Chapter 3
Biocatalytic Production of Hetero-Chitosan Oligosaccharides as Anti-oxidants

Swati Jaiswal, Pushplata Tripathi, and Sujata Sinha

Abstract Abundantly available chitin/chitosan and their derivatives are full of useful bioactivities. They have numerous applications in industries like food, wastewater treatment, pharmaceuticals, agriculture, cosmetics etc. However, their insolubility in water plays spoilsport in way of their use as cost-effective biomolecules for various sectors. Breakage of chitosan to smaller oligosaccharides solves this problem to larger extent preferably using highly specific enzymes. It is well known that that bioactivities of oligosaccharides improve upon hydrolysis to lower molecular weight chitosan i.e. chitooligosaccharides. Availability and production of anti-oxidant chitooligosaccharides by non-chemical approach is desirable for consumer satisfaction. Bioprocessing of chitin/chitosan generated from marine waste to be used as bioactive chitooligosaccharides, can reduce both environmental and human health hazards to a great extent.

Here we review (1) biocatalytic approaches for chitooligosaccharides production, (2) bioprocess strategies for large scale production, (3) functionalization and (4) anti-oxidant activity of chitooligosaccharides. Specific and non-specific biocatalysts are used for chitooligomer preparation either by hydrolysis and transglycosylation approaches. Cellulase enzymes have been found to be most frequently used non-specific enzymes for chitosan hydrolysis but microbial chitosanases show excellent performance for chitooligosaccharides production both in terms of yield and specificity. Transglycosylation also have been found to be promising for chitooligosaccharides production especially at small scale. Combination reactors have been found to be most suitable for upscaling of chitooligomer production. Immobilized packed column with ultrafiltration membrane reactors are used for simultaneous hydrolysis and separation of chitooligomers. Chemically synthesized derivatives of chitooligomers have been reported in many studies by introducing carboxyl, quaternized amino, amino ethyl, sulfate, gallyl and many more groups. Amino ethyl, Gallyl, sulphated,

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phenolic acid conjugated and carboxylated derivatized chitooligomers have shown anti-oxidant activity. Anti-oxidant activity of chitooligomers and relation with their structure and polymerisation has been well established. Chitooligomers longer than trimer show good activity while best activity has been reported in degree of polymerisation from 10 to 12. Acetylation of chitooligomers leads to improvement in anti-oxidant activity than their deacetylated version.

Keywords Anti-oxidant · Oligosaccharides · Chito-oligosaccharides · Chitosanase · Chitin · Chitosan · Radical-scavenging · DPPH · Bioactivity

Abbreviations

ABTS	2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
CAT	chloramphenicol acetyltransferase
COS	chitooligosaccharides
DCFH-DA	dichloro-dihydro-fluorescein-diacetate
DD	degree of deacetylation
DMPO	5,5-dimethyl-1-pyrroline N-oxide
DNA	deoxyribonucleic acid
DNA	deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ESR	electron spin resonance
FRAP	ferric reducing power
GSH-PX	glutathione peroxidase
KDa	kilo Dalton
PANNFM	polyacrylonitrile nanofibrous membrane
QCMCOS	quaternised carboxymethyl chitooligosaccharide
ROS	reactive oxygen species
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances
TBHQ	tertiary butyl hydroquinone

3.1 Introduction

Chitin is the second most abundant renewable biopolymer after cellulose in nature which could be used as starting material for various industries (Kaur and Dhillon 2015). Chitin and chitosan have been converted to oligosaccharides because lower solubility of chitosan in water possess difficulty in their application for various purposes and oligosaccharides are shown to have better bioactivity than their chitosan/chitin polymers. Chitosans with degree of polymerization less than 20 and an average molecular weight less than 3.9 KDa are called chitosan oligosaccharides (Mourya

et al. 2011). Chitooligosaccharides can be homochitooligomers or heterochitooligomers. Homochitooligomers contain either only glucosamine or D-glucosamine units, whereas heterochitooligomers contain both types of monomers and can be a mixture of various oligomers having various degree and position of polymerisation/acetylation. Hetero-chitooligosaccharides with degree of polymerisation less than 10 are soluble in water but for chitooligosaccharides having degree of polymerisation more than 10, solubility depends on pH of the solution and degree of acetylation. Pharmaceutical companies, food industries and researchers preferably uses chitooligosaccharide in hetero form (Il'ina and Varlamov 2015). Chitooligosaccharides have same or variation in degree of acetylation and the sequence of acetylated/deacetylated residues, however these can be considered as homologs (Kim 2011).

Chitin/chitosan conversion to their oligosaccharides can be achieved physically, chemically or bio-catalytically (Aam et al. 2010). Ultrasonic and gamma irradiation have been used for physical depolymerisation. Fully deacetylated chitosan was depolymerised with ultrasonic irradiation to produce chitosan oligos with degree of polymerisation between 2 and 11 and maximum concentration of trimers (Popa-Nita et al. 2009). Gamma irradiation has been used for reducing the viscosity of chitosan in acetic acid solution and producing dimers, trimers and tetramers of chitin/chitosan polymer (Choi et al. 2002). Chitosan hydrolysis using various acids like hydrochloric acid, electrolyte acid, nitrous acid, phosphoric acid, fluoric acid etc. and hydrogen peroxide or persulfate using oxidative/reductive methods have also been demonstrated (Lodhi et al. 2014). Milder acids like lactic acid, trichloroacetic acid, formic acid, acetic acid etc. has also been studied for their degradative action on chitosan (Ando and Kataoka 1980; Yamaguchi et al. 1982; Il'ina and Varlamov 2004). However, chemical reactions are difficult to control leading to synthesis of spurious or secondary products which hinder the downstream processing and therefore are not eco-friendly. Enzymatic processes are considered to be most feasible and attractive for chitooligosaccharide preparation either by hydrolysis of chitin/chitosan and related substrates or by synthesis of larger oligomers by transglycosylation methods. Biocatalytic methods reduce the use of toxic chemicals, are easier to control and do not generate any harmful waste as in case of chemical methods. Various specific and non-specific biocatalysts used for chitooligosaccharide production and their reported anti-oxidant activity has been covered in this chapter (Fig. 3.1).

3.2 Biocatalysts of Chitosan Oligosaccharides

3.2.1 Specific Biocatalysts

Chitosan enjoys broad substrate specificity as it is susceptible to number of carbohydrases, proteases and chitinases reported from fungi and bacteria (Kim and Rajapakse 2005; Sinha et al. 2016a). Microbial chitosanases from fungi and bacteria have shown excellent performances in hydrolysing chitosan of various degree of deacetylation and these enzymes have also been reported in virus, animals and plants (Hamed et al. 2016). However, chitosanases reported from microbial sources

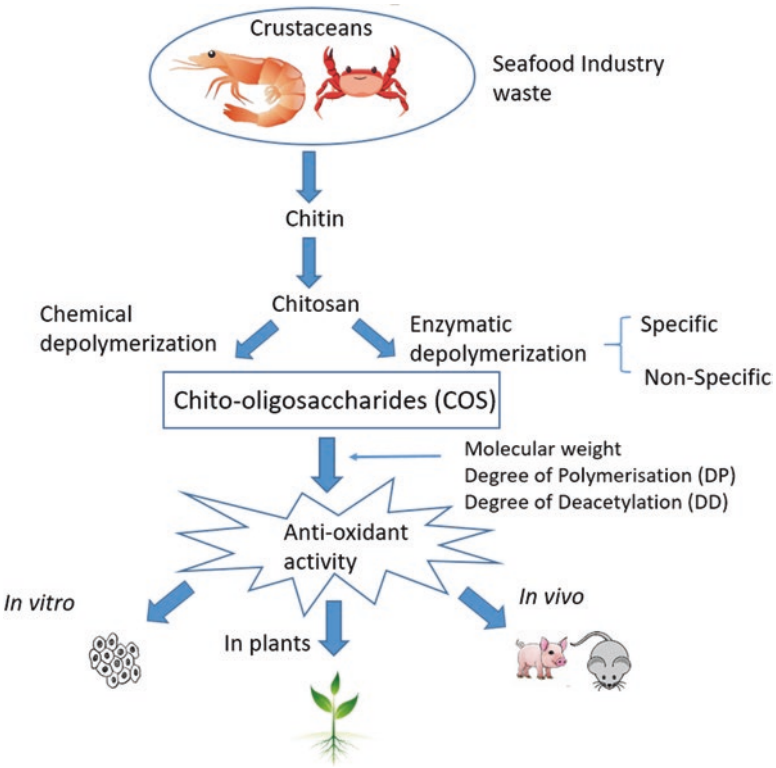

Fig. 3.1 Biocatalytic production of chitooligosaccharides and their anti-oxidant activity. Chitin from waste from seafood industry is the source of chitosan which is depolymerized (enzymatic or chemical) to produce chitooligosaccharides with a range of molecular weight, degree of polymerization and deacetylation. These chitooligosaccharides have been found to exert antioxidant activity in vitro, in vivo and in plants

are very few and are expensive to be used at industrial scale due to high cost of extraction, concentration and purification. There are specific and non-specific enzymes which affect the types and structure of glycosidic bonds in chitosan hydrolysis. Random distribution of four types of glycosidic bonds in the structure of chitosan determines their hydrolysis by enzymes. These could be between two deacetylated units (D-D), one acetylated and one deacetylated (A-D), one deacetylated and one acetylated (D-A) and two acetylated units (A-A) and their hydrolysis depends on presence of reducing/non-reducing ends and degree of deacetylation. Lysozyme from egg white has been found to be specific towards two acetylated (A-A) units and *Bacillus* chitosanase has been found to be specific towards glycosidic bonds two deacetylated units (D-D) (Vårum et al. 1996). Chitinases act on partially deacetylated chitosan by detecting the presence of N-acetyl glucosamine (GlcNAc) moiety in the sequence of chitosan (Aiba 1994). Chitosan and other non-specific enzymatic hydrolysis with respect to microbial source of enzyme and product obtained, have been summarised in Table 3.1.

Table 3.1 chitooligosaccharide production as a result of chitosan specific and non-specific enzymatic hydrolysis, their sources and reported anti-oxidant activity

Enzyme	Microbial source	Chitooligosaccharides product	Anti-oxidant activity	References
Specific enzyme				
Chitosanase	Streptomyces sp	Dimer to hexamer	1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity	Sinha et al. (2012a) and Sinha et al. (2014)
Chitosanolytic enzymes	*Metarhizium anisopliae*	Dimer to hexamer	Not studied	de Assis et al. (2010)
Chitosanase	*Purpureocillium lilacinum* CFRNT12	Dimer to hexamer	Not studied	Nidheesh et al. (2015)
Chitosanase	Bacillus sp	10 KDa, 5 KDa, 3 KDa, 1 KDa	1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, Alkyl radical assay, superoxide radical, hydroxyl radical	Park et al. (2003)
Chitosanase	Commercial	Details not given	Improved total anti-oxidant capacity and activity of SOD (superoxide dismutase)	Yuan (2009)
Chitinolytic enzyme	Chitiniphilus sp. LZ32	Dimer, trimer, tetramer, pentamer, hexamer	Reducing power of chitooligosaccharide, superoxide, hydroxyl radical scavenging	Zhang et al. (2017)
Non-specific enzyme				
Glycosyl transferase (Branchzyme) Free and immobilized form	Not known	2-20 polymerisation with high conc of 3-8 mers	Not reported	Montilla et al. (2013)
Lysozyme, papain, cellulase	Papain showed highest mol wt reduction	1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, reducing power, metal chelating activity	(0.003% w/w) compared between three enzyme	Laokuldilok et al. (2017)
Cellulase	Commercial	Hydrogen peroxide, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, ferrous ion chelation	Negative correlation between mol wt of COS and anti-oxidant activity was established	Chang et al. (2018)

3.2.2 Non-Specific Enzymatic Hydrolysis

As specific enzymes are not available in bulk for commercial preparation and are not cost effective hence non-specific biocatalytic preparations of chitooligomers are being explored for this purpose. Lipases, cellulases (Wu and Tsai 2004), papain, lysozyme, hemicellulases, protease, pectinases, pepsin, pronase, chitinases and many other non-specific enzymes have been reported for hydrolysis of chitosan (Abdel-Aziz et al. 2014). Cellulase is the frequently used non-specific enzyme being reported for chitosan hydrolysis and this is explained by similarity between structure of chitin, chitosan and cellulose due to presence of β, 1-4 glycosidic bond between glucose subunits. The presence of acetamide group in chitin and amino group in chitosan at C-2 hydroxyl position is found to have no role in enzyme substrate reaction. Wide variety of cellulases, especially bifunctional chitosanase-cellulase from various microbial sources have been reportedly used for production of chitooligomers from chitosan (Xia et al. 2008). All these enzymes belong to glycosyl hydrolase family mainly GH-5, GH-7 and GH-8 and few have been found to be superior to even chitosanases enzymes for chitosan hydrolysis.

3.2.3 Transglycosylation Activity for Synthesis of Chitooligosaccharides

Apart from hydrolysis of chitosan, chitooligomers are also synthesised using transglycosylation activity of biocatalysts. Chemical and enzymatic methods of chitooligosaccharides synthesis have been proposed and has been reviewed extensively (Yang and Biao 2014; Li et al. 2016). Large number of steps related to protection and deprotection make chemical glycosylation methods cumbersome as steps increases with the size of oligosaccharides, so synthesis of trimer and larger oligosaccharides are not considered feasible. However, biocatalytic methods allow regioselectivity, milder reaction and hassle-free ways (no protection and deprotection step) of glycosidic bond formation. Formation of new glycosidic bonds between donor and acceptor saccharides can also be established by few glycosyl hydrolases apart from their usual activity of glycosidic bond hydrolysis (Li et al. 2016). Active-site architecture of transglycolytic enzymes in their full efficiency hinders correct positioning of water molecule and promotes/favours binding of incoming carbohydrate molecule through strong interaction of aglycon subsites. Released chitooligosaccharides is transferred to suitable acceptor to form a new glycosidic bond to synthesize chitooligosaccharide. Stereo- and size specific preparation of higher chitooligosaccharide have been achieved using this activity of chitosanase/chitinolytic enzymes and other glycosidases. An exo-chitosanase enzyme having transglycosylation activity was isolated from *Aspergillus fumigatus* IIT-004 which was immobilized on nanofibers and employed for chitosan hydrolysis. However, synthesis of chitodimer was also achieved using this enzyme when reaction conditions were

changed (Sinha et al. 2016b). Purified chitinase from *Trichoderma reesei* KDR-11 has been shown to convert dimer and hexamer of N-acetyl glucosamine $(GlcNAc)_2$ (55.7%) and $(GlcNAc)_6$ (39.6%) from tetramer of N-acetyl glucosamine $(GlcNAc)_4$ (100%) using a transglycosylation reaction (Usui et al. 1990). Lysozyme from hen egg-white lysozyme has been used for synthesis of chitooligosaccharide (4-12) polymerisation (Akiyama et al. 1995).

3.2.4 Bioprocess Strategies for Chitooligosaccharides Production/Synthesis

Upscaling of COS production has been tried mainly in three types of bioreactor settings like batch, column and ultrafiltration reactor (Vidanarachchi et al. 2010). Batch reactor is most common where enzyme (chitosanase from *Bacillus pumilus* BN-262) is mixed with substrate (1% chitosan) and glycosidic bonds are allowed to break under optimized pH, temperature and time. Nonetheless, it has certain drawbacks like lower yield, lack of continuous production and higher cost due to lack of enzyme reusability. Packed immobilized enzyme in a column reactor through which substrate is passed continuously, has been suggested for continuous production but its use has been limited by poor affinity of immobilized enzyme towards substrates. Enzyme immobilization has been tested with various carriers for column packing but chitosanase bonded on chitin has shown better activity than other matrices (Kim and Rajapakse 2005). Ultrafiltration membrane with cut off of 3 KDa has been used for production of relatively higher oligomers of chitosan (trimer to hexamer) (Jeon and Kim 2000). Eleven batches of hydrolysis could be achieved with same amount of enzyme used in batch condition, thus at relatively lower cost, chitooligosaccharide with higher degree of polymerisation (chitotrimer to chitohexamer) could be achieved. In continuous reactor, a dual reactor system was proposed where ultrafiltration membrane was attached to chitin packed column with immobilized chitosanase enzyme system (Jeon and Kim 2000). Chitosanase enzyme was physically adsorbed on the chitin but showed less affinity and lower reaction rate towards substrates than free enzyme. Optimized permeation rate of 4 ml/min was determined where the 80% of product contained larger chitooligosaccharides (trimer to hexamer). Membrane of 10 KDa was used in ultrafiltration membrane reactor for selective fractionation of chitooligosaccharide which resulted from hydrolysis of partially hydrolysed chitosan and this was controlled by changing flow rate. Monomer production from chitooligosaccharide was stopped by controlling the chitosan hydrolysis which in turn halted product inhibition. In this study, membrane fouling in ultrafiltration membrane was removed by partially hydrolysing chitosan before applying to the reactors. Chitooligosaccharides preparation by continuous hydrolysis of chitosan in ultrafiltration membrane reactor along with immobilized column reactor utilising *Bacillus* chitosanase has also been reported from a different study where the product was used for radical scavenging activity studies (Park et al. 2003).

A chitosanolytic α-amylase enzyme from *Bacillus amylolyquefaciens* was covalently immobilized on glyoxal agarose beads and was assessed in batch and fixed bed reactors for continuous production of chitooligosaccharide with activity recovery of 25% (Moriano et al. 2016). Here, improved thermostability of the immobilized enzyme and conversion yield of 73% was obtained. Also, chitotriose and chitobiose were found to be the major products and conversion yield dropped by an increase in the dilution rate. In yet another study, polyacrylonitrile nanofibrous membrane (PANNFM) based chitosanase enzyme from *Aspergillus* sp. was used for selective fractionation of chitodimer to hexamer by varying the reaction temperature (Sinha et al. 2012b).

3.2.5 Production of Functionalized Chitooligosaccharides

Not only chitooligosaccharide but their derivatives have also shown antioxidant activity in different biological systems (Table 3.2). Various functional groups like hydroxyl and amino groups in the chitosan backbone have been added by enzymatic approaches to improve its application in various fields as it leads to changes in physicochemical properties. Amide coupling reaction has been used for conjugates preparation like phenolic and gallic acid conjugates (Liaqat and Rengin 2018). Phenolic acid compounds have tendency to donate H atom which enhanced the potential of conjugated anti-oxidant compounds, similarly, gallic acid conjugated chitooligosaccharides have also shown anti-oxidant capacity (Vo et al. 2017) Also, quaternization, alkyltion, thiolation, hydroxyalkylation, carboxyalkylation are some of the methods by which chitooligosaccharides can be modified (Mourya and Inamdar 2009). In a study on functionalized chitooligosaccharide, it was observed that chitooligosaccharides have better anti-oxidant activity than their O- and N-carboxymethyl substituted counterparts, while reducing power was greatest in O-carboxymethyl substituted chitooligosaccharide. In case of quaternized carboxymethyl chitooligosaccharide (QCMCOS), anti-oxidant activity was directly related to degree of substitution. Substitution with quaternary ammonium and carboxymethyl group also enhanced their thermal stability and degree of crystallinity (Li et al. 2012).

3.3 Anti-Oxidant Activity

Antioxidant molecules play an important part in neutralization of oxidative stress in living systems since they bind free radicals. Free radicals, also known as Reactive Oxygen Species or ROS, are chemical species with an unpaired electron in its outer orbital and therefore they are highly reactive e.g. hydroxyl free radical (OH°). ROS harms the cell membrane by lipid peroxidation and impairs cellular machinery by DNA and protein oxidation. Free radicals are produced under normal as well as pathological conditions and levels of free radicals are balanced by the endogenous

Table 3.2 Preparation of COS conjugates by functionalization using various derivatives and their anti-oxidant activity

Derivative	Preparation Method	Biological System	Antioxidant capacity assay	References
Carboxyl	Chemical synthesis	Cell free system	Ferrous ion chelating activity 1,1-diphenyl-2-picrylhydrazyl (no) radical scavenging Carbon-centered radicals scavenging by electron-spin resonance (ESR) Hydroxyl radical scavenging	Huang et al. (2006)
Amino	Chemical synthesis	Cell free system	Ferrous ion chelating activity 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging Carbon-centered radicals scavenging by electron-spin resonance (ESR) Hydroxyl radical scavenging	Huang et al. (2006)
Gallic acid	Chemical synthesis	Human chondrosarcoma (SW1353 cells)	1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity Superoxide anion radical scavenging activity Hydroxyl radical scavenging activity Reactive oxygen species (ROS) detection by Dichloro-dihydro-fluorescein-diacetate Dichloro-dihydro-fluorescein-diacetate (DCFH-DA)assay	Ngo et al. (2011)
N-Maleoyl chitosan and N-succinyl	Chemical synthesis	Cell free system	Superoxide anion radical scavenging activity Hydroxyl radical scavenging activity and ferric reducing power (FRAP)	Sun et al. (2011)
Phenolic acid conjugate	Chemical synthesis	Cell free system	1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity Hydroxyl radical scavenging activity Nitric oxide radical scavenging assay	Eom et al. (2012)
QCMCOS (quaternized)	Chemical synthesis	Cell-free system	Hydroxyl radical scavenging/ Fe^{2+} chelation assay, reducing power, superoxide anion free radical assay	Li et al. (2012)
Aminoethyl	Chemical synthesis	RAW 264.7 cells	ROS detection by Dichloro-dihydro-fluorescein-diacetate (DCFH-DA) Intracellular glutathione level	Ngo et al. (2012)

(continued)

Table 3.2 (continued)

Derivative	Preparation Method	Biological System	Antioxidant capacity assay	References
Aminoethyl	Chemical synthesis	BV-2 cells	1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity Lipid peroxidation assay Protein oxidation assay	Ngo et al. (2012)
Sulphated	Chemical synthesis	MIN6 cells	Reactive oxygen species detection by Dichloro-dihydro-fluorescein-diacetate (DCFH-DA) Measurement of enzymes such as superoxide dismutase, chloramphenicol acetyltransferase (CAT), glutathione peroxidase (GSH-PX)	Lu et al. (2012)
Hydroxy benzaldehyde	Chemical synthesis	BV-2 cells	ROS detection by Dichloro-dihydro-fluorescein-diacetate (DCFH-DA) measurement of NF-κB and Nrf2 proteins deoxyribonucleic acid (DNA) oxidation assay	Oh et al. (2017)

Abbreviations: *DPPH* 2,2-Diphenyl-1-picrylhydrazyl, *QCMCOS* quaternised carboxymethyl chitooligosaccharide, *ESR* Electron spin resonance, *DCFH-DA* Dichloro-dihydro-fluorescein-diacetate, *FRAP* ferric reducing power, *ROS* reactive oxygen species, *SOD* superoxide dismutase, *CAT* Chloramphenicol acetyltransferase, *GSH-PX* Glutathione peroxidase, *NF-κB* Nuclear Factor kappa beta, *Nrf2* Nuclear factor (erythroid-derived 2)-like 2

antioxidants produced by the living systems during normal physiological state (Valko et al. 2007). Imbalance between their level leads to damage to the biological components which is one of the underlying cause of diseases and disorders such as cancer, arteriosclerosis, stroke, heart attack, Alzheimer's, ageing etc. (Pham-Huy et al. 2008).

Initiation of free radical injury can be caused by ionising radiations, inflammatory conditions, excess metal ions in the body and drugs/chemicals such as acetaminophen, carbon-tetrachloride. Generally, these are overcome by body's defence mechanism which comprises of antioxidants (vitamins A, C, E, glutathione), enzymes (superoxide dismutase; catalase; glutathione peroxidase (GSH-PX) and metal carrier proteins (transferrin, ceruloplasmin) (Yu 1994). However, it has been postulated that external supply of antioxidants to the body will help in relieving it from diseases and disorders caused by oxidative stress (Sindhi et al. 2013). Thus, there is ongoing research towards discovery and synthesis of both artificial and natural antioxidant molecules which may assist patients in conquering their symptoms. Chitosan and chito-oligosaccharides have been extensively researched as natural antioxidants which are not only inexpensive but also biodegradable. The various antioxidant capacity assay along with their principle and end-product determination have been summarised in Table 3.3.

Table 3.3 Antioxidant assay, principle of the method and end product determination

Antioxidant capacity assay	Principle of the method	End-product determination
1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity	Antioxidant reaction with DPPH free radical which loses its violet colour	Colorimetry/ESR spectroscopy
ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) assay	Antioxidant reaction with the long-lived ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) radical	Colorimetry/ESR spectroscopy
Metal chelating activity	Chelation of ferrous ions by the inhibition of ferrozine–Fe^{2+} complex formation due to antioxidant	Colorimetry
FRAP (ferric reducing power)	Potassium ferricyanide reduction by antioxidants and subsequent reaction of potassium ferrocyanide with Fe^{3+}	Colorimetry
Superoxide anion radical scavenging activity	Superoxide anion production by a luminol-enhanced auto-oxidation of pyrogallol or by ultra violet (UV) irradiated riboflavin/ethylene diamine tetra acetic acid (EDTA) system followed by incubation with antioxidant.	Chemiluminescence quenching /ESR spectroscopy
Hydroxyl radical scavenging activity	Antioxidant capacity to quench OH radicals generated by a Co(II) based Fenton-like system	Loss of fluorescence of fluorescein/ESR spectroscopy
Carbon-centered radical spin adduct analysis	Carbon-centered radicals generated by AAPH (2,2-azobis(2-amidinopropane dihydrochloride)	ESR spectroscopy
TBARS (thiobarbituric acid reactive substances) assay	Hydroxyl radicals attack deoxyribose leading to the formation of thiobarbituric acid reactive substances (TBARS)	Colorimetry
Lipid peroxidation assay	Malondialdehyde (MDA) and other aldehydes are formed during lipid oxidation	Colorimetry
ROS (reactive oxygen species) detection by Dichloro-dihydro-fluorescein-diacetate (DCFH-DA) assay	Oxidation-sensitive dye Dichloro-dihydro-fluorescein-diacetate (DCFH-DA) detects formation of intracellular ROS	Fluorimetry
Measurement of enzymes such as superoxide dismutase (SOD), chloramphenicol acetyltransferase (CAT), glutathione (GSH)	Enzymes involved in free radical defence mechanism of living cells	Enzyme activity measurement
Measurement of NF-κB and Nrf2 proteins	Role of NF-κB and Nrf2 proteins in redox balance maintenance	Western blot analysis

(continued)

Table 3.3 (continued)

Antioxidant capacity assay	Principle of the method	End-product determination
Measurement of mRNA expression of antioxidant enzymes (GPx1, GPx4, and Mn-SOD) or pro-inflammation cytokines, tumor necrosis factor (TNFα)	Mn and Cu/Zn-SOD (superoxide dismutase), chloramphenicol acetyltransferase (CAT), glutathione (GSH), glutathione peroxidase (GSH-PX) are antioxidant enzymes	Real time polymerase chain reaction (PCR)
DNA oxidation assay	Hydrogen peroxide mediated DNA oxidation is performed by reacting Fe(II) and H_2O_2 on genomic DNA which is inhibited by the antioxidant.	Agarose gel electrophoresis
Protein oxidation assay	Oxidation of cellular proteins by the Fenton reaction products produces carbonyl groups such as aldehydes and ketones in proteins	Spectroscopy

Abbreviations: *DPPH* 2,2-Diphenyl-1-picrylhydrazyl, *QCMCOS* quaternised carboxymethyl chitooligosaccharide, *ESR* Electron spin resonance, *DCFH-DA* Dichloro-dihydro-fluorescein-diacetate, *FRAP* ferric reducing power, *ROS* reactive oxygen species, *SOD* superoxide dismutase, *CAT* Chloramphenicol acetyltransferase, *GSH-PX* Glutathione peroxidase, *NF-κB* Nuclear Factor kappa beta, *Nrf2* Nuclear factor (erythroid-derived 2)-like 2

3.3.1 Anti-Oxidant Activity of Chitooligosaccharides

In view of growing interest in identification of natural anti-oxidants, anti-oxidant activity of chitooligosaccharides has been explored in various biological systems (Table 3.4). Chitooligosaccharide and their conjugates possess greater anti-oxidant activity as compared to chitosan. Although, molecular mechanism of antioxidant activity of chitooligosaccharides is unclear but it is suggested that amino group in chitooligosaccharides react with unstable free radicals in order to make them stable, resulting in its anti-oxidant activity. Radical scavenging activity or anti-oxidant activity of chitooligosaccharide has been correlated with molecular weight, degree of polymerisation, degree/fraction of deacetylation and N-acetylation and chitosan substrate source (Anraku et al. 2018). In a systematic study on seven chitosan samples with molecular weight in the range of 2 to 300 KDa it was concluded that anti-oxidant properties of chitosan and chitooligosaccharide were inversely proportional to their molecular weight (Laokuldilok et al. 2017; Chang et al. 2018). Chitooligosaccharides with active hydroxyl and amino groups helps to scavenge free radicals and low molecular weight chitooligosaccharide with lower degree of polymerisation have more such groups available for reaction. Chitobiose and chitotriose were found to possess better anti-oxidant and reducing activity than chitooligosaccharides with higher degree of polymerisation (Chen et al. 2003). Chitooligosaccahrides with degree of polymerisation (10-12) show best anti-oxidant

Table 3.4 Molecular weight/degree of polymerisation and their *in vitro/in vivo* anti-oxidant activity of chitooligosaccharides

Biological System	Antioxidant capacity assay	MW, DD and DP of highest activity	References
In vitro			
Cell free system	1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity Hydroxyl radical scavenging activity Superoxide anion radical scavenging activity Carbon-centered radical spin adduct analysis	5–10 KDa 90% DD	Je et al. (2004)
Polymorphonuclear leukocytes	Superoxide radical Reactive oxygen species (ROS) detection by dichloro-dihydro-fluorescein-diacetate (DCFH-DA)	1.1 KDa, degree of polymerisation DP = 7; DD 92.3%	Yang et al. (2006)
Cell free system	1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity Superoxide anion radical scavenging activity Hydroxyl radical scavenging activity Thiobarbituric acid reactive substances (TBARS) assay	Not defined	Rao et al. (2006)
Cell free system	Ferrous ion chelating activity 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity Carbon-centered radical spin adduct analysis Hydroxyl radical scavenging activity	DD 76.54% Activity decreased with increased degree of substitution	Huang et al. (2006)
Rabbit neutrophils	Superoxide anion radical scavenging activity	DD 95%	Dou et al. (2007)
B16F1 cell line	Hydroxyl radical scavenging activity superoxide anion radical scavenging activity Carbon-centered radical spin adduct analysis Intracellular ROS detection by dichloro-dihydro-fluorescein-diacetate (DCFH-DA) Intracellular glutathione (GSH) level	<1 KDa with DD 90%	Mendis et al. (2007)
Cell free system	2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) assay Superoxide anion radical scavenging activity Hydroxyl radical scavenging activity Metal chelating activity Scavenging of hydrogen peroxide	1.7 KDa with DD 47.9%	Feng et al. (2007)

(continued)

Table 3.4 (continued)

Biological System	Antioxidant capacity assay	MW, DD and DP of highest activity	References
Salmon tissue homogenate	1,1-diphenyl-2-picrylhydrazyl(DPPH)radical scavenging Thiobarbituric acid reactive substances (TBARS) assay	30 KDa, DD 84.71%	Kim and Thomas (2006)
ECV304 cell line	ROS detection by Dichloro-dihydro-fluorescein-diacetate (DCFH-DA)	DP 2–6; DD 95%	Liu et al. (2009)
RAW 264.7 cell line	ROS detection by Dichloro-dihydro-fluorescein-diacetate (DCFH-DA) Intracellular glutathione (GSH) level	DD <10%; MW 1–3 KDa	Ngo et al. (2009)
HepG2	ROS detection by Dichloro-dihydro-fluorescein-diacetate (DCFH-DA)	Not defined	Cho et al. (2010)
Erythrocytes and bacteriophages	2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) assay hemolysis	<3 and <5 KDa; DD 80–85%	Fernandes et al. (2010)
HITT15 cell line	Reactive oxygen species (ROS) detection by dichloro-dihydro-fluorescein-diacetate (DCFH-DA)	3–5 KDa	Karadeniz et al. (2010)
Cell free system	Hydroxyl radical scavenging activity superoxide anion radical scavenging activity Metal chelating activity	DD 85%	de Assis et al. (2012)
L02 cells	ROS detection by Dichloro-dihydro-fluorescein-diacetate (DCFH-DA) Lipid peroxidation assay Intracellular glutathione (GSH) level	DD ≥95%; <1 KDa	Luo et al. (2014)
Cell free system and blood mononuclear cells	Hydroxyl radical scavenging activity superoxide anion radical scavenging activity 1,1-diphenyl-2-icrylhydrazyl (DPPH) radical scavenging activity	DP 2	Salgaonkar et al. (2015)
SH-SY5Y cells	ROS detection by Dichloro-dihydro-fluorescein-diacetate (DCFH-DA) Superoxide anion radical scavenging activity Measurement of Nrf2 proteins	DP <10	Huang et al. (2015)
Cell free system	1,1-diphenyl-2-picrylhydrazyl((DPPH) radical scavenging activity, ABTS (2,2′-azino-bis-3-thylbenzthiazoline-6-sulfonic acid)assay FRAP (ferric reducing power)	<1 KDa	El-Sayed et al. (2017)
Cell free system	1,1-diphenyl-2-picrylhydrazyl(DPPH) radical scavenging activity Metal chelating activity FRAP (ferric reducing power)	5.1KDa; DD 90%	Laokuldilok et al. (2017)

(continued)

Table 3.4 (continued)

Biological System	Antioxidant capacity assay	MW, DD and DP of highest activity	References
In vivo			
Male albino rats	Lipid peroxidation assay	150 KDa; DD 82%	Koryagin et al. (2006)
Sprague-Dawley rats	Reactive oxygen species detection by dichloro-dihydro-fluorescein-diacetate (DCFH-DA)	2.3 KDa; DD 92.7%	Yang et al. (2006)
Male Wistar rats	Measurement of enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) Lipid peroxidation assay	1.2 KDa; DD 90%	Yuan (2009)
Sows	Measurement of mRNA expression of several antioxidant enzymes GSH-PX (glutathione peroxidase), total-superoxide dismutase (SOD) and chloramphenicol acetyl transferaase (CAT) Lipid peroxidation assay	DP 2–7	Xie et al. (2016)
ICR mice	Measurement of enzymes such as superoxide dismutase, chloramphenicol acetyl transferase, glutathione 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity Hydroxyl radical scavenging activity Superoxide anion radical scavenging activity	1.5 KDa	Qu and Han (2016)

Abbreviations: *DD* degree of deacetylation, *DP* degree of polymerization, *DPPH* 2,2-Diphenyl-1-picrylhydrazyl, *QCMCOS* quaternised carboxymethyl chitooligosaccharide, *ESR* Electron spin resonance, *DCFH-DA* Dichloro-dihydro-fluorescein-diacetate, *FRAP* ferric reducing power, *ROS* reactive oxygen species, *SOD* superoxide dismutase, *CAT* Chloramphenicol acetyltransferase, *GSH-PX* Glutathione peroxidase, *NF-κB* Nuclear Factor kappa beta, *Nrf2* Nuclear factor (erythroid-derived 2)-like 2

activity while trimers and higher degree of polymerisation shows good anti-oxidant activity (Li et al. 2012). Partially acetylated version of chitotrimer was compared with deacetylated version and it was seen that acetylated chitosan has greater anti-oxidant activity proving that degree of acetylation has a role in anti-oxidant activity (Liaqat and Rengin 2018).

Chitooligosaccharides of five types were prepared using reactor where membrane of cut off 10, 5, 3 and 1 KDa was used for fractionation of various molecular weight chitooligosaccharide. Size of chitooligosaccharides varied according to pore size of membrane used. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging ability was found to be present in all types of chitooligosaccharide and mechanism involved pairing of odd electrons of the DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals. They also showed radical scavenging activities for all the free radicals but showed most effectiveness on DMPO (5,5-dimethyl-1-pyrroline N-oxide)-OH scavenging. Among all the fractions, those containing mol wt. 3–10 KDa showed

highest radical scavenging activity. In another study, radical scavenging ability of chitosan of various molecular weight (30, 90 and 120 KDa) was compared with that of an established synthetic anti-oxidant butylated hydroxytoluene (BHT) and an equivalent efficiency of 85% was obtained. Here also the lowest molecular weight chitosan showed highest activity (Hamed et al. 2016). Radical scavenging ability of chitooligosaccharides has also been studied in *in vivo* condition in a mouse model on high fat diet. Chitooligosaccharide showed potent anti-oxidant activity by protecting mice from oxidative stress (Qu and Han 2016) by reducing level of certain enzymes (Glutathione peroxidase, superoxide dismutase, catalase) in liver, serum and stomach which increases considerably in case of stress due to high fat diet. Gallic-acid conjugated chitooligosaccharides were been found to exert anti-inflammatory and anti-cancer effect on human lung epithelial cells (A549). It also depicted DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging and H_2O_2 induced DNA damage protection (Vo et al. 2017). Effects of chitooligosaccharide supplementation on performance, blood characteristics, relative organ weight, and meat quality in broiler chickens was analysed by Zhou et al. (2009). Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertiary butyl hydroquinone (TBHQ) are some of the reported synthetic anti-oxidants which are used in food industry to prevent oxidation and reduce rancidity and loss of flavours. However, because of health hazards related with these compounds natural and safe compounds are preferable and chitooligosaccharides has been found to be a suitable alternative of these compounds (Kim and Thomas 2006). Supplementation of chitooligosaccharide was reviewed on processing and storage quality of foods of animal and aquatic origin such as milk, meat, fish, eggs, sea foods, etc. (Singh 2016). Therefore, there is an increasing interest in antioxidants, particularly in those intended to arrest the presumed harmful effects of free radicals in the living systems, as well as the deterioration of fats and other constituents of foodstuffs (Rao et al. 2006).

3.3.2 Anti-Oxidant Activity of Chitooligosaccharides in Plants

Due to their strong antioxidant activity, chitooligosaccharide has been studied in plants for their agricultural application. Chitooligosaccharides at 1–10 mg/L with acetylation degree of 65% and molecular weight of 5–10 KDa significantly activated OPD (o-phenylenediamine) oxidation by wheat seedlings (Khairullin et al. 2001). Cabrera et al. (2006) reported effect of the degree of polymerization, degree of acetylation and concentration of chitooligosaccharide on defence activation in *Arabidopsis thaliana* suspension-cultured cells. Similarly chitooligosaccharides were able to induce nitric oxide (NO) generation followed by up-regulation in the activities of defence-related enzymes through an oligochitosan induced Ser/Thr protein kinase (OIPK)-dependent or independent pathway (Zhang et al. 2011). Plant

mineral nutrient dynamics was studied in hydroponically grown plants by Chatelain et al. (2014) and their use in phytoremediation and biofortification programs is promoted (Vasconcelos 2012). Application of chitooligosaccharide as a commercial preservative to improve the longevity of cut roses has also been studied (Jing and Li 2015). This was due to the decreased superoxide anion, hydrogen peroxide and malondialdehyde levels in the cut roses which protected them from withering.

3.4 Conclusion

In spite of making a lot of progress in the research area of "COS as an anti-oxidant" very few industries have used them in the area of food, pharmaceuticals and cosmetics. Their appearance as promising anti-oxidant biomolecules are being hindered by their non-availability on larger scale as obtaining chitooligosaccharide in highly purified form in bulk is still considered a difficult task. Downstream processing of enzymatically produced chitooligosaccharides is one of the key areas which is attracting the attention of researchers for getting purified fraction of chitooligosaccharide in terms of defined degree of polymerization and exact known sequence of monomer (both acetylated and deacetylated). More studies are required to establish relation between the anti-oxidant activity and degree of deacetylation/polymerisation and sequence in order to predict the connection between biological activity and their structure. Studies on *in vitro* and *in vivo* activity of chitooligosaccharides after characterisation are required for their establishment as an anti-oxidant molecule. However, most of these studies have been done on chitooligosaccharide mixtures which contained chitooligosaccharides molecule with various degree of polymerisation. Establishment of anti-oxidant activity due to any particular type of chitooligosaccharide was found to be difficult and needs to be explored further. Efficient biocatalytic production of chitooligosaccharides with good yield and cost effectiveness are desirable. Downstream processing studies leading to separation and purification of chitooligosaccharide with single degree of polymerization along with their *in vitro* and *in vivo* effect is another area where more investigation is needed. Apart from antioxidant activity, chitooligosaccharides has been implicated in medicinal uses due to their antimicrobial effect (Park et al. 2004), neuroprotective effect (Huang et al. 2015) and anti-tumour effect (El-Sayed et al. 2017). Recently nanoparticles of chitooligosaccharides and their conjugates have shown a promise as a drug delivery vehicle (Lu et al. 2015; Xu et al. 2016) and more research is needed for evaluating the anti-oxidant potential of nanoparticulate forms of chitooligosaccharide.

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