it may be dependent on the degree of deacetylation of native chitosan. The molecular weight of oligosaccharides produced using pepsin enzyme was the lowest while papain produced highest molecular weight oligosaccharides. This molecular weight directly influenced the water solubility of oligosaccharides derived. Oligosaccharides produced using pepsin enzyme had higher solubility followed by β -amylase-COS, α -amylase-COS and papain-COS. Structural variation among chitosan and their oligosaccharides were studied by FTIR analysis and it revealed the existence of various differences among the oligosaccharides and chitosan. Thermal behavior of chitosan and oligosaccharides are examined using DSC analysis, and it exhibited difference among the four oligosaccharides and native chitosan. Among the four COS, pepsin-COS had the highest DPPH radical scavenging and reducing power activity. According to the patterns of molecular weight reduction, antioxidant properties, thermal behavior properties, α -amylase, β -amylase, pepsin and papain found suitable for hydrolyzing chitosan. These results explain the production of chitooligosaccharides from chitosan using different non-specific enzymes with antioxidant properties can be can be utilized in food industry as bio-preservatives to enhance the quality, safety and shelf life of the products.

Declarations

Disclosure

The authors declare no conflict of interest.

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