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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,*]

[a] Department of Biochemistry and Nutrition, Central Food Technological Research Institute (Council of Scientific and Industrial Research Laboratory), Mysore 570020, Karnataka State, India
[b] Department of Meat, Fish and Poultry Technology, Central Food Technological Research Institute (Council of Scientific and Industrial Research Laboratory), Mysore 570020, Karnataka State, India

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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 ^{1}H NMR and ^{13}C NMR spectroscopy revealed that both have (2 → 1) β-D-fructofuranosyl bonds linked to a terminal glucose at the non-reducing end and β-D-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.

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-D-fructofuranosyl units], and graminan [both (2 → 1)-linked and 2 → 6)-linked β-D-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; Roller et al., 2004; Wu et al., 2006).

* Corresponding author. Address: Department of Biochemistry and Nutrition, Central Food Technological Research Institute (CFTRI), KRS Road, Mysore-570020, Karnataka State, India. Tel.: +91 821 2514876; fax: +91 821 2517233.
E-mail addresses: venkatyp@yahoo.com, ypv@cftri.res.in (Y.P. Venkatesh).

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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 dialyzed 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 sucrose 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).

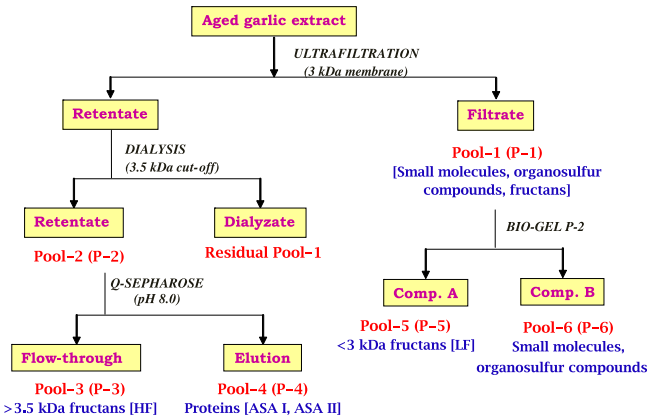

Fig. 1. Flow chart for the isolation of low molecular weight fructans (<3 kDa; LF) and high molecular weight fructans (>3.5 kDa; HF) from aged garlic extract. ASA I and ASA II denote garlic lectins (*Allium sativum* agglutinins I and II) present in both aged garlic extract (Chandrashekar and Venkatesh, 2009) and raw garlic (Clement et al., 2010).

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. ^{1}H NMR and ^{13}C 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 ^{1}H and ^{13}C 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