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Research Note

Antioxidant Properties of Chitooligosaccharides Produced by Enzymatic Degradation of Chitosan

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Chitooligosaccharides (COS) are the degraded products of chitosan produced by the depolymerization process, which is receiving greater attention due to their array of beneficial health properties. Compared to chitin and chitosan, COS has low molecular weight, low viscosity, high solubility, biodegradability and biocompatibility. Physical, chemical, and enzymatic methods are available to produce COS, and all these methods have their own merits and demerits. Enzymatic methods using specific chitinolytic enzymes give controlled hydrolyzed products, but the high costs of chitinolytic enzymes and lower yields hamper their practical application and feasibility compared to other methods (Liang et al., 2018). Considering the demerits, researchers have reported the alternative low-cost, high-productivity, and feasible commercial production process using non-specific enzymes available in plenty at low cost (Pantaleone et al., 1992). Proteolytic enzymes are most commonly used for the depolymerization of chitosan. In this study, the non-specific enzyme, papain was used to depolymerize the chitosan and the antioxidant properties of Chitooligosaccharides were determined.

COS was produced from chitosan as per the method described by Laokuldilok et al. (2017). Initially, the chitosan was dissolved in 1 % acetic acid and filtered through the Buchner funnel to remove impurities. Papain (Himedia, India) was used to depolymerize the chitosan. The initial pH of the solution was 4.3 ± 0.2. The solution was pre-equilibrated to attain the desired temperature of 40 °C before adding the enzyme. The optimum pH of 4 was attained using 1 M NaOH solution. Papain enzyme (1 %) was added at the enzyme-substrate ratio of 1:1 and the solution was incubated for a period of 16 h. The COS formed due to the depolymerization reaction were adjusted to pH 6.5 using 3 M NaOH, then centrifuged (Biofuge Stratos, Thermo fisher scientific, USA) at 5,000 g for 10 min and filtered through Whatman No. 4 filter paper. Under reduced pressure, the filtrate was concentrated in a rotary evaporator (Heidolph Instruments, Germany). This concentrated COS was precipitated by adding 70 % methanol and centrifuged at 5,000 g for 10 min and the process was repeated with 80 % and 90 % methanol to reduce the impurities. The final precipitate from 90 % methanol was freeze-dried and named as papain-COS. The yield of COS was calculated using the following formula:

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

The average molecular weight of COS was determined using the viscometric method of Roberts & Domszy (1982) 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 (2005).

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

Where K= 3.5×10^4, MW= molecular weight and ^{a} = 0.76

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COS 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. (2008). An aliquot of 1.5 ml of COS solution prepared in 0.001 % acetic acid at different concentrations (1, 2, 3, 4 and 5 mg/ml), was mixed with 1.5 ml of 0.1 mM DPPH prepared in 99.5 % ethanol. Similarly, 1.5 ml of chitosan dissolved in 1 % acetic acid was tested to compare the activity. Acetic acid (1 %) solution was used as a 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:

DPPH free radical scavenging activity (%) =

$$1 - \left(\frac{Abs_{sample}}{Abs_{control}}\right) \times 100$$

Ferric reducing power of COS was determined according to the method of Oyaizu (1986). 1 ml of chitosan and COS of different concentrations were 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 an equal volume of distilled water. Finally, 0.5 ml of 0.1 % $FeCl_3$ 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).

DPPH radical scavenging activity of chitosan and COS at different concentrations (1, 2, 3, 4 and 5 mg/ml), is shown in Fig. 1 & 2. The radical scavenging activity of COS was 70.59 % at 5 mg/ml. Chitosan had a very low radical scavenging activity of 27.85 % at the same concentration. The higher amount of freely exposed amino groups in the COS could be responsible for the higher reducing ability (De Assis et al., 2012). The lower activity observed for chitosan compared to COS could be due to the steric hindrance posed by larger molecules (Pavinatto et al., 2013). The solvent system also modifies the radical and antioxidant reactions differently. In this study, chitosan was dissolved in 1 % acetic acid whereas COS samples were dissolved in 0.001 % acetic acid. These results indicate that the COS formed by the enzyme hydrolysis could donate electrons or protons and possess bioactive properties like free radical scavenging activity.

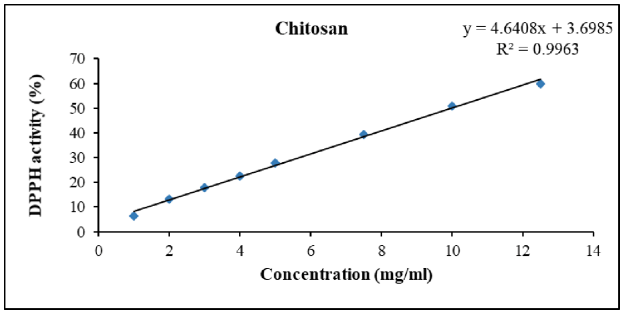

Fig. 1. DPPH free radical scavenging activity of chitosan at different concentrations

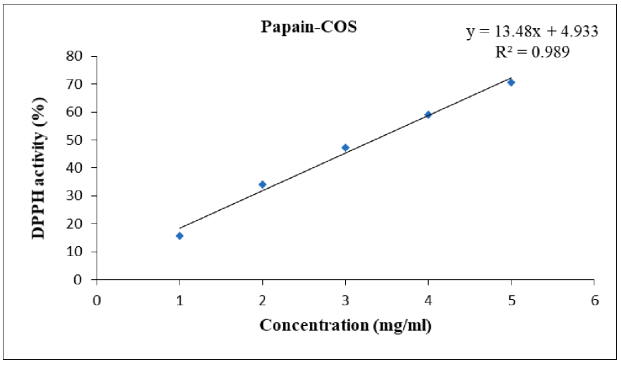

Fig. 2. DPPH free radical scavenging activity of papain-COS at different concentrations

Several studies have suggested that low molecular weight COS had a higher potential to scavenge the free radicals (Li et al., 2012; Park et al., 2003). In this study, papain-COS had a molecular weight of 7.45 ± 0.08 kDa with a degree of deacetylation of 79.91 ± 0.67 and exhibited EC_{50} value at 3.34 mg/ml for radical scavenging activity. Similar results were reported for low molecular weight chitosan and COS having a molecular weight of <3 kDa and <5 kDa respectively (Yen et al., 2009; Fernandes et al., 2010). Though the exact mechanism of radical scavenging activity of COS is not fully elucidated, the amino groups and hydroxyl groups, which are present in the pyranose ring of COS can scavenge free radicals and convert them into stable macromolecule radicals. Likewise, COS scavenges the free radical DPPH compounds by donating a hydrogen ion and reducing the DPPH (Brand-Williams et al.,

1995). Many antioxidants that quickly react with peroxyl radicals in vivo may slowly react or even be inert with DPPH (Prior et al., 2005).

The effective concentration of COS needed to scavenge 50 % of radicals was calculated to know the EC_{50} values of papain-COS (Fig. 3). The EC_{50} values of papain-COS were lower than that of chitosan. A lower EC_{50} value indicates a higher potency of radical scavenging. The COS exhibited an EC_{50} value at 3.34 mg/ml, which indicates an effective radical scavenging nature than the chitosan. Similarly, Kim & Thomas (2007) reported that a 30 kDa size chitosan exhibits higher DPPH scavenging ability compared to 90 and 120 kDa chitosan. Tomida et al. (2009) explained that the strong intramolecular hydrogen bonding present in the high molecular weight chitosan polymer forms a compact and rigid structure. The free amino groups (NH_2), which are present in the COS react with the hydrogen ion from the solution to form the ammonium groups (NH^{3+}) which in turn react with free radicals by an addition reaction (Laokuldilok et al., 2017; Yuan et al., 2009). The hetero-chito-oligosaccharide radical scavenging activity depends on their degree of deacetylation and molecular weight (Je et al., 2004).

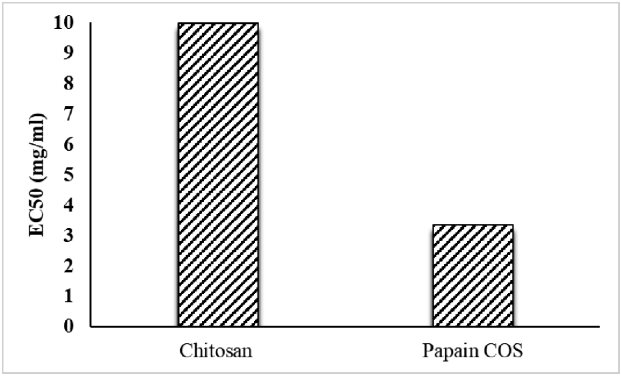

Fig. 3 Effective concentration for DPPH activity of Chitosan and Papain COS

The compounds which could reduce iron (III) to iron (II) are detected using FRAP assay. The reducing power of bioactive compounds is linked with electron-donating capacity (Dorman et al., 2003). The reducing power of papain COS and chitosan at different concentrations (1–5 mg /ml) is given in Fig. 4. The results indicate the reducing power of papain COS and chitosan, which proportionally correlated with the increased concentrations. The papain COS had an absorption of 0.45 at 5 mg/ml concentration. However, the chitosan had the low absorption (0.27 for 5 mg/ml) at 700 nm. The results indicate that the higher concentration had a greater ability to reduce Fe_3^+ ions. This may be because of the higher exposure of the readily available free amino groups in the smaller COS (De Assis et al., 2012). Li et al. (2012) observed that the degree of polymerization lesser than 6 homo COS showed a higher reducing power with increasing concentration.

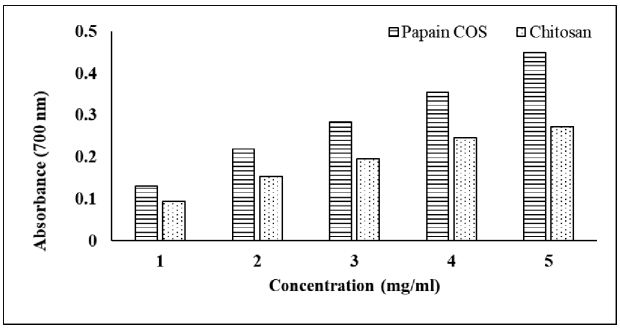

Fig. 4. FRAP assay of Chitosan and Papain COS

In the present investigation, COS from chitosan using papain enzyme exhibited radical scavenging activity as assessed using DPPH free radical activity and ferric reducing power. Results obtained clearly demonstrate that COS produced by enzymatic hydrolysis using papain had the ability to donate the electrons or protons while reacting with the DPPH free radical and greater ability to reduce ferric ions thus could act as an antioxidant.

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