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Structural analyses and immunomodulatory properties of fructo-oligosaccharides from onion (*Allium cepa*)☆

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ABSTRACT

Onion (*Allium cepa*) is an immune-boosting food rich in fructans. The major aim of this study is to characterize and investigate the immunomodulatory properties of onion fructo-oligosaccharides (FOS). FOS was isolated from onion bulbs by hot 80% ethanol extraction (yield: ~4.5 g/100 g fw) followed by gel permeation chromatography. NMR of onion FOS revealed unusual β-D-Glc terminal residue at the non-reducing end. TLC and ESI–MS analyses showed that onion FOS ranged from trisaccharides to hexasaccharides. Onion FOS (50 μg/mL) significantly increased (~3-fold) the proliferation of mouse splenocytes/thymocytes vs. control. Further, onion FOS enhanced (~2.5-fold) the production of nitric oxide by peritoneal exudates cells (PECs) from Wistar rats; intracellular free radicals production and phagocytic activity of isolated murine PECs were also augmented. Our structural and in vitro results indicate that onion FOS comprising of tri- to hexasaccharide units belongs to inulin-type fructans, and possess immunostimulatory activities towards murine lymphocytes and macrophages.

1. Introduction

Certain foods not only provide energy and nutrients to the body, but also induce modulation of immune response in multiple ways and bring about host resistance to infection by their bioactive components (Huang, Zourdos, Jo, & Ormsbee, 2013). Diet contains various plant sources like garlic, onion, artichoke, chicory and a few others, which are rich in fructo-oligosaccharides (FOS)/inulin (IN). Dietary supplementation of neutral and acidic oligosaccharides for six weeks enhanced the systemic Th1-dependent immune responses (fecal bifidobacteria and lactobacilli) in a murine vaccination model (Vos et al., 2007). A study in infants at high risk for allergy showed that supplementation with a mixture of short-chain galacto-oligosaccharides and FOS induces a beneficial antibody profile, and modulates the immune response towards cow's milk proteins without altering the immune response to vaccination (Van Hoffen et al., 2009).

Onion (*Allium cepa* L.; Alliaceae family) is a perennial herbaceous plant closely related to garlic (*Allium sativum*). It is a commonly used bulb spice in daily food preparations in most of the Indian/international cuisine. It contains water (~89%), fibre (~1.7%), protein (~1.1%) and flavonoids (~0.02%) based on fresh weight (Lanzotti, 2006). In traditional Chinese medicine as well as Ayurveda (traditional Indian medicine), onion has been classified as a body-warming food. Till date, potential health benefits of onion such as antiviral activity, antimicrobial activity and anti-carcinogenic activity have been attributed to its bioactive components like organosulfur compounds (diallyl sulfide, 146.2; diallyl sulfoxide, 130.2 Da), peptides, proteins and flavonoids (Bystrická, Musilová, Vollmannová, Timorácká, & Kavalcová, 2013; Suleria, Butt, Anjum, Saeed, & Khalid, 2015).

FOS/IN are generally referred to as fructans belonging to the category of non-digestible carbohydrates. Fructans have been classified into three types based on the type of linkage present in the polysaccharide, viz., inulin, levan and graminan (Roberfroid, 2005). Fructans have texture improvement and organoleptic properties, and provide health benefits such as fat and sugar replacement (Apolinário et al., 2014). Recent animal studies clearly suggested that non-digestible carbohydrates including fructans have a strong influence on the immune system (Nauta & Garssen, 2013;

Abbreviations: A.Gal, arabinogalactan; CD, cluster of differentiation; DP, degree of polymerization; FOS, fructo-oligosaccharides; fw, fresh weight; LPS, lipopolysaccharide; NO, nitric oxide; PBMCs, peripheral blood mononuclear cells; PMA, phorbol 12-myristate 13-acetate; PECs, peritoneal exudates cells.

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Watzl, Girrbach, & Roller, 2005). The prebiotic function of fructans reduce the pathogenicity of pathogenic bacteria and leads to decreased inflammatory markers like phagocytosis and IL-6 production by increasing the $CD3^+$, $CD4^+$ and $CD8^+$ cell populations (Guigoz, Rochat, Perruisseau-Carrier, Rochat, & Schiffrin, 2002).

FOS/IN are widespread in various families of plants as storage oligo/polysaccharides. Garlic and onion belong to a monocotyledonous family, and contain considerable amounts of FOS/IN (Benkeblia, 2013). The nonstructural carbohydrate content of onion varieties (per 100 g fresh weight) range from 5.8 to 7.3 g including fructose (~0.6 g), glucose (~1.65 g) and sucrose (~1.1 g). FOS present in total nonstructural carbohydrate of onion is ~40%; the degree of polymerization (DP) of onion FOS ranges from DP 3 to DP 11 and provides osmotic adjustment during bulbing (Darbyshire & Henry, 1978). Additional medicinal values of onion include their prebiotic properties, lowering of blood lipid and insulin levels (Sabater-Molina, Larqué, Torrella, & Zamora, 2009). Onion has been considered as a functional food due to the prebiotic nature of its FOS (Watzl et al., 2005).

Fructans from various plants like garlic, chicory (*Cichorium intybus*), greater burdock (*Arctium lappa*) and others possess immunomodulatory activity, i.e., the proliferation of various immunoresponder cells and activation of macrophages (Chandrashekar, Prashanth, & Venkatesh, 2011; Koo et al., 2003; Kardosová et al., 2003). Earlier, it has been demonstrated that fructans from aged garlic extract have potent immunomodulatory activity (Chandrashekar et al., 2011). A delayed immunoadjuvant response to ovalbumin antigen in BALB/c mice was produced by fructans isolated from aged garlic extract (Chandrashekar & Venkatesh, 2012). In view of the abundance of FOS in onion and its likely similarity to garlic fructans, it appeared interesting to isolate fructans from onion and to investigate their structure and the immunomodulatory activity of onion FOS towards immune-responder cells, namely, murine lymphocytes and peritoneal exudates cells.

2. Materials and methods

Bio-Gel P-2 (fine, 40–90 μm) was procured from Bio-Rad Laboratories, Inc., Hercules, CA. Arabinogalactan (A.Gal; from larch wood), LPS from *E. coli*, phorbol 12-myristate 13-acetate (PMA), zymosan, antibiotic and antimycotic solutions were products of Sigma-Aldrich Co., St. Louis, MO, USA. Fetal bovine serum (FBS), RPMI-1640 medium and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide A.R.] were products of HiMedia Laboratories Ltd., Mumbai, India. Inulin (pyrogen- and starch-free, approx. molecular mass 5000 Da; synonyms: dahlin, alantin) was obtained from ICN Pharmaceuticals, Inc., Cleveland, OH. Flat-bottom 96-well microtiter plates (gamma sterilized) were purchased from Tarsons Products Ltd., Kolkata, India. TLC plates (100-μ thick silica gel adsorbent with fluorescent indicator, Cat. No. 13181) were obtained from Eastman Kodak Co., Rochester, NY.

2.1. Experimental animals

Thymus and spleen were obtained from 8- to 10-week-old Swiss albino mice (25 ± 2 g) for the isolation of thymocytes and splenocytes, respectively; peritoneal exudate cells (PECs) were obtained from 12- to 14-week-old CFT Wistar rats (275 ± 2 g). All animals were housed and maintained on a standard commercial diet at ambient temperature in a clean environment as per the ethical guidelines. Following approval from the Institutional Animal Ethics Committee (IAEC Approval # 235/12), all experimental procedures involving the handling and caring of animals have been carried out in accordance with the ethical guidelines.

2.2. Preparation of soluble saccharides from onion

Onion bulbs (cv. Arka kalyan) were purchased from the local market. Saccharides were extracted from selected onion leaves (number 4 from outermost to inner youngest leaf of onion bulbs; outermost leaf is numbered 1) as described previously (Darbyshire & Henry, 1978). The selected leaf bases (100 g) were homogenized in 100 mL of 80% ethanol using a blender. The homogenate was placed in a water bath at 90 °C for 5 min, and then subjected to centrifugation at 7000 × *g* at 25 °C for 10 min. The pinkish supernatant was concentrated by flash evaporation and used for further studies. The yield of the soluble saccharides was found to be ~5.7 g/100 g onion fw.

2.3. Purification of onion saccharides and analysis by TLC

The mono-, di- and oligosaccharides present in the supernatant of hot 80% ethanol extract of onion were separated by using Bio-Gel P-2 column (0.6 × 110 cm). Triple distilled water was used as the eluant at a flow rate of 12 mL/h; 1 mL fractions were collected. All fractions were analyzed by phenol-sulfuric acid assay (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956) for neutral sugar content. The saccharide-containing fractions were concentrated for further studies and the total fructose content was measured by cold anthrone method (Van Handel, 1967).

The individual saccharides of onion fractionated by Bio-Gel P-2 were analyzed by TLC. The TLC chamber was saturated with solvent (propanol: ethyl acetate: water, 6:1:3 v/v) overnight. Samples were spotted on TLC plate and allowed to run until the solvent front reached the top edge of the plate, then allowed to dry using a hair drier. Finally, the chromatogram was developed using urea-phosphoric acid spray reagent (specific detection solution for glucose, fructose and fructans) and kept in the oven until the appearance of blue–gray coloured spots on the chromatogram as described earlier (Wise, Dimler, Davis, & Rist, 1955).

2.4. ESI–MS, ^1H NMR and ^{13}C NMR spectroscopy of onion FOS

The molecular mass of individual saccharide pools was determined by using Q-TOF Ultima Globa mass spectrometer (Waters Associates, Manchester, UK) instrument using positive electrospray ionization with the following operational conditions: capillary voltage 3.5 kV, core voltage 100 V, source temperature 80 °C, desolvation temperature 150 °C, core gas (argon) 35 L/h and desolvation gas (nitrogen) 500 L/h. The ESI–MS data were evaluated as per Matamoros Fernández, Obel, Scheller, and Roepstorff (2004), and the calculation is as follows: the molecular mass of the ESI–MS peak in Da = [molecular mass of monosaccharide × number of monomers] − [18 (H_2O) × number of glycosidic bonds] + [atomic mass of Na^+ (23) or K^+ (39) adduct].

^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded using a 500 MHz NMR instrument (Bruker Avans, Fallanden, Switzerland) equipped with a dual probe in the FT mode at 20 °C. The inulin and samples were prepared in D_2O at a concentration of 10 mg/mL.

2.5. Lymphocytes proliferation assay

The effect of onion FOS on cell proliferation or mitogenicity of murine splenocytes and thymocytes was assessed by MTT assay (Mosmann, 1983). The splenocytes and thymocytes were isolated from spleen and thymus, respectively, from 8- to 10-week-old Swiss albino mice (25 ± 2 g) as described earlier (Chandrashekar et al., 2011). Thymocytes were treated with a co-stimulatory molecule like LPS (0.05 μg/mL) before plating the cells and splenocytes were plated without LPS. The thymocytes or splenocytes

(2×10^6 cells/mL) were cultured with various concentration of onion FOS in 80% humidified atmosphere containing 5% CO_2 in a CO_2 incubator at 37 °C for 24 h and 72 h. After incubation, 20 μL of 5 mg/mL MTT reagent were added and allowed to incubate for 4 h. Crystals were observed under a microscope; in order to dissolve the purple crystals, 100 μL of 0.04 N acidified isopropanol were added and incubated at −20 °C for 10 min. Finally, the microtiter plate was centrifuged at 750 × *g* at 4 °C for 15 min to remove the precipitated proteins and cells. The supernatant was transferred to another 96-well microtitre plate, and the absorbance at 570 nm was measured in an ELISA reader (model 680, Bio-Rad Laboratories, Hercules, CA).

2.6. Determination of nitric oxide (NO) release from rat PECs

The effect of onion FOS on the function of macrophages was assessed by quantification of nitric oxide (NO) production as described earlier (Clement, Pramod, & Venkatesh, 2010). NO is a unique secondary gaseous signalling molecule derived from L-arginine by the action of NO synthase. NO is produced by macrophages, and is involved in inflammation. NO release can be used as a quantitative index of macrophage activation. Since NO is very unstable, it gets converted into nitrite in an oxidized environment. Therefore, quantification of nitrite is an indicator for the release of NO in to the cell culture media.

Murine peritoneal macrophages were isolated from adult male Wistar rats (12-week-old weighing ~250–300 g) following the standard procedure (Clement et al., 2010). The peritoneal macrophages (2×10^6 cells/mL) were cultured with positive controls (LPS and A.Gal) and sample (onion FOS) at various concentrations in 80% humidified atmosphere containing 5% CO_2 in a CO_2 incubator at 37 °C for 24 h and 72 h. The culture supernatant was treated with 5% $ZnSO_4$ (to remove the proteins and dead cells which interfere in absorbance), vortexed for 1 min, centrifuged at 10,000 × *g* at 4 °C for 10 min, and 100 μL supernatant was transferred to different wells of the 96-well plate. Each well received an equal volume of Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% *o*-phosphoric acid) followed by incubation at room temperature for 10 min. The absorbance of pink coloured azo-derivative was measured using a microplate reader at 540 nm. The concentration of NO was determined using sodium nitrite as a standard.

2.7. Measurement of phagocytic activity

Phagocytosis assay was performed according to the method described by Roy and Rai (2009). The collected murine PECs were suspended in complete medium, poured on a clean surgical slide (cell density: 1×10^6 cells/mL). Macrophages were incubated with various concentrations of onion FOS for 90 min. Following the activation of macrophages, slides were washed twice with PBS, and yeast cells with density of 1×10^8 cells/mL were added and kept for 90 min. After incubation, slides were subjected to differential Giemsa stain and observed under microscope; at least 15 fields were counted for each slide. The phagocytic index was determined by calculating the average number of yeast cells engulfed by or adhering to macrophages. The phagocytic index for control was taken as 100% phagocytosis.

2.8. Quantification of intracellular free radicals in murine PECs

The production of intracellular free radicals is a characteristic phenomenon in activated macrophages. The quantification of intracellular free radicals was followed by nitroblue tetrazolium (NBT) assay as described (Choi, Kim, Cha, & Kim, 2006). Murine PECs (5×10^5 cells/well) were seeded in 96-well plate in the presence of positive controls (PMA, LPS), sample (onion FOS) and saturated

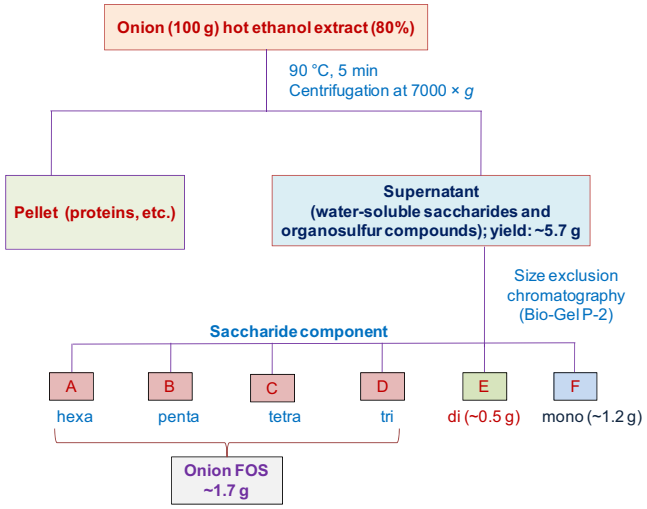

Fig. 1. Flow chart for the isolation of onion FOS from onion bulbs. Components A (hexasaccharide) to D (trisaccharide) were pooled as onion FOS for all the experiments described in this study. The yield of onion FOS (DP 3–6) is ~1.7 g/100 g onion.

Y–NBT (yellow coloured–nitroblue tetrazolium) for 45 min at 80% humidified atmosphere containing 5% CO_2 at 37 °C in cell culture incubator. Next, cells were washed with warm PBS to remove extracellular NBT crystals, and then once with methanol to fix the cells and allowed to air dry. Intracellular NBT deposits were dissolved by adding 120 μL of 2 M KOH and 140 μL of DMSO. The NBT crystals were dissolved at 25 °C for 10 min by shaking the plate. Absorbance at 620 nm was measured using a microplate reader.

2.9. Statistical analysis

All assays were performed in triplicate. The statistical analysis was done using Prism 5 software (Graph Pad, San Diego, CA). Single 'ANOVA' (analysis of variance) was done to determine the statistical significance. A p value of <0.05 was taken as statistically significant.

3. Results

3.1. Separation of saccharide components from onion extracted with hot 80% ethanol

The extraction and separation of saccharides from onion are depicted as a flow chart in Fig. 1. Among *Allium* species, fructans are found to be the major component in the category of water–soluble carbohydrates. Onion bulbs were found to contain 5.7 ± 0.2% neutral sugars and 4.2 ± 0.2% fructose and fructose-containing saccharides (fresh weight basis). Mono-, di- and oligosaccharides present in the supernatant of hot ethanol extract (80%) of onion were separated by size exclusion chromatography on Bio-Gel P-2, and the elution profile is shown in Fig. S1, panel a (Supplementary data). The eluted fractions were analyzed by phenol–sulfuric acid assay. The yield and percentage of onion saccharides were found to be as follows: monosaccharides, ~1.2 g (~21%); disaccharides, ~0.5 g (~8.7%); oligosaccharides DP 3–6, ~1.7 g (~30%). The separated component pools were analyzed by TLC, and the components were visualized using urea–phosphoric acid reagent which indicated the presence of individual oligosaccharides (Fig. S1, panel b; Supplementary data).

The molecular mass (in Da) of the individual pools in sodiated form determined by ESI (positive)—mass spectrometry are as follows (Fig. S2 of Supplementary data): component F, 203.35 (monosaccharide; 219.2 for potassium adduct); component E,

Table 1
^{13}C NMR spectra assignments[a] (δ in ppm) for standard inulin, onion DP 3 and DP 4.

	Inulin	Onion DP 3	Onion DP 4
(→ 1)-Fru*f*-(2→)			
C-1	60.6	61.1	60.1
C-2	102.9	103.4	103.4
C-3	76.7	76.5	76.5
C-4	74.0	74.1	74.1
C-5	80.8	81.0	80.8
C-6	61.9	62.1	61.9
Fru*f*-(2→)			
C-1		60.6	–
C-2		102.9	–
C-3		76.3	–
C-4		74.0	–
C-5		80.8	–
C-6		62.0	–
α-D-*Glc*-(1→)			
C-1	92.3	91.7 (92.2)	91.7
C-2	–	71.2	–
C-3	73.8	73.6	70.8
C-4	70.9	70.8	68.9
C-5	72.2	72.2	72.1

Note: "–" indicates not assigned.
[a] Spectrum was recorded in D_2O at 20 °C.

365.16 (disaccharide); component D, 527.25 (trisaccharide); component C, 689.32 (tetrasaccharide); component B, 851.42 (pentasaccharide) and component A, 1013.46 (hexasaccharide). Components A to D were pooled and designated as onion FOS, and used for all the studies described herein.

3.2. NMR analysis of onion FOS indicates predominantly β-(2 → 1) linkage

The chemical shift values and the relative intensities of ^{1}H NMR and ^{13}C NMR signals (spectra not shown) of C-2 atoms from onion FOS showed δ values of 103.4, 102.9 and 102.7, which confirmed the presence of fructofuranosyl residues. The appearance of different signals for C-2 of β-D-fructofuranosyl residues showed δ 102.7 which is typical for C-2 of (2 → 1) linked residues, those at δ 103.4 and 102.9 are assigned for terminal and branched fructose residues, respectively. The ratio of these intensities indicates that onion FOS consists of 70% (2 → 1) terminal fructose units, ≥20% (2 → 1) linked fructose units and very less branched β-D-fructofuranosyl residues. The presence of unusual β-D-glucopyranosyl units in abundance was seen at δ 95.6 compared to α-D-glucopyranosyl units (δ 92.2, 91.8 and 91.7); this indicates that β-D-glucopyranosyl units occur at the non-reducing end of fructo-oligosaccharides in onion.

The deduced structures of standard inulin and purified onion oligosaccharides (DP 3 and DP 4) as analyzed by ^{13}C NMR (Fig. 2, panels a–c) showed respective chemical shift values, and confirmed the identity of DP 3 and DP 4 as 1-kestose and nystose, respectively (Table 1). DP 3 oligosaccharide consists of two units of fructose moieties linked by β-(2 → 1) linkage depicting the typical structure of 1-kestose, and is indicated by mass signal data *m*/*z* 527 $[M + Na]^+$. DP 4 is composed of one terminal reducing fructose moiety linked by β-(2 → 1) linkage and a non-reducing α-D-glucopyranosyl unit. The chemical shift values of C-2 at δ 103.4, and C-3, C-4, C-5, C-6 and C-1 at δ 76.5, 74.1, 80.8, 61.9 and 60.1, respectively, confirmed DP 4 as nystose and has a mass signal *m*/*z* 689.

3.3. Mitogenic activity of onion FOS towards murine splenocytes

The effect of onion FOS on cell proliferation of murine splenocytes was studied in vitro, and the results are shown in Fig. 3 The

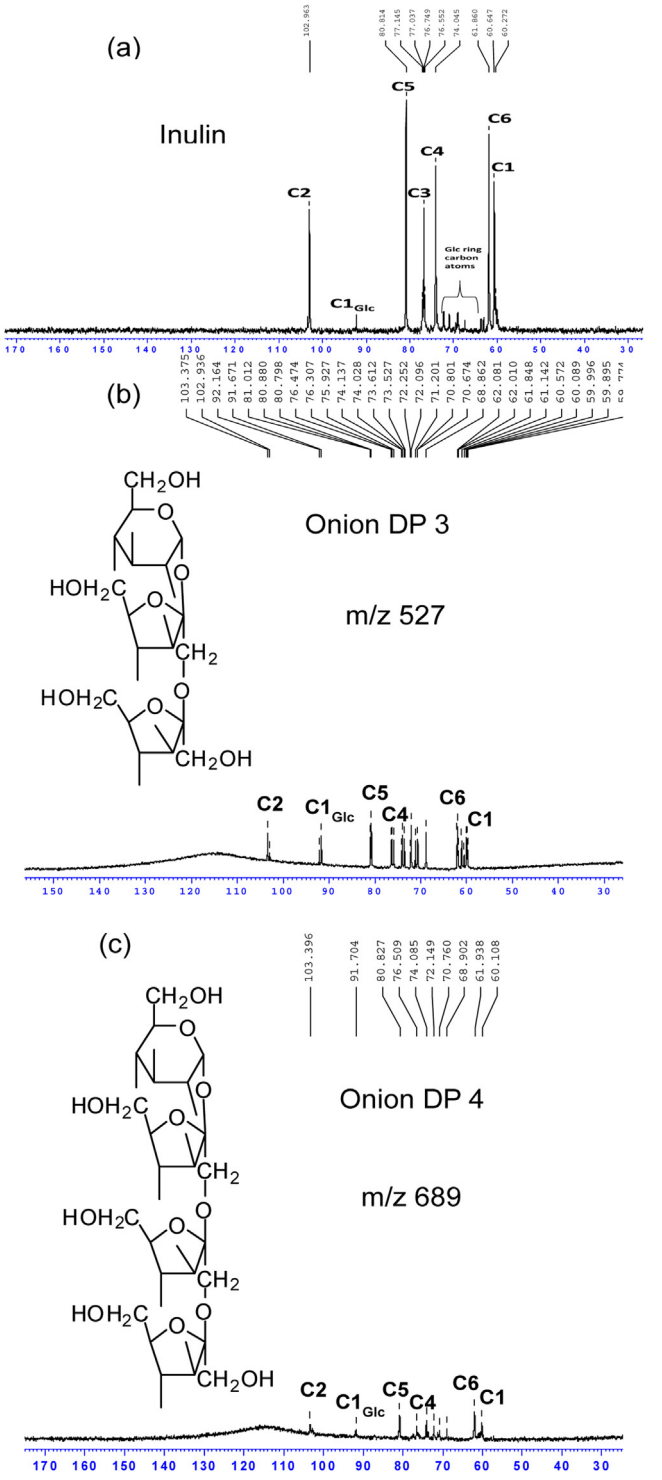

Fig. 2. ^{13}C NMR spectra of (a) inulin (approx. molecular mass 5000 Da), (b) onion FOS trisaccharide (DP 3) and (c) onion FOS tetrasaccharide (DP 4) in D_2O. Onion FOS DP 3 and DP 4 were obtained from Bio-Gel P-2 chromatography of onion saccharides shown in Fig. S1, panel a (Supplementary data).

well-known polysaccharide mitogens like arabinoagalactan (A.Gal) and zymosan were used as positive reference controls. Onion FOS at concentrations of 5 and 50 μg/mL showed a significant increase (2.5–3.5-fold) in mitogenic activity of murine splenocytes when compared to untreated cells after 24 h incubation (Fig. 3, panel a). However, at 72 h, onion FOS showed only 1.7–2.2-fold increase in mitogenic activity compared to untreated cells (Fig. 3, panel b).

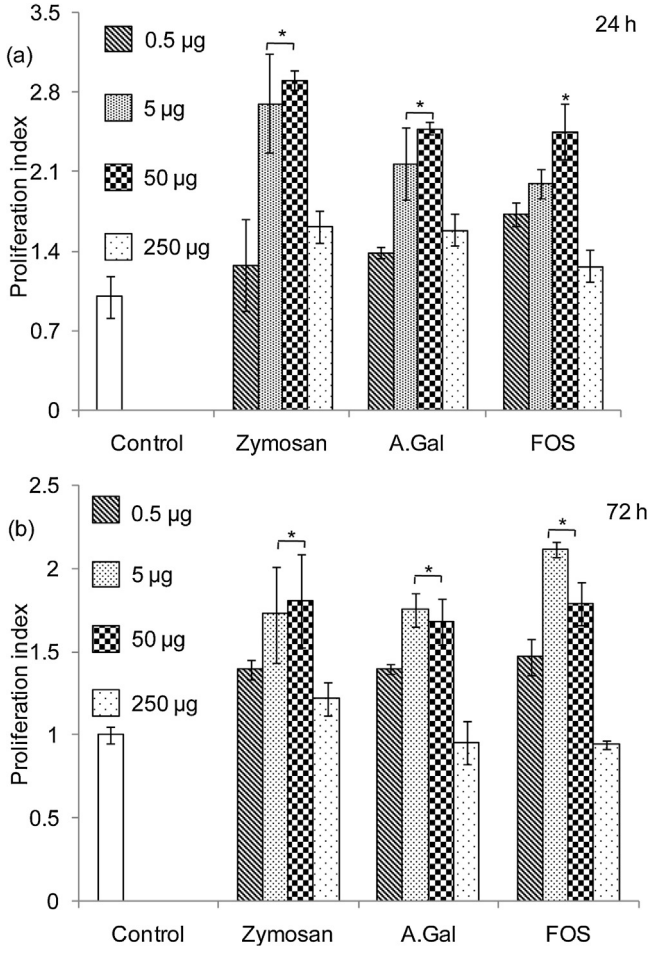

Fig. 3. Mitogenic activity of onion FOS towards mouse splenocytes (2×10^6 cells/mL) at various concentrations (0.5, 5, 50 and 250 μg/mL) at 24 h (panel a) and 72 h (panel b). A.Gal (arabinogalactan) and zymosan served as positive polysaccharide controls for mitogenicity. Each value is represented as mean ± S.D ($n = 3$). Level of significance: * $p < 0.001$ compared to unstimulated cells (Control).

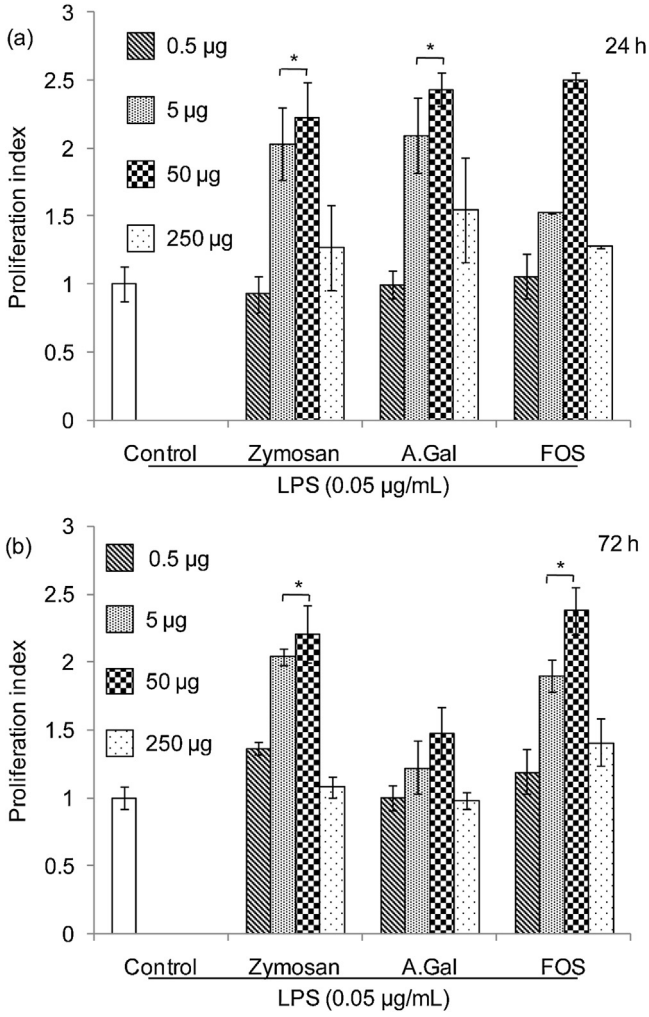

Fig. 4. Mitogenic activity of onion FOS towards mouse thymocytes (2×10^6 cells/mL) at various concentrations (0.5, 5, 50 and 250 μg/mL) in the presence of co-stimulatory molecule (50 ng/mL LPS) at 24 h (panel a) and 72 h (panel b). A.Gal (arabinogalactan) and zymosan served as positive polysaccharide controls for mitogenicity. Each value is represented as mean ± S.D ($n = 3$). Level of significance: * $p < 0.001$ compared to unstimulated cells (Control).

3.4. Onion FOS also display mitogenic activity towards murine thymocytes

Murine thymocytes were subjected to various concentrations of onion FOS and the results are shown in Fig. 4. Positive controls (A.Gal and zymosan) and sample (onion FOS) showed a 2–2.5 fold increase in mitogenic activity at 5 and 50 μg/mL concentrations compared to control (in the presence of co-stimulatory molecule LPS at 50 ng/mL) at 24 h incubation (Fig. 4, panel a). The mitogenic activity of onion FOS after 72 h incubation with thymocytes also showed a significant (1.8–2.3-fold) increase in cell proliferation compared to control cells (in the presence of 50 ng/mL LPS) (Fig. 4, panel b). Both the lowest (0.5 μg/mL) and highest (250 μg/mL) concentrations of A.Gal and zymosan (known immune-stimulators) and onion FOS showed only a marginal increase in proliferation activity towards splenocytes and thymocytes compared to control after 24 h and 72 h incubation (Figs. 3 and 4).

3.5. Onion FOS activate murine peritoneal macrophages to release NO

NO is one of the important mediators to promote the inflammation in order to eliminate the antigen. In the present study, the release of NO by murine peritoneal macrophages upon incubation with different concentrations of onion FOS for various periods is shown in Fig. 5. PECs incubated with A.Gal and onion FOS at concentrations of 5 μg/mL and 50 μg/mL showed a significant increase (~2.5-fold) in the production of NO compared to untreated cells after 24 h as depicted in Fig. 5, panel a.

The effect of onion FOS on NO production by PECs upon 72 h incubation is shown in Fig. 5 (panel b). A.Gal and onion FOS show similar responses in the production of NO. At concentrations of 5 μg/mL and 50 μg/mL of A.Gal and onion FOS, ~3-fold increase in NO production compared to untreated cells is seen. However, LPS induced ~3.5-fold increase compared to untreated cells at 5 μg/mL concentration at both 24 h and 72 h (Fig. 5, panels a and b). As seen at 24 h, the 72 h incubation with PECs at the lowest (0.5 μg/mL) and highest (250 μg/mL) concentration of LPS, A.Gal and onion FOS showed a similar magnitude of NO production when compared to untreated cells. Overall, the pattern of onion FOS-induced release of NO by PECs is similar to their effect on lymphocyte cell proliferation.

3.6. Onion FOS significantly stimulate the phagocytic activity of murine PECs

The effect of onion FOS on phagocytosis of yeast cells by murine PECs is shown in Fig. 6. Onion FOS significantly induces, in a

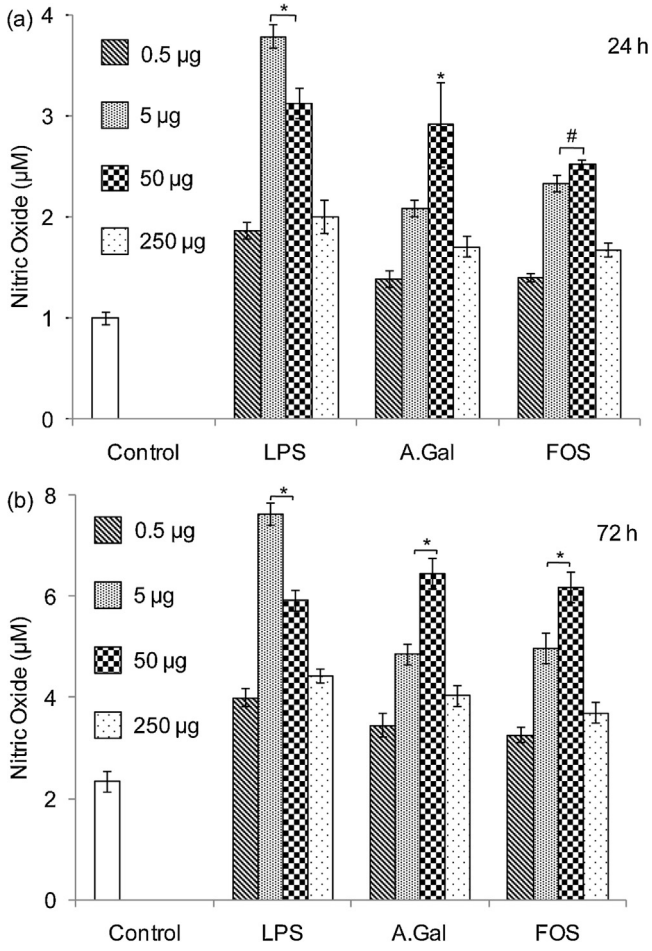

Fig. 5. Effect of onion FOS on production of NO by rat peritoneal exudates cells in vitro at various concentrations (0.5, 5, 50 and 250 μg/mL) for 24 h (panel a) and 72 h (panel b). NO production was measured using Griess reagent (absorbance at 540 nm). Each value represents the mean ± S.D ($n = 3$). * $p < 0.001$ vs. control; # $p < 0.005$ vs. control.

dose-dependent manner, the phagocytosis of ghost yeast cells by murine peritoneal exudates cells. The percentage of phagocytosis is proportional to the amount of onion FOS tested in the range 5 to 250 μg.

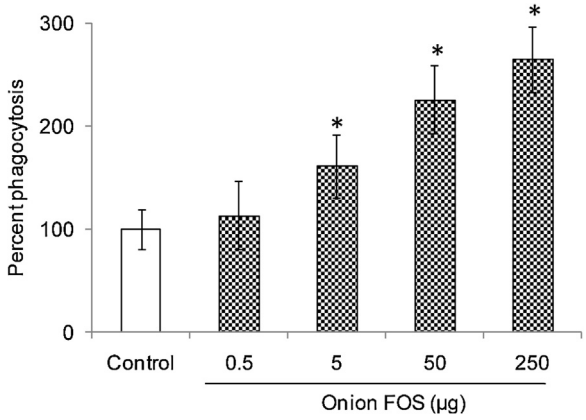

Fig. 6. Effect of onion FOS at different concentrations on the phagocytic activity of rat peritoneal macrophages. The phagocytic index for control is taken as 100% phagocytosis. Phagocytosis by onion FOS is significantly higher (*$p < 0.001$) compared to control cells.

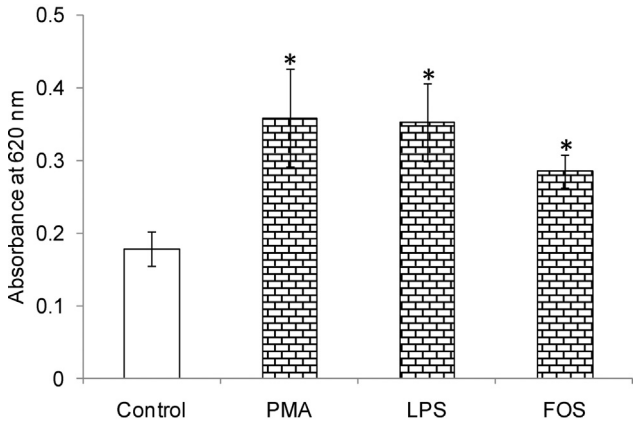

Fig. 7. Effect of onion FOS on the production of intracellular free radicals by murine PECs. Murine PECs were incubated with PMA (0.5 ng/mL), LPS (1 μg/mL), FOS (10 μg/mL) for 45 min. * $p < 0.001$ compared to Control.

3.7. Onion FOS induce intracellular free radicals by murine PECs

Phagocytes undergo oxidative respiratory burst in response to various stimuli resulting in the production of superoxide anion (O_2^-) and other reactive oxygen species, which serve as inflammatory mediators in killing the invading pathogens. The effect of onion FOS on intracellular free radicals production by murine PECs is shown in Fig. 7. Onion FOS, at 10 μg/mL, induce a significant ~2-fold increase compared to unstimulated cells in the production of intracellular free radicals by murine PECs, similar to those observed in the case of PMA and LPS, which are used as positive reference controls.

4. Discussion

Various immunostimulators (synthetic molecules or cytokines) are known to reverse the suppressed immune system in immunosuppressive diseases to normal/healthy state. Although these immunostimulators are known to be effective, they also cause considerable side effects. However, some natural dietary components which have the capability to stimulate the suppressed immune system could be ideal candidates to boost the immune system, since they are derived from natural sources (Wichers, 2009).

Onion is commonly used as a spice/vegetable in culinary preparations worldwide. In this study, onion bulbs were found to contain ~5.7% total sugars of which ~4.2% is fructose-containing saccharides; these results compare well with the total carbohydrate content of ~5.5% (fw basis) reported previously (Yaguchi et al., 2008). ESI–MS analysis of onion FOS clearly indicated the presence of well-resolved oligosaccharides up to DP 6, similar to earlier reports on onion FOS with DP ranging from 3 to 11 (Benkeblia, 2013). Onion FOS (DP 3–6) was free of mono-/disaccharides and organosulfur compounds. Studies in the last decade have demonstrated that fructans exhibit prebiotic activity (Watzl et al., 2005). Prebiotic inulin-type fructans elicit additional direct effects such as immunomodulation along the gastrointestinal tract (Jeurink, van Esch, Rijnierse, Garssen, & Knippels, 2013; Delgado, Thomé, Gabriel, Tamashiro, & Pastore, 2012).

The immune system's response could be modulated by inulin-type fructans by an indirect mechanism through fermentation products of gut commensal bacteria or by a direct mechanism via interaction with toll-like receptors, membrane lipids, etc. of mostly innate immune cells (Vogt et al., 2013b). Further, one study has demonstrated that inulin-type fructans directly modulate the immune response through TLR activation (TLR2) in human PBMCs. The chain length of inulin-type fructans appeared to be

an important factor in the ability to switch between the induction of anti-inflammatory and pro-inflammatory cytokines (Vogt et al., 2013a).

In the field of cellular or clinical immunology, cell proliferation after antigenic activation is an important biological parameter used in the diagnosis of immunodeficiency. Several studies on low molecular weight fructans from dietary sources showed mitogenic activity towards murine lymphocytes (Kardosová et al., 2003). It can be concluded on the basis of our data that short chain inulin-type fructans (DP 3–6) promote the proliferation of splenocytes in a dose-dependent manner; a similar response was observed for thymocytes in the presence of LPS. Another study of fructans with DP ≥ 8 has shown increased growth of *Lactobacilli* which may contribute to the stimulation of cecal IgA secretion; further, IFN-γ and IL-10 production in the cecal $CD4^+$ T cells were enhanced solely by DP4 (Ito et al., 2011).

Macrophages are immune-responder cells that act as bridges between two major arms of the immune system, i.e., innate and adaptive immune response. Generally, activation of macrophages is assessed by production of NO and increased phagocytic activity (Kardosová et al., 2003; Clement et al., 2010). The results presented here suggest that onion FOS (DP 3–6) is a potent inducer of NO production from rat PECs similar to the reference polysaccharide, A.Gal; further, augmentation of the phagocytic ability of rat PECs was also observed. A similar study on fructans from aged garlic extract demonstrated that low molecular weight inulin-type fructans (DP ≤ 15) were found to be more potent inducers of NO production and phagocytosis by murine peritoneal macrophages than high molecular weight inulin-type fructans (DP ≥ 25) (Chandrashekar et al., 2011). The intracellular free radical production is an indicator of macrophage activation in response to a stimulus (James, Grinberg, & Swartz, 1998); onion FOS promotes enhancement of intracellular free radical production in murine PECs similar to PMA and LPS (positive controls). It would be interesting to investigate further the immunomodulatory properties of individual onion FOS and the molecular mechanism of immunomodulation.

5. Conclusions

Nearly 70% of the total neutral sugar content of onion is contributed by fructose, sucrose and FOS. The degree of polymerization of onion FOS ranges from DP 3 to 11; however, FOS comprising of tri- to hexasaccharide units have been pooled (yield: ~1.7 g/100 g onion fw) and used as onion FOS in this study. NMR studies revealed that onion FOS belongs to inulin-type of fructans with unusual β-D-*Glc*-terminal residue at the non-reducing end and has along with *(2 → 1)*-β-D-*fructofuranosyl* residues, *(→1)-Fruf-(2→)* linkage for trisaccharide (1-kestose), and α-D-*Glc-(1→)* terminal residue at the non-reducing end for tetrasaccharide (nystose). Onion FOS has lymphoproliferative activity for both murine splenocytes and thymocytes, and also activate the murine peritoneal macrophages by inducing the production of NO and intracellular free radicals; further, they also augment the phagocytic capability of murine peritoneal macrophages. In conclusion, this study demonstrates that onion FOS possess marked mitogenic activity and enhancement of phagocytic activity having potential utility in therapeutic immunomodulation, because they are derived from a safe dietary source consumed widely since ages.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.09.039.

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