Isolation, structural elucidation and immunomodulatory activity of fructans from aged garlic extract

Puthanapura M. Chandrashekar a, Keelara V. Harish Prashanth b, Yeldur P. Venkatesh a,∗

Abstract

Traditionally, garlic (Allium sativum) is known to be a significant immune booster. Aged garlic extract (AGE) possesses superior immunomodulatory effects than raw garlic; these effects are attributed to the transformed organosulfur compounds. AGE is also known to contain fructans; the amount of fructans in AGE represents a small fraction (0.22%) of the total fructans in raw garlic. In order to evaluate the biological activity of fructans present in AGE, both high molecular weight (>3.5 kDa; HF) and low molecular weight (<3 kDa; LF) fructans were isolated. The structures of purified HF and LF from AGE determined by 1H NMR and 13C NMR spectroscopy revealed that both have (2→1)β-D-fructofuranosyl bonds linked to a terminal glucose at the non-reducing end and β-α-fructofuranosyl branching on its backbone. Biological activity of fructans was assessed by immunostimulatory activity using murine lymphocytes and peritoneal exudate cells (source of macrophages). Both HF and LF displayed mitogenic activity and activation of macrophages including phagocytosis. These activities were comparable to that of known polysaccharide immunomodulators such as zymosan and mannan. This study clearly demonstrates that garlic fructans also contribute to the immunomodulatory properties of AGE, and is the first such study on the biological effects of garlic fructans.

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1. Introduction

Garlic (Allium sativum L.) belongs to the botanic family of Liliaceae. It contains water (62–68%), carbohydrate (26–30%), protein (1.5–2.1%), amino acids (1–1.5%), organosulfur compounds (1.1–3.5%), and fiber (1.5%), all based on fresh weight (Koch and Lawson, 1996). Carbohydrates are the most abundant class of compounds present in garlic bulbs and account for about 77% of the dry weight. The majority of the carbohydrate material in garlic cloves, as well as in other Allium species, consists of water-soluble fructose polymers called fructans or fructosans (Koch and Lawson, 1996). It has been established that approximately 65% of the dry weight of garlic consists of fructans; hence, fructans constitute ~84% of the carbohydrate content of garlic (Lawson and Wang, 1995). Most of the research on garlic carbohydrates has been related to the types of sugars and oligosaccharides, and their structural characterization (Darbyshire and Henry, 1981; Losso and Nakai, 1997; Baumgartner et al., 2000; Tsukamoto et al., 2008).

Fructans are widely distributed as carbohydrate storage polymers in the vegetative tissue of many families of plants, bacteria, and fungi (Hosono et al., 2003). According to the type of linkage, fructans are classified into three families, namely, inulin [(2→1)-linked β-D-fructofuranosyl units], levan [(2→6)-linked β-α-fructofuranosyl units], and graminan [both (2→1)-linked and 2→6]-linked β-α-fructofuranosyl units] (Roberfroid, 2005).

The immune-modulating effects of prebiotics such as inulin or oligofructose have recently received much attention (Vos et al., 2007; Bosscher, 2009). They have been described as food components that can modulate various metabolic processes. Most investigations have been carried out with unprocessed chicory inulin or fructans derived thereof. Inulin has been described to activate murine macrophage cell line (RAW264.7) (Koo et al., 2003). Supplementation with either short-chain or long-chain fructooligosaccharides (FOS) in control rats resulted in enhanced IL-10 production in splenocyte culture, splenocyte natural killer (NK)-activity, and a decreased ratio of CD4+/CD8+ T-cells, as well as IL-10 production from rat Peyer's patches after mitogen stimulation (Manhart et al., 2003; Roll et al., 2004; Wu et al., 2006).
Fructans seem to be involved in the positive modulation of the immune system, mainly in an increased resistance to infections and microbicidal activity as well as by the reduction of allergic reactions and cancer in experimental models (Choque Delgado et al., 2010).

Garlic contains a mixture of fructooligosaccharides and fructopolysaccharides ranging in molecular mass from <1000 Da to ~6800 Da corresponding to degree of polymerization (DP) as high as 38 (Losso and Nakai, 1997). A high molecular weight fructan (DP ~ 58) with branching has been isolated from raw garlic extract (RGE) and the structure determined by enzymatic, chemical and spectroscopic (NMR) methods (Baumgartner et al., 2000). Both the structural analysis and biological activity of fructans are areas of intense research in the field of non-digestible oligosaccharides and polysaccharides (referred to as dietary fiber) from a variety of sources; they are natural constituents of many foods (Kelly, 1999; Paulsen, 2001; Block and Mead, 2003).

Aged garlic extract (AGE) is an odorless product prepared by prolonged aqueous extraction of fresh garlic for approximately 20 months; garlic and AGE have been reported to have an array of pharmacologic effects, including immunomodulation (Arunkumar et al., 2005; Biren et al., 2006; Bongiorno et al., 2008). AGE contains stable, water-soluble organosulfur compounds that have been thought to be the bioactive principles for numerous health benefits (Gardner et al., 2007). The majority of the immunomodulatory actions of garlic have been well studied using purified organosulfur compounds. Recently, it has been shown that the immunomodulatory effects exerted by garlic proteins are due to the presence of garlic lectins or agglutinins (ASA I and ASA II) in both aged garlic extract (Chandrashekar and Venkatesh, 2009) and raw garlic (Clement et al., 2010). Further, it has been shown that garlic lectins (ASA I and ASA II) are highly stable and immunogenic under in vitro and in vivo conditions (Clement and Venkatesh, 2010).

In view of the aged garlic extract’s role as an important biological response modifier, and the role of fructans as prebiotics in immune modulation, it appeared very interesting to isolate fructans from aged garlic extract, and to study their immunomodulatory effects on immunoresponder cells, namely, murine lymphocytes, and peritoneal exudates cells. Both high molecular weight fructans (>3.5 kDa; HF) and low molecular weight fructans (<3 kDa; LF) have been isolated and studied for their immunomodulation in vitro.

2. Results and discussion

2.1. Isolation of HF and LF from aged garlic extract

The isolation of HF and LF from aged garlic extract is shown as a flowchart in Fig. 1. AGE was subjected to ultrafiltration using 3 kDa membrane followed by dialysis of the retentate using 3.5 kDa cut-off dialysis membranes. Ion-exchange chromatography of ultrafiltration retentate was carried out on Q-Sepharose (anion-exchanger) at pH 8; the flow-through pool represents HF. The amount of diazylated ultrafiltration retentate obtained from ~300 mL of AGE (derived from 1 kg raw garlic) ranged from 1.18 to 1.36 g based on five preparations; this includes both the HF and the proteins originally present in AGE. The yield of HF was ~303 mg from AGE prepared using 1 kg raw garlic. In contrast to this, the amount of fructans obtained from raw garlic is approximately 138 g from 1 kg raw garlic; this is in agreement with the yield of fructans from raw garlic reported earlier (Losso and Nakai, 1997). Thus, only a very small amount of fructans is recovered in AGE (0.22%) compared to the amount present in raw garlic.

The ultrafiltrate of AGE was subjected to Bio-Gel P-2 chromatography and the chromatographic profile is shown in Fig. 2 (panel a). Analysis of the column fractions by cold anthrone assay reveals the presence of two components, namely, A and B. Since component A elutes after the void volume (22.5 mL), the eluted fructan appears to be a fructooligosaccharide (<1800 Da), hereafter referred to as low molecular weight fructans (LF); mercaptan odor was evident in the fractions comprising component A. An organosulfur compound was observed in traces upon TLC analysis of component A, as detected by iodoplatinate reagent (Fig. 2, panel c). Component B appears to contain free sucrone and fructose as detected by cold anthrone assay. Since sucrose is a non-reducing disaccharide made up of glucose and fructose, the hydrolyzed fructose will react with cold anthrone under the assay conditions. It has been reported that glucose (0.1% fresh weight), fructose (0.2% fresh weight), and sucrose (1.9% fresh weight) are the known mono- and disaccharides of garlic (Praznik et al., 2004). Besides these free sugars, component B contains some transformed organosulfur compounds as detected by the mercaptan odor and RP-HPLC analysis (Fig. 2, panel b); among the three peaks detected by HPLC, the component eluting at an RT of 5.1 min matches with that of diallyl sulfide. Further, a well-defined spot corresponding to an unknown organosulfur compound is seen in TLC analysis of component B (Fig. 2, panel c).

In this study, LF isolated from AGE appears to have a molecular mass of ~1800 Da based on the elution position from Bio-Gel P-2. It is interesting to note that an oligosaccharide of 1800 Da has recently been isolated from raw garlic extracts, and characterized as having 10 fructose units connected by β-(1 → 2)-linkage to a terminal glucose (Tsukamoto et al., 2008). SDS–PAGE (silver staining) analysis of HF, LF and component B purified from AGE do not show the presence of protein or peptides (data not shown).

2.2. 1H NMR and 13C NMR analyses

Although fructans have been shown to be present in AGE, its isolation and further characterization has not been attempted previously. In the present study, fructans have been isolated from AGE, and separated into two fractions using 3 kDa ultrafiltration – a high molecular weight (HF) and low molecular weight (LF). The 1H and 13C NMR spectra of inulin standard, isolated raw garlic fructans (RGF), and components purified from AGE (HF, LF and component B) are shown in Figs. 3 and 4, respectively. The corresponding proton and carbon atoms assignments are consistent with those previously achieved for inulin and garlic fructans (Tsukamoto et al., 2008; Spies et al., 1992), and the chemical shift data are given in
Table 1 for comparison. The $^{13}$C spectrum of HF contained four different signals for C-2 of $\beta$-D-fructofuranosyl residues (Fig. 4).
The signal at δ 102.9 is typical for C-2 of a (2→1)-linked residue, those at δ 103.5 and 103.4 are assigned to terminal and branched residues, respectively, and that at δ 103.7 to a (2→6)-linked residue. The ratios indicate that the component with the highest DP (n/C0 9) consists of 42% (2→1)-linked, 9% (2→6)-linked, 24% terminal and 21% branched b-D-fructofuranosyl residues. It may be noted here that a high molecular weight fructan, having a highest DP of 58 (n/C0 9) has been isolated and characterized from raw garlic; its structure bears resemblance to inulin backbone (Baumgartner et al., 2000). Our NMR results are in agreement with the structure of fructans from raw garlic (Baumgartner et al., 2000). Fructooligomers were not present in component B of AGE as indicated by 1H and 13C NMR spectra (Figs. 3 and 4).

The deduced structure of HF from AGE is depicted in Fig. 5. HF retained its branched structure intact and also its molecular size, but we observed slight downfield shift of HF for C-2 signals compared to RGF and LF. The significant difference between RGF and HF in the C-2 relative intensities indicates a higher degree of branching and crystalline nature of purified HF. The raw garlic and aged garlic fructans showed that the signals belong to a residue α-D-glucopyranosyl unit in 1-kestose form (C-1, δ 91.9) at the non-reducing end of the chain as suggested by previous reports for fructooligosaccharides from raw garlic (Tsukamoto et al., 2008; Fu, 2009) and for fructans from Pucinella peisonis (Spies et al., 1992). RGF showed little higher intensity signal for the same compared to HF. However, LF has group of three anomeric glucosyl signals (δ 92.3, 92.0, and 91.8) in very low intensities due to heterogeneity (Fig. 4). Darbyshire and Henry (1981) suggested that, in Allium, carbohydrate supply in the form of substrate sucrose and their respective transferases plays a central role in fructan synthesis, and control the ability of the plant to synthesize...
polymers having different degrees of polymerization and branching. From this study, it is inferred that the ageing process for the preparation of AGE alters the degree of branching, composition and degree of polymerization, and consequently, the functionality of the isolated fructans.

2.3. Mitogenic activity of purified fructans

The mitogenic activity of HF isolated from AGE towards mouse splenocytes was studied in vitro, and the results are shown in Fig. 6 (panel a). Zymosan and mannan were used as reference positives; a 2-fold increase in mitogenic activity is seen compared to untreated cells. HF shows 2.5–3.5-fold increase in mitogenic activity compared to untreated cells. Also, in the case of rat splenocytes, 2–3-fold increase in mitogenic activity is observed for HF, whereas other polysaccharides like arabinogalactan, galactan and galactomannan showed ~1.5-fold increase compared to untreated cells (Fig. 6, panel b).

LF significantly induced proliferation of murine splenocytes and thymocytes. At 5 µg/mL concentration, LF as well as certain polysaccharides (arabinogalactan and galactan) showed roughly 2–3-fold increase in mitogenic activity compared to unstimulated murine splenocytes (Fig. 7); however, at 1 and 10 µg/mL concentrations, LF showed only marginal increase in proliferation activity. Component B obtained from Bio-Gel P-2 chromatography does not show any lymphoproliferation (Fig. 7). When tested toward murine thymocytes, LF and other polysaccharides showed a 2–3-fold increase in mitogenic activity compared to unstimulated cells (data not shown). HF displayed mitogenic activity towards mouse lymphocytes and rat splenocytes compared to appropriate unstimulated cells. Such a magnitude of response is similar to or slightly better than that induced by zymosan or mannan, which are used as positive controls representing polysaccharide immunostimulators. LF also significantly induced proliferation of murine splenocytes at concentrations of 1 and 5 µg/mL. In the case of mouse thymocytes

Fig. 5. Elucidated chemical structure for HF from aged garlic extract.
too, LF produced somewhat similar response in mitogenic activity at the same concentrations. It is worth noting here that a low molecular weight fructofuranan of the inulin-type from the roots of Arctium lappa (a Compositae member used as an edible vegetable in the Orient) was found to exhibit mitogenicity and comitogenicity comparable to that of the commercial zymosan immunomodulator (Spies et al., 1992; Kardošová et al., 2003). Further, a fructan from Radix ophiopogonis has also been shown to stimulate the proliferation of cultured lymphocytes (Wu et al., 2006).

2.4. Effect of garlic fructans on activation of macrophages

The release of nitric oxide (NO) from rat peritoneal exudate cells (PECs: used as a source of macrophages) upon incubation with aged garlic fructans or other polysaccharides is shown in Fig. 8. NO is an important signaling molecule synthesized from l-arginine by NO synthase. NO is known to play a key role during the course of infections (Coleman, 2001). HF shows a 4-fold increase in NO release compared to untreated cells at two different concentrations tested, namely, 1 and 5 µg/ml. (Fig. 8, panel a). The release of NO from rat PECs in the presence of LF and peak B component was measured at 24 and 48 h. At 24 h of incubation, both LF and peak B showed a similar response compared to lipopolysaccharides (LPS) and arabinogalactan (Fig. 8, panel b). Both LF and peak B component, at a concentration of 1 µg/ml, release NO to the same extent as LPS and arabinogalactan (Fig. 8, panel c) at 48 h; only LF, but not peak B component was active at 5 µg/ml (Fig. 8, panel c). HF showed activation of macrophages present in rat PECs compared to untreated cells, as seen by NO release. Similarly, LF also produced similar increase in NO release at 1 µg/ml concentration, while at 5 µg/ml concentration LF produced much higher increase in NO release. NO release could be ascertained as early as 12 h of incubation. Overall, LF produced a similar magnitude of response compared to HF in both mitogenic activation and NO release. The NO release seen with peak B component (Fig. 8, panels b and c) may be contributed by an organosulfur compound or other small molecules present.

Macrophages are considered the pivotal immunocytes of the host defense. Many polysaccharides from plants have been shown to activate macrophages by binding to the receptors on the surface of immune cells (Schepetkin and Quinn, 2006). It is interesting to note in this context that inulin from other sources also stimulates NO synthesis in a macrophage cell line (Koo et al., 2003). It is known that macrophages can interact with botanical polysaccharides and/or glycoproteins via toll-like receptor 4 (TLR4), complement receptor 3, the scavenger receptor, dectin-1 and the mannose receptor (Schepetkin and Quinn, 2006). Our results on NO release from rat PECs are similar to those seen with other polysaccharide immunomodulators (Vos et al., 2007; Schepetkin and Quinn, 2006), demonstrating the immunostimulatory property of garlic fructans. In the present study, we have shown that garlic fructans stimulated macrophages (present in rat PECs) to produce NO, which is important for the cytotoxic activity exerted by macrophages.

2.5. Effect of garlic fructans on the phagocytic activity

The present study also examined the effect of garlic fructans on the phagocytic ability of macrophages present in rat peritoneal exudate cells. Percent phagocytosis was significantly higher (p < 0.001) for LF (fructooligosaccharides) sample isolated from AGE compared to control. However, phagocytic activity was less significant (p < 0.01) in HF and component B samples obtained from chromatographic separation of AGE (Fig. 9). Thus, it is clear that garlic fructans activate macrophages to induce yeast cell phagocytosis.

Based on the immunomodulatory effects of garlic fructans described above, they appear to be potent T-cell and B-cell stimulators similar to other bioactive α-(1 → 4), (1 → 6)-glucans isolated from medicinal plants such as Aloe vera and guduchi (Tinospora...
research on garlic fructans may throw light on the underlying mechanisms of immunomodulation and should aid in identifying potential uses of garlic fructans in various therapeutic applications.

4. Experimental

4.1. Materials

Q-Sepharose FF anion exchange resin (bead size: 24–44 μm), Bio-Gel P-2 and Bio-Gel P-6 resin, D2O (99.9 atom% D), arabinogalactan (from larch wood), galactan (from gum arabic), galactomannan (locust bean gum from Ceratonia siliqua seeds), lipopolysaccharide (LPS), zymosan, mammalian allyl methyl sulfide (AMS), allyl sulfide or allyl disulfide (ADS), diallyl disulfide (DADS), L-methionine (Met), chloroplatinic acid hexahydrate (ACS reagent; >37.5% Pt basis) and RPMI-1640 medium were products of Sigma–Aldrich Co., St. Louis, MO, USA. Inulin (from chicory root; mol. wt. ~5000) was obtained from ICN Pharmaceuticals Inc., Cleveland, OH, USA. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazo- lium bromide A.R.] was a product of HiMedia Laboratories Ltd., Mumbai, India. Fetal bovine serum (FBS) was obtained from Sera-Lab (Sussex, England). Flat-bottom 96-well microtiter plates (MIRCROM) were purchased from Greiner Bio-One GmbH, Frickenhausen, Germany. Tissue culture plates (24-well, gammastereilized) were procured from Tarsons Products Ltd., Kolkata, India. Garlic bulbs were obtained from local grocery. All other chemicals and reagents used were of analytical grade.

4.2. Experimental animals

Thymus was obtained from 8 to 10-week-old albino and BALB/c mice (25 ± 2 g) for the isolation of thymocytes; spleen was obtained from 12 to 14-week-old C57/129 rats (275 ± 2 g) for the isolation of splenocytes. 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), all experimental procedures involving the handling and caring of animals have been carried out in accordance with the ethical guidelines.

4.3. Isolation of fructans from raw and aged garlic extracts

One hundred grams of raw garlic was homogenized using 10 mM Tris–HCl buffer, pH 8, centrifuged, and filtered through Whatman No. 1 filter paper. The resultant filtrate was adjusted to pH 8, and then passed through Q-Sepharose FF column equilibrated with 10 mM Tris–HCl buffer, pH 8. The flow-through from Q-Sepharose was collected and lyophilized. The lyophilized material represents RGF, and the yield of fructans from raw garlic was calculated after correcting for the weight contributed by the buffer.

Aged garlic extract was prepared by extracting fresh garlic in 25% ethanol for a period of 20 months as described by Hirao et al. (1987). One hundred milliliters of decanted solution was lyophilized to powder form and stored at −20 °C. It was envisaged to remove the low molecular weight organosulfur compounds by ultrafiltration using 3 kDa cut-off membrane disc filter (OMEGA 3K, Pall Life Sciences, Ann Arbor, MI, USA) in a Amicon stirred ultrafiltration cell (Model 8050; Millipore Corporation, Bedford, MA, USA), and the retentate was dialyzed using 3.5 kDa cut-off dialysis membranes. AGE (lyophilized material) was subjected to Q-Sepharose FF (0.8 × 8 cm) chromatography, and the flow-through from this ion-exchanger was collected, lyophilized to fine powder and stored at −20 °C; this is referred to as Pool-3, and has been identified as fructose-containing polysaccharide (HF) based on the positive reaction
with cold anthrone reagent (Van Handel, 1967). The flowchart for the isolation of HF and LF from aged garlic extract is shown in Fig. 1.

4.4. Detection of fructose or fructose-containing saccharides

Cold anthrone assay is used for the determination of fructose and fructose-yielding carbohydrates (Van Handel, 1967). The reagent was prepared fresh by dissolving 150 mg anthrone in 100 mL of 71.7% sulfuric acid. Since the assay reagent consists of concentrated sulfuric acid, any disaccharide/oligosaccharide and polysaccharides containing fructose will be hydrolyzed to yield free fructose under the assay conditions. Each fraction was diluted to 50 μL with water, mixed with 1.5 mL cold anthrone reagent, and incubated at 25 °C for 1–1.5 h. The absorbance was read at 620 nm.

4.5. Purification of garlic LF by size exclusion chromatography (SEC)

Pool P-1 obtained by ultrafiltration of aged garlic extract using 3 kDa membrane was concentrated by lyophilization. Fifteen milligrams of this material was subjected to size exclusion chromatography on Bio-Gel P-2 column (0.9 x 106 cm; bed volume: 67.5 mL; exclusion limit: 1800 Da for dextrans) using water as the eluant. Fructose or fructose-containing oligosaccharides were detected by cold anthrone assay (Van Handel, 1967).

4.6. HPLC analysis (reverse-phase)

Analytical reverse-phase HPLC analysis was carried out using a GraceSmart C18 column (4.6 mm x 250 mm, 5 μm) in a Shimadzu LC-6A HPLC system. The sample was eluted using methanol/water (50/50) with a flow rate of 1 mL/min.

4.7. Detection of organosulfur compounds in LF

Bio-Gel P-2 components A and B were analyzed by TLC on Silica gel 60 F254 plate (20 x 20 cm; E. Merck AG, Darmstadt, Germany) using the solvent system methanol/glacial acetic acid/12 N HCl/water (80:10:2:18, v/v). Briefly, 5 μL of comp. A, and comp. B (lyophilized material dissolved in water to 1 μg/μL concentration) were spotted onto the TLC plate. The sulfur-containing amino acid methionine, and allyl sulfides were used as positive controls (~1 μg). The chromatograms were dried in a stream of cold air. The modified iodoplatinate spray reagent was prepared by mixing 0.1 mL of aqueous 10% chloroplatinic acid, 7 mL of 1.1% potassium iodide solution, and 7 mL of water, to which 80 mL of 0.5% starch solution was added immediately prior to spraying the TLC plate (Njaa, 1963). Organosulfur compounds appear as clear spots on a pink background. All organosulfur compounds with the exception of R-SO3H and R-S-SO3H are detected in the iodoplatinate reaction (Cavallini et al., 1959).

4.8. 1H NMR and 13C NMR spectroscopy

1H and 13C 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 samples were prepared in DMSO-d6 to a concentration of 20 mg/mL.

4.9. Preparation of cells from spleen and thymus

The mice and rat were sacrificed by cervical amputation under light anesthesia; spleen and thymus were removed using aseptic techniques. The organs were passed through a sterilized stainless steel sieve (150 mesh) to obtain a single-cell suspension. Erythrocytes were destroyed by 0.85% NH4Cl/20 mM Tris–HCl buffer and the remaining cells were washed twice with PBS. The cells were suspended in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL streptomycin at a density of 2 x 10⁶ cells/mL.

4.10. Cell count and determination of viability

The isolated thymocytes and splenocytes from mice and rat were counted using Trypan blue (0.2%) stain. An aliquot (10 μL) of cell suspension was taken and diluted with 250 μL of diluent buffer (PBS with 1% BSA) to which 10 μL of Trypan blue stain was added. The mixture was charged to an improved Neubauer (0.1 mm deep) Bright-Line hemocytometer (Reichert Scientific Instruments, Buffalo, NY, USA) using a clean fine pipette tip. The cells were observed under eyepiece (10× and 40×) and counted in the outer four chambers of the hemocytometer. The cell concentration was adjusted to 1 x 10⁶ cells/mL in the case of thymocytes or 2 x 10⁶ cells/mL in the case of splenocytes, and used for cell proliferation assay.

Percentage viability of thymocytes and splenocytes in the prepared cell suspension was checked by Trypan blue exclusion method as described earlier (Chandrashekar and Venkatesh, 2009; Clement et al., 2010). For cell viability determination, an aliquot of cell suspension was taken and mixed with an equal volume of 0.2% Trypan blue, and kept at 25 °C for 10 min. The cell suspension with Trypan blue was charged to a hemocytometer and observed under microscope. Cells, which are dead or partially damaged, appear as dark blue against a light blue background, since they take up the dye. The viable cells appear clear without any stain against the light blue background.

4.11. Measurement of cell proliferation

Cell proliferation or mitogenicity was measured by MTT assay (Mosmann, 1983). Splenocytes and thymocytes (2 x 10⁶ and 1 x 10⁶ cells/mL, respectively) were cultured with samples (1, 5 or 10 μg/mL), and polysaccharide mitogens, i.e., arabinogalactan, galactan, galactomannan, zymosan and mannan (1, 5, or 10 μg/mL) in 96-well plates for 3 days at 37 °C humidified atmosphere of 5% CO2 in a incubator. Four hours before culture termination, 20 μL of 5 mg/mL MTT solution (MTT dissolved in 0.1 M Tris-buffered saline and filtered to remove any insoluble matter) was added and incubated for an additional 4 h under the same conditions. After removing the culture plates, the samples were centrifuged at 750g at 4 °C for 15 min. Supernatant was removed and the blue formazan crystals were resolubilized in 200 μL of acidic isopropanol (0.04 N HCl) under agitation. After dissolving the crystals, 100 μL of each sample was taken in microtiter plates which were then read in a microtiter plate reader (Model 680, Bio-Rad Laboratories Inc., Hercules, CA, USA) at 570 nm. Proliferation activity was represented by absorbance at 570 nm in comparison to control.

4.12. Isolation of rat peritoneal exudates cells (PECs)

Peritoneal fluid from BALB/c mice and Wistar rat was harvested from peritoneal cavities by infusing ice-cold sterile PBS. After centrifugation at 1000 rpm for 10 min, the cell pellets were suspended in RPMI-1640 supplemented with 10% (v/v) FBS, streptomycin 100 U/mL, and seeded in 96-well plate at a cell density of 2 x 10⁶ cells/mL, containing various concentrations of fructans or polysaccharide mitogens or LPS (at 1, 5, and 10 μg) and allowed to culture in 5% CO2 humidified incubator at 37 °C for 48 h. The viability of the cells was assessed by Trypan blue exclusion test, and the proportion of macrophages was determined by cell morphologic study under a microscope.
4.13. Determination of nitric oxide (NO)

NO release can be used as a quantitative index of macrophage activation. As an indicator of nitric oxide production, nitrite concentration in the culture medium was determined by Griess reagent (Morihara et al., 2002). The culture supernatant (100 µL volume) was incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H3PO4) at room temperature for 10 min. The absorbance of the chromogenic azo-derivative molecule was measured using microplate reader at 540 nm. NO2− was determined by using sodium nitrite as the standard.

4.14. Phagocytic activity of macrophages by garlic fructans

Rate PECs were isolated from male Wistar rats (adult; 4 weeks-old; weighing ~250–300 g) using PBS, pH 7.4 containing 0.1% BSA (Clement et al., 2010). After injecting sterile buffer into the peritoneal cavity, the fluid containing PECs was collected after 5 min, washed three times in PBS, and finally resuspended in RPMI-1640 medium supplemented with 5% FBS and 1 µg/mL LPS. Viability and cell count was assessed by Trypan blue dye exclusion.

Phagocytic assay was performed according to the method described by Roy and Rai (2009). Briefly, peritoneal cells (1 x 10^6 cells/mL) were flooded onto prewashed clean slides (200 µL) to which 5 µL of PBS containing aged garlic fructans (2 µg) were added. The macrophages were allowed to adhere by incubating at 25 °C in a CO2 incubator for 90 min. Non-adherent cells were washed off with PBS. In the adherent cell population, more than 90% of the cells were macrophages as judged by their morphology following Giemsa staining. Yeast cell suspension was made by mixing 30 mg of commercial baker's yeast (Saccharomyces cerevisiae) in 10 mL of PBS. Yeast cells were heat killed at 80 °C for 15 min. The suspension was washed three times with PBS and finally suspended in culture medium supplemented with 4% FBS to get a concentration of ~1 x 10^6 cells/mL.

Each slide with adhered peritoneal macrophages was flooded with heat-killed yeast cell suspension and phagocytosis was allowed to proceed. After 90 min incubation at 25 °C, the slides were rinsed three times in PBS, fixed in methanol, and stained with Giemsa. For each slide, 100 macrophages were observed without any predetermined sequence or scheme. The phagocytic index was calculated by dividing the total number of yeast cells engulmed by or adhering to 100 macrophages. The phagocytic index for control is taken as 100% phagocytosis.

4.15. Statistical analysis

Assays were performed in triplicate. Results were expressed as mean ± standard deviations (S.D). The statistical analysis was performed using SPSS Software Package 10.0 version (SPSS Inc., Chicago, IL, USA). T-test was done to determine the statistical significance. A p-value of less than 0.05 was taken as significant.

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