Dietary garlic (*Allium sativum*) lectins, ASA I and ASA II, are highly stable and immunogenic

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**Abstract**

The immunomodulatory proteins present in garlic have recently been shown to be identical to the garlic lectins ASA I and ASA II [Clement F, Pramod SN, Venkatesh YP. *Int. Immunopharmacol*. 2010; 10: 316–324]. In this study, the stability of garlic lectins as a function of pH, temperature and denaturants has been examined in relation to biological activity (hemagglutination and hagocytosis). Stability of garlic lectins in simulated gastric fluid (SGF) was assessed by their hemagglutination activity, immunoreactivity, and intactness by SDS-PAGE. Garlic lectins were moderately stable in SGF for up to 30 min; while they retained hemagglutination activities, immunoreactivity with the respective rabbit antiserum decreased immediately (0.5 min) to 10–30%. ASA I retained ≈ 80% hemagglutination activity in the pH range 2–12; however, ASA II retained only 40% in the pH ranges 2–4 and 10–12. Garlic lectins exposed to 60 °C (30 min) and pepsin (1 and 2 min) retained hemagglutination and phagocytic activities. Urea (4 M) and Gdn.HCl (2 M) did not affect hemagglutination. The immunogenicity of garlic lectins upon oral feeding in BALB/c mice was examined. A lectin-specific serum IgG response was seen in mice comparable to the oral immunogen, phytohemagglutinin. The recovered lectin in feces of mice administered with garlic lectins showed antigenicity identical to that of the administered proteins. The stabilities of the garlic lectins, their ability to withstand the gastrointestinal passage, and their recognition by the immune system upon oral feeding reinforce the reported presence of natural antibodies to garlic proteins in normal human sera.

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**1. Introduction**

Orally-administered antigens interact with the gut-associated lymphoid tissue (GALT) to produce an immune response that would give rise to, depending on the physiological status of the animal, one of the following: (i) oral tolerance, (ii) allergy and (iii) immunomodulation [1]. An important class of oral immunomodulators includes the dietary lectins, many of which are stable to gastrointestinal conditions and are able to enter into systemic circulation. Tomato lectin (LEA), for example, is known to resist digestion in the alimentary canal and bind to intestinal villi [2], and wheat germ agglutinin (WGA) has been detected in human intestinal contents [3]. Intact peanut agglutinin (PNA) has also been detected in blood samples of subjects who consumed peanuts [4]. Lectins include all plant proteins possessing at least one non-catalytic domain, which binds reversibly to a specific mono- or oligosaccharide [5]. Most commonly consumed plant foods contain lectins, and many of these foods are eaten raw, while others contain lectins that are active even after cooking and processing. A delicate equilibrium exists within the alimentary canal between lectins, dietary saccharides, immunoglobulins, viruses, bacteria and host cells [6].

Despite some of them being known for their anti-nutritional properties, lectins have a plethora of physiological and immunological implications [7–10]. There is evidence to suggest that most lectins given orally are immunogenic. It has been shown that phytohemagglutinin (PHA) is a powerful oral immunogen and produces a high titre of monospecific anti-PHA IgG antibodies in animals, including ruminants [11]. Both oral and intranasal delivery of five plant lectins investigated — mistletoe lectin 1, LEA, PHA, WGA and *Ulex europaeus* I lectin stimulated the production of specific serum IgG and IgA antibody [12]. Some lectins have been demonstrated to have adjuvant activity. The jacalin lectin (from seeds of *Artocarpus integrifolia*) is seen to induce a humoral immune response to a co-administered hapten, trinitrophenyl group and to *Trypanosoma* antigens in mice [13]. Mistletoe lectins (MLI, MLII, and MLIII) are potent mucosal adjuvants and intranasal co-administration of these lectins with herpes simplex virus glycoprotein D2 led to both mucosal and systemic immune responses to the viral antigen [14].

Natural antibodies to dietary proteins which were never administered as a component of medication have been detected in human serum. High titre anti-banana lectin (BanLec-1) IgG4 antibodies have been detected in pooled human sera [15]. Antibodies to the legume lectins, *Erythrina corallodendron* lectin (ECoL, the seed lectin of coral tree), PNA, and soybean agglutinin (SBA) and to the cereal lectin, WGA...
have been purified from normal human sera obtained randomly [16].
Natural antibodies (mostly IgG type) to two garlic proteins, alliinase (110 kD) and a mannos-specific Allium sativum agglutinin (ASA) have been purified by affinity chromatography from sera of healthy individuals [17]. The anti-alliinase antibodies were highly specific whereas, the anti-ASA antibodies were polyclonal as they interact with many other proteins [17].

Garlic, an important medicinal spice has immune-stimulating properties, many of which are contributed by the oraganosulfur compounds present in them [18]. Recently, two proteins have been identified as the major immunomodulatory proteins of both raw garlic and aged garlic extract, and have been shown to be identical to the well-characterized garlic lectins or agglutinins, ASA I and ASA II [19,20]. The garlic lectins belong to the GNA (Galanthus nivalis agglutinin) family; they bind weakly to mannos but exhibit a strong affinity toward oligomannosides and high-mannose N-glycans [5]. ASA I (heterodimer of 11.5 and 12.5 kD subunits) and ASA II (hodomer of two 12 kD subunits) are present in raw garlic in a ratio of 4:1 by weight [20–22]. A recent study has shown that consumption of garlic stimulated production of nitric oxide (NO), and in turn, increased IFN-α levels in humans [23]; it has been suggested that the protein component of garlic could be the immunomodulatory agent.

In view of the abundant presence of lectins in garlic, and the presence of natural antibodies in human sera, it appeared very interesting to investigate the immunogenic potential of garlic lectins upon oral administration in mice. An essential prerequisite for oral immunogenicity of proteins is that they should withstand the highly acidic conditions of the stomach, resist the action of several digestive enzymes and bypass the mucosal lining of the GI tract, thereby stimulating the immune cells of the GALT or gaining entry into systemic circulation. The present study was therefore undertaken to examine the stability of the potent garlic immunomodulatory proteins (ASA I and ASA II) in simulated gastric fluid (SGF), exposure to trypsin, and as a function of pH in relation to their biological activity (hemagglutination) and immunoreactivity to rabbit polyclonal antiserum. The immunogenicity of garlic lectins in the absence of an adjuvant upon oral feeding in BALB/c mice has also been examined.

2. Materials and methods

Garlic bulbs were procured from the local grocery. OMEGA 30 K cut-off membrane disc filters were procured from Pall Life Sciences, Ann Arbor, MI, USA. Garlic powder was a product of The Nilgiri Dairy Farm Pvt. Ltd., Bangalore. Amicon stirred ultrafiltration cell (Model 8050) was a product of Millipore Corporation, Bedford, MA, USA. Q-Sepharose (high performance) anion-exchange resin (bed size: 24-44 μm), agar (electrophoresis grade), bovine serum albumin (BSA), pepsin, trypsin, chymotrypsin, urea (electrophoresis grade), guanidine hydrochloride (Gdn.HCl), fetuin, concanavalin A (Con A), PHA, ovalbumin (OVA), goat anti-rabbit IgG-alkaline phosphatase (AP) and goat anti-mouse IgG-AP conjugates, detoxified bacterial lipopolysaccharide (LPS) and brilliant blue G-colloidal concentrate were products of Sigma-Aldrich Co., St. Louis, MO, USA. Mannose, horseradish peroxidase (HRP), p-nitrophenyl phosphate, fertil bovine serum (FBS) and RPMI-1640 medium were purchased from HiMedia Laboratories Ltd., Mumbai, India. Flat-bottomed 96-well microtiter plates (MICROLON) were bought from Greiner Bio-One GmbH, Frickenhausen, Germany. Avi-din-AP was procured from Bangalore Genei, Bangalore, India. All other chemicals/reagents used in this study were of analytical grade.

This study was undertaken 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. Ten-month-old New Zealand white male rabbits (Oryctolagus cuniculus) were used for generation of polyclonal antisera to garlic lectins ASA I and ASA II as described in Clement et al., [20]. For oral immunogenicity studies, BALB/c mice were used. Peritoneal exudate cells (PECs) were isolated from Wistar rats, after sacrificing the animals. All animals were housed and maintained on a standard commercial diet at ambient temperature in a clean environment as per the ethical guidelines.

2.1. Protein estimation and SDS-PAGE

Protein concentration was determined following the method of Bradford [24] using BSA as the standard. SDS-PAGE under reducing conditions was carried out as described by Laemmli [25], in 17% polyacrylamide gels followed by staining with brilliant blue G-colloidal concentrate.

2.2. Preparation of raw and heat-processed garlic extracts

Extracts of raw garlic, garlic powder and heat-processed garlic were prepared. The hemagglutination activity and the glycoprotein-binding activity of the lectins in these extracts were measured by hemagglutination (HA) assay and enzyme-linked ligand sorbent assay (ELISA), respectively, as described later in sub-Sections 2.3 and 2.4.

2.2.1. Raw garlic extract (RGE)

Fresh garlic bulbs (25 g), following removal of the thin outer peel, were homogenized by blending in 50 mL of 10 mM sodium phosphate buffer/140 mM NaCl (PBS; for neutral pH extract) or distilled water (for aqueous extract) or 50 mM sodium acetate buffer, pH 4.0 (for acidic pH extract) to obtain a 50% w/v extract. After stirring at 4 °C for 2 h, the extract was passed through a porous gauze, filtered through muslin cloth, and the filtrate was centrifuged (5000 × g at 4 °C) for 15 min. The clear pale yellowish supernatant obtained was designated RGE.

2.2.2. Garlic powder extract (GPE)

Commercially available garlic powder (25 g) was suspended in 50 mL of PBS to obtain 50% w/v extract. Other steps were as described for the preparation of RGE; the clear supernatant obtained was designated GPE.

2.2.3. Heat-processed garlic extract (HPGE)

Twenty-five grams of peeled, fresh garlic bulbs was suspended in 50 mL of PBS. The contents were boiled for 20 min, cooled to room temperature and blended using a mortar and pestle. The extract was initially filtered through porous gauze and the filtrate was subjected to centrifugation (5000 × g at 4 °C). The supernatant obtained was concentrated by refrigerated Speed VAC concentration system (Model: RCT 60, Jouan, CR4-12, St. Herblain, France), and designated as HPGE.

2.3. Hemagglutination assay

HA assay was carried out at 25 °C following the method of Burger [26] using freshly prepared, trypsin-treated rabbit erythrocytes. Briefly, a 2% suspension of trypsin-treated rabbit erythrocytes (0.15 mL) was added to an equal volume of serially (two-fold) diluted protein solution in an agglutination plate, gently mixed and incubated at 37 °C for 2 h, and the agglutination was visualized. The amount of protein present at the highest dilution showing visible agglutination represents the minimum quantity of protein necessary for agglutination and is taken as the titer. One HA unit is equivalent to the concentration of protein in the last well that completely agglutinates an equal volume of standardized RBC suspension. HA titer is calculated by dividing the initial protein concentration (mg/mL) by (2^n), where 2^n represents the dilution in the last well having visible agglutination. The specific HA activity is calculated as the reciprocal of HA titer, and expressed in HA units/mg protein.
2.4. Glycoprotein-binding activity by ELISA

Microtiter plate wells were coated with 10–20 μg protein present in various garlic extracts or purified garlic lectins (ASA I and ASA II) in coating buffer (100 mM carbonate–bicarbonate, pH 9.6) at 4 °C overnight. After blocking with 3% gelatin in PBS, the microtiter wells were incubated with HRP (100 μL of 0.1 mg/mL), or avidin-AP conjugate (100 μL of 1:2000 dilution) in PBS containing 1% BSA/0.05% Tween-20 at 37 °C for 2 h. Following the addition of the respective substrate (o-phenylenediamine/H₂O₂ for HRP or p-nitrophenyl phosphate for AP), the relevant absorbance was measured in a microplate reader (Model 680, Bio-Rad Laboratories, Hercules, CA).

2.5. Purification of garlic lectins

Garlic lectins were purified from raw garlic as described earlier [20]. Briefly, an extract of fresh garlic cloves was prepared in 20 mM unbuffered 1,3-diaminopropane (1:10 w/v) and the clear extract subjected to ultrafiltration using a 30 kD cut-off membrane in an Amicon stirred-cell filtration unit. The ultrafiltrate was subjected to anion-exchange chromatography on Q-Sepharose (High Performance) column equilibrated with 20 mM Tris–HCl buffer, pH 8. Step-wise elution of the bound proteins was carried out using different concentrations of NaCl in the starting buffer. The proteins eluting at 0.1 M and 0.25 M NaCl concentrations, in a ratio of 1:4 by weight, represent ASA II and ASA I, respectively. They were dialyzed against water (3 kD cut-off dialysis membrane), lyophilized, and stored at −20 °C until further use.

2.6. Stability of garlic lectins to various physicochemical conditions

Garlic lectins, ASA I and ASA II were studied for their stability to varying pH and temperature conditions by assessing their hemagglutination activities. Effect of pH was studied by incubating the lectins, (90 μg of ASA I or ASA II) in appropriate buffers (0.1 M) of varying pH (glycine–HCl, pH 2; sodium acetate, pH 4; sodium phosphate buffer, pH 6; Tris–HCl, pH 8; carbonate buffer, pH 10 and 12) for 1 h at ambient temperature (25 °C). At the end of the incubation time, the protein samples were tested for their hemagglutination activities.

The effect of temperature on the lectins was studied by incubating the proteins in an oven fitted with thermostat in PBS for 30 min at different temperatures (20 °C, 40 °C, 60 °C, 80 °C and 100 °C). At the end of the incubation period, the samples were gradually cooled to room temperature and measured for their hemagglutination activities.

The effects of chemical denaturants, urea and guanidine hydrochloride (Gdn.HCl) on the lectins were assessed by incubating the proteins in varying concentrations of urea (2 M, 4 M, 6 M and 8 M) or Gdn.HCl (2 M, 4 M and 6 M) in PBS, for 30 min at room temperature followed by HA assay.

2.7. Stability of garlic lectins to simulated gastric fluid (SGF)/simulated intestinal fluid (SIF) digestion

Garlic lectins were studied for their stability in SIF, according to the method described by Ofori-Ani et al. [27]. Accordingly, 0.1 mL test protein (5 mg/mL) was mixed with 1.9 mL SGF (0.084 N HCl, 35 mM NaCl, pH 1.5 along with 4000 U pepsin) and incubated at 37 °C. At incubation time periods of 0, 0.5, 2, 5, 10, 20, 30, 60, and 120 min, 200 μL aliquots of the digest were taken out and quenched with 200 μL of 200 mM NaHCO₃. Zero min digest is the solution of pepsin quenched before adding the test protein. The aliquots were analyzed by SDS-PAGE and hemagglutination assay.

Immunereactivity of SGI–digests of the two garlic lectins to polyclonal antiserum raised in rabbits was measured by ELISA as follows. Two micrograms of protein (ASA I or ASA II) were coated on to ELISA plates in 100 μL 0.1 M carbonate–bicarbonate buffer, pH 9.6 (coating buffer) and left at 4 °C overnight. After washing three times with PBS containing 0.1% Tween-20 (PBS-T), the wells were blocked with 2% gelatin in PBS-T at 37 °C for 1 h. Subsequent to washing thrice with PBS-T, 100 μL of 1:1000 dilution of specific rabbit antiserum in PBS-T were added to the wells and the plate was incubated at 37 °C for 2 h. The wells were washed with PBS-T and goat anti-rabbit IgG-AP conjugate (100 μL of 1:2000 dilution) was added to the wells and incubated at 37 °C for 2 h. Following the addition of the substrate p-nitrophenyl phosphate (100 μL; 1 mg/mL), the reaction was stopped by adding 3 N NaOH (50 μL) and the absorbance at 405 nm was recorded in a microplate reader.

Intestinal digestion was simulated by a mixture of trypsin and chymotrypsin according to the procedure described by Sen et al. [28]. Purified ASA I or ASA II was diluted in 65 mM Tris–HCl buffer, pH 8.3 containing 1 mM EDTA to a final concentration of 0.5 μg/μL. Protein samples were mixed with enzymes in a ratio of 34.5 U trypsin activity/mg protein: 0.44 U chymotrypsin activity/mg protein, and incubated at 37 °C. Aliquots were taken after incubation for 1, 3, 6 and 24 h. The reaction was immediately quenched with SDS sample buffer and samples were stored at −20 °C.

2.8. Phagocytic activity of macrophages by garlic lectins exposed to pH, heat or pepsin treatment

Rat PECs were isolated from male Wistar rats (adult; 4 weeks-old; weighing ~250–300 g) using PBS, pH 7.4 containing 0.1% BSA [20]. After injecting sterile buffer into the peritoneal cavity, the fluid containing PECs was collected after 5 min, washed thrice in PBS, and finally resuspended in RPMI-1640 medium supplemented with 5% PBS 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 [29]. Briefly, peritoneal cells (1 × 10⁷ cells/mL) were flooded onto prewashed clean slides (200 μL), to which 5 μL of PBS (control) or PBS containing treated/untreated garlic lectins (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% PBS to get a concentration of ~1×10⁸ 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 determined by calculating the average number of yeast cells engulfed by or adhering to 100 macrophages. The phagocytic index for control is taken as 100% phagocytosis.

2.9. Oral immunization of mice for immunogenicity of garlic lectins

Twelve-week-old BALB/c mice were grouped according to their body weights such that each group had about 5–8 mice and the average body weights of mice in each group was approximately the same. For oral immunogenicity studies, mice were administered 10 or 100 μg of ASA I or ASA II in 100 μL PBS, in the absence of any adjuvant, on days 1, 14, 21, 28, 35, 42 and 49 by oral gavage using a blunt-ended steel syringe. Ovalbumin (OVA) was used as the test antigen and PHA, an orally immunogenic lectin in rodents, as the positive control. Control mice were administered 100 μL of PBS. Blood was drawn on days 12, 35 and 55 from the retro-orbital plexus using heparinized capillary tubes, and the sera collected were stored at −20 °C.
Serum antibody (IgG) response to the administered proteins was determined by direct ELISA. Microtiter plate wells were coated with 1 μg protein in 100 μL coating buffer at 4 °C overnight. After washing three times with PBS-T, the wells were blocked with 2% gelatin in PBS-T at 37 °C for 1 h. Subsequent to washing thrice with PBS-T, mouse serum (100 μL of 1:20 dilution) in PBS-T was added to the wells and the plate was incubated at 37 °C for 2 h. The wells were washed with PBS-T and goat anti-mouse IgG-AP conjugate (100 μL of 1:2000 dilution) was added to the wells and incubated for 2 h. Dilution buffer (PBS-T) was used with 0.2 M mannose for immunogenicity studies of garlic lectins, and 1% fetuin for immunogenicity study of PHA in order to prevent the agglutinin–sugar interactions [12,30]. Following the addition of the substrate p-nitrophenyl phosphate (100 μL; 1 mg/mL), the reaction was stopped by adding 3 N NaOH (50 μL) and the absorbance at 405 nm was recorded in a microplate reader.

2.10. Recovery and detection of copro-antigens

Two groups of 12-week-old BALB/c mice (n = 3) were administered with either 200 μL PBS (control group) or 100 μg of an equal mixture of ASA I and ASA II in 200 μL PBS (test group) by oral gavage for 7 consecutive days. Mice feces was collected on the last three days of feeding and stored at −20 °C until further use.

Recovery of biologically active protein in feces of mice administered with lectin was determined by HA assay and Ouchterlony double diffusion (ODD). Extracts of the feces collected were prepared in PBS along with 0.2 M mannose to release any bound lectins. The clear solution was dialyzed using 3.5 kD cut-off dialysis membrane against water. ODD was performed as described by Higuchi et al., [31]. Antiserum against the purified garlic lectins were raised in rabbits, as described previously [20]. The assay was carried out in 1.5% agarose gel in plastic petri plates in a humidified chamber at 37 °C for 24 h. The precipitin lines were stained with Coomassie brilliant blue stain for 30 min and destained with 7% glacial acetic acid to visualize the stained precipitin lines against the clear background.

2.11. Statistical analysis

Results are expressed as mean of triplicate values ± standard error of mean (S.E.M.). Data were analyzed by Student’s t-test to determine the statistical significance using SigmaStat-3.5 software (Systat Software Inc., Chicago, IL). A p-value of <0.05 was considered to be statistically significant.

3. Results

3.1. Hemagglutination activity and glycoprotein-binding activity in various garlic extracts

In order to standardize the extraction method for obtaining 50% w/v extract, 25 g peeled garlic bulbs were extracted with (a) distilled water, or (b) 50 mM sodium acetate buffer, pH 4.0 or (c) phosphate-buffered saline, pH 7.2. The protein amounts, glycoprotein-binding activity and hemagglutination activity of these extracts are shown in Table 1. It is clear that extraction with PBS results in a slightly higher protein yield as compared to aqueous or acidic pH extracts. HPGE has very low protein yield (0.74 mg/g) compared to RGE and this could probably be due to precipitation of proteins during heat processing of garlic bulbs.

In the glycoprotein-binding assay as assessed by HRP and avidin-AP binding, the neutral pH extract shows considerable binding, and is higher compared to other extracts. GPE shows almost similar binding as that of RGE (neutral pH). The trend in hemagglutination assay is also found to be similar to that seen in glycoprotein-binding assay (Table 1). HPGE shows ~30% reduction in the specific hemagglutination activity (units/mg total protein) compared to other extraction procedures.

3.2. Influence of physicochemical treatments on the stability of garlic lectins

Garlic lectins, ASA I and ASA II were tested for their stabilities under varying conditions of pH, temperature and denaturants in relation to their biological activity (hemagglutination). One hundred percent hemagglutination activity for ASA I and ASA II is the specific activity displayed by the proteins upon incubation in PBS at 25 °C for 1 h, and corresponds to 3200 and 1400 U/mg protein, respectively. The hemagglutination activity of ASA I and ASA II as a function of pH is shown in Fig. 1 (panel a). ASA I was found to retain 80–100% activity at all pH values tested. On the other hand, ASA II retains 100% activity at pH 6 and 8, 40% at pH 10 and 12, and 25% at pH 2 and 4. The hemagglutination activity of both ASA I and ASA II was retained at 60 °C for 30 min, but was completely lost at 100 °C incubation (Fig 1, panel b). However at 80 °C, ASA II seems to retain ~60% hemagglutination activity compared to ~5% for ASA I.

The effect of denaturants, urea and Gdn.HCl on the hemagglutination activity of ASA I and ASA II is shown in Fig. 2. Both ASA I and ASA II seem to retain approximately 60–90% HA activity in Gdn.HCl concentration ranging from 2 to 4 M (Fig. 2, panel a). ASA I and ASA II retain 50% and 25% hemagglutination activity, respectively, at 6 M Gdn.HCl. With respect to urea as a denaturant, there is no loss of hemagglutination activity at 2 M urea in the case of ASA I; at concentrations of 4 M and 6 M urea, approximately 70% activity is retained (Fig 2, panel b). The HA activity drops to 40% at 8 M urea in the case of ASA I. A somewhat similar trend was observed for ASA II, although only 30% activity was retained at 8 M urea.

3.3. Influence of simulated gastric/intestinal digestion on the stability of garlic lectins

To simulate the proteolytic digestive conditions of the gastrointestinal tract in vitro, pepsin in a buffering solution akin to gastric fluid was used to study the resistance of the garlic lectins. Samples of the digestes were taken at different times and analyzed by SDS-PAGE, hemagglutination assay, and ELISA for examining the immunoreactivity to specific rabbit antisera. The biological activity and cross-reactivity of '0 min' digests is taken as 100% for hemagglutination and immunoreactivity studies, respectively. Under the conditions tested for SGF digestion, Con A, OVA and BSA showed stability towards pepsin up to 30, 10 and 0.5 min, respectively (data not shown), similar to the results obtained by Herman et al. [32]. In the case of ASA I, resistance to digestion was seen up to 60 min as analyzed by SDS-PAGE (Fig. 3, panel a). Purified ASA II resisted SGF digestion up to 60 min.
30 min, with a decrease in the intensity of the protein bands at 60 and 120 min (Fig. 3, panel b).

Hemagglutination activity was retained completely up to 0.5 min in SGF for both ASA I and II, followed by a gradual decline up to 60 min; roughly 30% hemagglutination activity is seen at 60 min of digestion (Fig. 4, panel a). However, immunoreactivity of garlic lectins to rabbit polyclonal antisera decreased remarkably even as early as 0.5 min of digestion; while ASA I retained 30% immunoreactivity, ASA II retained only 10% (Fig. 4, panel b). From 2 min onwards, the decrease was more gradual, and both garlic lectins retained only 5–10% immunoreactivity at 60 min of digestion.

ASA I is stable to simulated intestinal digestive conditions (trypsin–chymotrypsin) up to 3 h; after 6 h, the intensity of the original protein band decreases on SDS-PAGE analysis (Fig. 5, panel a). ASA II has relatively lower stability, compared to ASA I, to simulated intestinal conditions and the original band intensity decreases after 3 h (Fig. 5, panel b).

3.4. Effect of low pH, heat or protease-treated garlic lectins on the phagocytic activity

Percentage phagocytosis was significantly increased (p<0.001) in the presence of ASA I or ASA II compared to control (Fig. 6, panel a).

Significant phagocytic activity was also retained with heat-treated ASA I (p<0.001) and ASA II (p<0.01) at 60 °C. However, phagocytic activity was lost in samples exposed to 80 °C, and acidic pH values – pH 2 and 4 (Fig. 6, panel a). SGF-treated garlic lectins retained phagocytic activity (p<0.01) at 1 and 2 min of digestion (Fig. 6, panel b).

3.5. Effect of orally-administered garlic lectins on humoral response

The immunogenicity of garlic lectins ASA I and ASA II in the absence of an adjuvant at 10 and 100 μg doses is shown in Fig. 7. Administration of garlic lectins by oral route did not lead to weight loss or gain and induced no changes upon physical examination. The time-course of humoral response follows the normal course of immunization with an IgG response by twelve days after the prime oral dose. Further oral exposure results in booster effects to the administered test proteins as evidenced by IgG responses on days 35 and 55. Compared to control, the garlic lectins ASA I and ASA II shows a 3–4 fold increase in IgG response for both dose groups and at different days. The model antigen OVA shows a very weak IgG response, while PHA shows a 3-fold IgG response compared to control. Thus, garlic lectins are as immunogenic as PHA, and there is no significant difference in the IgG response between the two doses (10 and 100 μg) or between booster doses.
3.6. Detection of copro-antigens in mice administered garlic lectins orally

The fecal extract of mice administered with a mixture of both agglutinins showed hemagglutination activity on a qualitative basis (data not shown). Ouchterlony double diffusion results show the presence of ASA I in the fecal extract as indicated by the continuous line of precipitation with the purified ASA I arc (Fig. 8, panel b) in comparison to the control fecal extract (Fig. 8, panel a). Similar results were seen when ODD was carried out with rabbit anti-ASA II antiserum (data not shown).

4. Discussion

The lectins (ASA I and ASA II) and alliinase are the most abundant proteins in raw garlic. Natural antibodies to these proteins have been demonstrated in healthy human sera screened at random [17]. Similarly, antibodies to other dietary proteins like avidin, WGA, PNA, SBA and ECorl have been reported in normal human sera by various investigators [16,33]. A landmark study by de Aizpurua and Russell-Jones [34] has revealed that among the classes of proteins tested for eliciting antibody responses upon oral administration, proteins with “lectin or lectin-like” binding activities were most effective. Generally these proteins, by virtue of their ability to bind to glycolipids and/or glycoproteins on the intestinal mucosal cells stimulate them to transport the proteins into the systemic circulation, thereby eliciting a systemic immune response. Since garlic is consumed universally, and natural antibodies to garlic lectins have been reported, it appeared interesting to investigate the immunogenic response of orally-administered garlic lectins in BALB/c mice.

Proteolytically active antigens such as bromelain can stimulate both systemic and mucosal immune responses following repeated oral exposure [35,36]. Some studies have suggested that either carbohydrate-binding activity or proteolytic activity as an important parameter for the immunogenicity of proteins. It is of interest to note here that a garlic protein with sequence similarity to the lectin ASA II has been shown to possess both hemagglutination and cysteiny protase activity [37]. Our results clearly show that both ASA I and ASA II, upon oral administration, produce a remarkable humoral immune response compared to control.
Since stability of proteins in the GI tract could be another important parameter for immunogenic response, we have looked into the stability of garlic lectins ASA I and ASA II in SGF. Garlic lectins are resistant to peptic digestion in vitro as determined by SDS-PAGE and hemagglutination assay. It is interesting to note that hemagglutination activity, although reduced, is retained after SGF digestion, while the SGF-digests are recognized to an extent of only 10–30% in 0.5-min digests by polyclonal antisera raised against the garlic lectins. Earlier studies on natural antibodies to dietary lectins revealed that recognition of these proteins by specific antibodies did not interfere with the agglutination properties of the lectins [16]. This suggests that although the highly acidic conditions of digestion in SGF may be altering the conformation of reactive (conformational) epitopes as a result of unfolding, the sugar-binding sites of garlic lectins appear to remain intact retaining most of their hemagglutination activity during the early stages of SGF digestion.

The folding and unfolding properties of ASA I as a function of temperature and pH, and in the presence of chemical denaturants (urea and Gdn.HCl) have been studied by calorimetry and spectroscopy [38]. In the present study, the stability of ASA I and ASA II based on their biological (hemagglutination) activity has been tested. Although it would be difficult to directly compare the results obtained in our study with the study done in real time by Bachhawat et al. [38], it is compelling to conclude that the garlic lectin ASA I is moderately stable in a broad range of pH, up to 60 °C, and at 4 M urea and 2 M Gdn.HCl concentrations. It appears that even under extremes of pH and chemical denaturants, garlic lectins retain part of their biological (hemagglutination) activity after returning to physiological conditions. The high degree of reversibility of denaturation in case of ASA I studied by calorimetric and spectroscopic studies is in accordance with the results obtained in the present study, and may apply to ASA II as well, considering the high degree of sequence homology between the two garlic lectins [39].

Garlic as a spice is consumed in various forms besides raw — cooked, dried or pickled wherein the bioactive components, specially the proteins, may lose their biological activities. Abrus agglutinin (from the non-edible seeds of jequirity bean, Abrus precatorius) having specificity for galactose, is a potential immunomodulator both in native and heat-denatured forms when tested in mice [40–44]. The heat-denatured agglutinin is seen to stimulate T and B cells, induce production of

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cuits, affect macrophage functions, production of NO and hydrogen peroxide and has anti-tumor and immunoadjuvant activities comparable to that of the native agglutinin [40–44]. The present study also examined the effect of variously treated garlic lectins on the phagocytic ability of rat peritoneal macrophages. From the present study it is apparent that garlic lectins stimulate yeast cell phagocytosis. It is, however, interesting to note that heat treatment up to 60 °C and SGF digestion at 1 and 2 min do not destroy the phagocytic ability as compared to immunoreactivity which drops instantaneously at 0.5 s of digestion. The ability of garlic lectins subjected to brief periods of SGF digestion appears to follow the trend in hemagglutination activity indicating that the initial cleavages by pepsin under acidic conditions do not affect the biological activities. The stability of the garlic lectins to highly acidic or alkaline conditions, or at higher temperatures, as observed in this study, reinforces the immunomodulatory potential of garlic as a therapeutic food even in cooked or processed forms.

Recently, it was shown that oral administration of the Enoki mushroom agglutinin (FVE) displayed anti-tumor activity in hepatoma-bearing mice [45]. The effect of feeding the high molecular weight lectin (ASA110) to Swiss albino rats, in native and heat-denatured states, showed binding of the lectin to GI tract and changes in intestinal mucosal ATPase [46,47]. In the present study, feeding of ASA I and ASA II gave rise to a humoral lectin-specific IgG response in mice.

Alternatives to traditional vaccine delivery include mucosal (oral) delivery. Oral immunization of an animal is generally hard to achieve unless large quantities of antigen are administered. There is likelihood that orally delivered immunogenic proteins will be degraded after ingestion and that some immunogens may not be recognized efficiently [48]. As lectins are multifunctional molecules, the potential of using them as both targeting and therapeutic agents is viable. The sugar specificity of lectins makes them suitable agents for oral delivery of antigens to specific sites of the GI tract or targeting drugs within the oral cavity [49–51]. In general, the uptake of lectins via the Peyers patches favors the secretory IgA-mediated response, and the transport across the enterocytes facilitates the systemic IgG-mediated response [51]. Tomato lectin, for example, is considered a potential agent for oral delivery of drugs, especially so because it is from a non-toxic dietary source [52]. In this context, our results indicating the stability of garlic lectins, ASA I and ASA II, to gastric digestion in vitro and their intrinsic immunogenicity upon oral administration in mice is of considerable significance, since garlic is also a non-toxic dietary source.

Fig. 7. Oral immunogenicity of garlic lectins in BALB/c mice. Mice were administered two doses of garlic lectins or PHA by gavage feeding: (a) 10 μg and (b) 100 μg. Ovalbumin (OVA; 100 μg dose) represents a weak immunogen and PHA a prototype antigen representing the lectin category. Anti-protein IgG response in the sera (1:20 dilution) of BALB/c mice was measured by ELISA using the individual administered proteins as coating antigens. Serum IgG response to PHA(a) representing 10 μg protein is identical to PHA(b), and hence is not shown. Values represent mean of triplicates ± SE. Significant difference (p<0.05) was observed in IgG response of ASA I and ASA II groups at both doses vs. control group.

Natural antibodies to PNA, WGA and garlic lectins have been reported to be polyreactive [16,17]. Many polyreactive antibodies have been shown to possess broad antibacterial and antiviral activities [53–55]. In conclusion, garlic lectins ASA I and ASA II seem to be important immunomodulatory proteins of garlic that are able to withstand the harsh conditions of the GI tract, perhaps taken up by the enterocytes, and modulate the immunoresponder cells to evoke immunogenicity, and could possibly represent one of the critical components responsible for a host of beneficiary immunomodulatory responses described in garlic till date.

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