

Adjuvant Effect of Garlic Lectins (Asa I and Asa II) on Mucosal Immunity Induction Following Intranasal Immunization with Ovalbumin Antigen

Siddanakoppalu N Pramod¹⁺ and Yeldur P Venkatesh²

¹ Department of Biochemistry, Sahyadri Science College, Kuvempu University, Vidyanagar, Shimoga-577203, Karnataka, India.

² Department of Biochemistry and Nutrition, Central Food technological research institute (CFTRI), Mysore-570020, India. Email:snpramod20@yahoo.com

Abstract. Several dietary components are known to affect various functions of the immune system and to interfere with immune regulatory circuits. The immunological activity of carbohydrate-binding proteins from garlic, the lectins has been identified to modulate immune functions. In this study the immunogenicity and adjuvant property of the two purified garlic lectins (ASA I and ASA II) has been examined in BALB/c mice. Lectins were administered by intradermal and intranasal routes and the anti-lectin IgG response were assessed. No reduction in the body weight was observed, however there was significant increase in spleen and thymus indices of test groups. ASA I showed an increased anti-Ova IgG response compared to ASA II for co-administered ovalbamin. Anti-lectin antibody response by both systemic and mucosal routes of administration increased after each booster dose, and the titer was found to be very significant. These observations indicate that only ASA I was found to have significant adjuvant activity whereas ASA II does not. It appears that garlic lectins are potent immunogens, in boosting the immune response to co-administered antigens. Since garlic lectins are non toxic and lymphoid activator, these lectins make a best choice for the mucosal delivery of oral or nasal targeted vaccines.

Key words: Agglutinin/lectin, immunogen, adjuvant, Food antigen, Garlic, immunomodulatory, BALB/c mice

1. Introduction

The health effects of dietary lectins have been addressed by several investigators and currently only limited information is available on the immunomodulatory effects of lectins [1]. Lectins are typical globular proteins that are mostly resistant to digestion in the gastrointestinal tract and are known to affect the integrity of the intestinal epithelium and the absorption of dietary antigens, and may further become internalized and circulate intact in the peripheral blood [2]. Lectins provoke diverse biological consequences in mammals such as hyperplasia and hypertrophy of the small intestine and could increase the permeability of the intestinal wall, resulting in enhanced absorption for dietary co-administered antigens [1]. Relatively few molecules have been identified that are able to induce a strong immune response when delivered by the oral or other mucosal route [3]. Lectins are some of the few proteins that when given by the mucosal route induces an antibody response [4]. Although still sparsely documented, the type of immune response (local vs. systemic, tolerance vs. immunity) may be strongly dependent on the site of absorption in the gut. Uptake across Peyer's patches (PP) might induce immunity, whereas, absorption through enterocytes might induce tolerance. Apart from indications that the binding activity of lectins can confer immunogenicity [3] and [4], there are very few investigations on what determines mucosal immunogenicity of plant lectins. However,

⁺ Corresponding author. Tel.: + 91-8182-242098; fax: +91-8182-241793.
E-mail address: snpramod20@yahoo.com.

mucosal delivery of non-replicating antigens generally does not stimulate strong immune response, requires multiple doses [5] and may result in systemic unresponsiveness [6]. A number of strategies may be used to enhance responses to mucosally delivered vaccines including bacterial vectors, biodegradable microparticles, or liposomes. The most powerful mucosal adjuvants identified to date are cholera toxin (CT), heat-labile enterotoxin (LT) [7] and [8]. Stimulation of toxin-specific local and systemic responses and responses to co-administered antigens after mucosal application distinguish these molecules from most soluble proteins [9]. Although toxicity prevents clinical application, molecules with retained adjuvanticity and low toxicity have been generated.

Garlic is present in many foods as a flavor enhancer and is not a component in any vaccine or drug administered to humans. Garlic cloves contain a limited amount of protein, two of which (alliinase and mannan-specific lectins) together constitute more than 50% of the total amount of proteins [4] and [9]. Therefore, in the present study an attempt has been made to examine the two purified garlic lectins (ASA I and ASA II) to assess their ability to induce anti-lectin antibody response by both intradermal and intranasal routes of administration using BALB/c mice. The study will provide insight into the systemic and mucosal effect of immune responses for these lectins.

2. Materials and Methods

This study was undertaken after clearance by the Institutional Animal Ethics Committee (IAEC). The animals were maintained in the animal house facility. All the necessary precautions and care were taken during the experiments as per the guidelines provided by the IAEC. Garlic lectins used in the present study were purified in the laboratory. Ovalbumin (OVA; type V, hen egg), concanavalin A (Con A), and goat anti-mouse IgG-alkaline phosphatase conjugate were products of Sigma-Aldrich Co., St. Louis, MO, USA. All other chemicals and reagents used in this study were of analytical grade.

2.1. Experimental Animals and Grouping of Animals for Immunization

Young adult (8-12 weeks old) female BALB/c strain mice were used throughout these studies. Well-maintained hygienic quality food and water were provided to the animals. All experiments were carried out under the provisions of the Indian Animals Act. The average weights of the animals in each group were almost similar. The random distribution of animals was done to ensure the same response from animals in each group receiving the antigens. All the antigens were administered by either intradermal or intranasal route at the same concentration following the standard protocol.

2.2. Purification of Garlic Lectins and Preparation of Antigens

Garlic lectins ASA I and ASA II purified from raw garlic bulbs. 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 ultra filtration using a 30 kD cut-off membrane. The filtrate was subjected to anion-exchange chromatography on Q-Sepharose 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. The dialyzed and lyophilized lectins were adjusted to 1 mg/mL based on Bradford protein assay. Commercially procured ovalbumin (OVA) was prepared in PBS at 1 mg/mL concentration

2.3. Intradermal (Systemic) and Intranasal (Mucosal) Immunization Schedule

BALB/c mice (n=6) received an intradermal injection of 30 μ L of 1 mg/mL antigen on the dorsum of each ear on days 0 and 7; blood was drawn from the animals of control and treated groups on day 7 and 14 by retro-orbital vein puncture using heparinized capillary tube. Serum was separated and stored at -20°C. For Intranasal administration of antigens groups of mice (n=6) were mucosally immunized by intranasal (i.n.) route of administration. On days 1, 7, 14, 21, 28, 35 and 42 with OVA and garlic lectins (ASA I and ASA II), which were all prepared in PBS at a concentration of 1 mg/mL. Thirty micrograms of OVA and plant lectins were delivered in 30 μ L of PBS (15 μ L was applied to each nostril during dosage administration. Fifty days following the initiation of the antigen exposure, all animals were exsanguinated by cardiac puncture. Blood

was drawn from control and treated groups on day 13, 34 and 50 day by retro-orbital vein puncture using heparinized capillary tube. Serum was separated and stored at -20°C.

2.4. Body weights of Experimental Animals and Calculation of Spleen and Thymus Index

The body weight of each individual animal in the experimental groups was monitored at specific intervals of time during the experiment. The weights were taken prior to the administration of each dose by intradermal or intranasal routes of administration. The animal weight was monitored at intervals and the final weight was recorded at the end of the treatment (14th day for i.d. group and 50th day for i.n. group). The spleen and thymus of the individual animals of each group was collected on the final day after sacrificing the animals. Spleen and thymus were weighed both in control and treated animal groups. Based on the spleen and thymus weights and body weight of the individual animals, spleen index and thymus index were calculated using the following formulae: Spleen index = Spleen weight / Body weight, whereas for Thymus index = Thymus weight / Body weight; the index was expressed as mg/g.

2.5. Detection of Lectin and Antigen-Specific IgG Antibodies by ELISA

ELISA was performed for the detection of specific IgG antibodies. Sera at 1: 10 dilution were titrated in the appropriate dilution buffer. For the assay, microtiter plates were coated with 100 µL per well of 0.1 mg/mL antigens in 0.1 M carbonate-bicarbonate buffer, pH 9.6, and incubated at 4°C overnight. After washing with PBS containing 0.05 % Tween (PBS-T), plates were blocked with 2% gelatin/dilution buffer and incubated at 37 °C for 2 h. Plates were washed, and samples (mouse serum diluted 1:10 with a dilution buffer containing PBS-T with 1% BSA) were added and incubated at 4°C overnight. Following the incubation, plates were washed, and incubated with 100 µL/well of goat anti-mouse IgG conjugated to alkaline phosphatase at 1:1000 dilution as a source of secondary antibody. The secondary antibody diluent used was PBS-T containing 1% BSA and 0.5% gelatin. The plates were incubated at 37°C for 2 h. After extensive washing, plates received 100 µL of alkaline phosphatase substrate (p-nitrophenyl phosphate at 1 mg/mL prepared in 10 % diethanol amine buffer, pH 9.4). The plates were further incubated for 20 min and the reaction was arrested by the addition of 100 µL 3 N NaOH. The absorbance of the plates was read at 405 nm using microtiter plate reader.

2.6. Statistical Analysis

Data are expressed as the arithmetic mean and standard deviation. Unpaired two-tailed t-test was used to test for significance between the groups. A paired t-test for means was used to test for significance in the same group at different time points.

3. Results and Discussion

3.1. Body Weights of Animals in the Systemic and Mucosal Immune Response

The body weights of the animals in the control and treated groups were carefully monitored. Animals were weighed prior to each dose administration. The average weight of the animals in the groups fell in the range of 25 ± 1 g. By intradermal route of administering the garlic lectins or OVA, there is no significant change in the body weights of animals in the treated group as compared to control group. Though there is a slight increase in the body weight of ~ 0.5 to 1 g in the control group, and only a slight change in the treated group, the results are not very significant. This clearly shows that the dietary garlic lectins and OVA do not have any effect on the growth and body weight of animals when administered by intradermal route.

By intranasal route of administration of garlic lectins and OVA, only control group shows a significant (P< 0.05) increase in weight of 1-2 g. The treated group does not show any significant increase or decrease in body weights. The slight changes in the weights of treated group from day 0 to day 50 may be due to difference in the metabolic activity of animals in different treated groups. These observations clearly indicates that the body weights of animals remain fairly constant after the administration of garlic lectins by intranasal route suggesting that the garlic lectins are not growth-retardant, and are not toxic (Tables not shown).

3.2. Systemic and Mucosal Anti-Lectin Immune Response

The effect of OVA and garlic lectins (ASA I and ASA II) was studied without adjuvant on the systemic immune response, by intradermal administration in BALB/c mice. OVA is a well-known weak antigen and hence serve as a control protein. The anti-lectin IgG response in comparison to the response from OVA provides a measure of the systemic response of garlic lectins. The anti-lectin IgG response by intradermal route of administration is shown in Fig. 1A. The IgG response to OVA on days 7 and 14 are similar, and there is no significant increase in anti-OVA IgG on day 14 as compared to day 7. However, both lectins ASA I and ASA II showed a significant increase in anti-lectin antibodies on day 14 compared to day 7. The anti-lectin antibody response on day 14 was found to be higher (significant at the level of $P < 0.005$) for both ASA I and ASA II as compared to control protein OVA indicating that garlic lectins are strong systemic immunogens. IgG response to ASA I on day 14 is very marked as it shows ~3.5 - 4 fold increase in comparison to OVA response. However, ASA II shows only a 2.5 fold higher IgG response compared to the response shown by OVA.

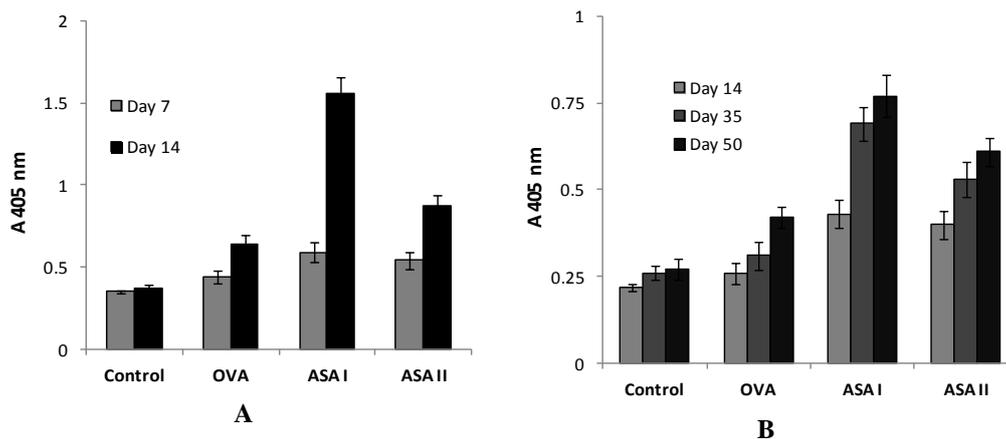


Fig. 1: A. OVA and anti-lectin IgG response after immunization of antigens in BALB/c mice. A. Systemic immune (IgG) response against proteins (OVA, ASA I and ASA II) measured from the serum of animals (BALB/c mice) administered with OVA, ASA I, or ASA II by intradermal route. Amount of antigen: 10 $\mu\text{g/well}$. Serum dilution, 1:10 (volume: 100 $\mu\text{L/mL}$). B. Anti-protein IgG response against proteins (OVA, ASA I and ASA II) measured in the serum of animals (BALB/c mice) administered with OVA, ASA I and ASA II by intranasal route. Antigen amount: 10 $\mu\text{g/well}$. Serum dilution, 1:10 (volume 100 $\mu\text{L/mL}$).

OVA and garlic lectins were administered by intranasal route to study the mucosal immune response. Serum obtained at different time periods was estimated for anti-protein IgG response and the results are shown in Fig. 1A. The anti-lectin IgG response on day 50 is represented in Fig. 1B. OVA shows a marginal increase in anti-OVA IgG response compared to naïve control group and there is a slight increase in the response from day 14 to day 35 and 50, which indicates that the anti-OVA response increase in relation to dose of administration; the increase in response to dose is significant at $p < 0.01$ and is not significant at the level of $p < 0.001$. The anti-lectin IgG response to garlic lectins is significantly higher as compared to the response for OVA (significant at $p < 0.001$). The mucosal IgG response to ASA I is markedly strong (2-2.5 fold increase), whereas in the case of ASA II, the response is 1.75 to 2-fold higher as compared to OVA group. These results indicate that ASA I is a stronger mucosal immunogen compared to ASA II. This may be due to the difference in their binding ability to cell surface glycoproteins involved in immune response.

3.3. Spleen and Thymus Weights and Their Indices in Control and Treated Groups

The spleen and thymus were isolated on day 14 for i.d group and day 50 for i.n group after sacrificing the animals in the control and treated groups. Their weights were measured to assess the activation of spleen or thymus by the treated antigens (OVA, ASA I or ASA II). The results are summarized in Table 1. The spleen and thymus weights were slightly found increased between control and treated groups. However, no significant difference was found between OVA and garlic lectin group indicating the possibility of stimulation in the OVA and lectin groups. The spleen and thymus indices were measured and are shown in Fig. 2 respectively. The indices are known to measure the difference accurately among the control and

treated groups. The ASAI group is known to stimulate both spleen and thymus compared to OVA and ASAII.

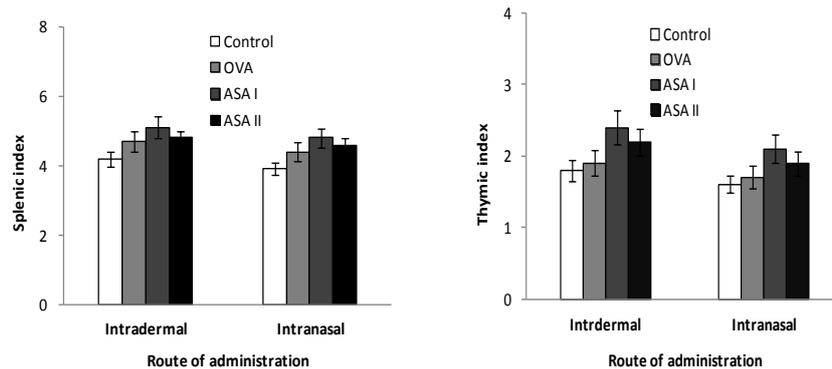


Fig. 2: Splenic and thymic indices of the control and tested groups after intradermal and intranasal immunization of antigens

Table 1. Spleen and thymus weights (in mg) after administration of test samples by intradermal and intranasal routes

Samples	Intradermal route*		Intranasal route [#]	
	Thymus (in mg) Mean ± SD	Spleen (in mg) Mean ± SD	Thymus (in mg) Mean ± SD	Spleen (in mg) Mean ± SD
Control	43.66 ± 1.25	105.33 ± 08.37	39.20 ± 5.58	106.03 ± 10.53
OVA [†]	47.33 ± 7.03	133.83 ± 07.77	41.70 ± 2.65	119.28 ± 08.91
ASA I [†]	54.66 ± 2.49	136.16 ± 05.01	49.37 ± 4.85	127.28 ± 25.66
ASA II [†]	52.33 ± 3.29	129.16 ± 11.60	43.53 ± 1.64	118.15 ± 19.10

*spleen and thymus obtained on day 14; n=6 in each group; the body weight is expressed as mean weight of animals in a group. [#]spleen and thymus obtained on day 50; n=6 in each group; the body weight is expressed as mean weight of animals in a group significant at $p < 0.01$.

3.4. Mucosal Adjuvant Activity of Garlic Agglutinins ASA I and ASA II

Garlic lectins ASA I and ASA II were examined for their ability to induce both systemic and mucosal immune responses by intradermal and intranasal routes of administration. Both the lectins were found to be highly immunogenic as compared to protein OVA. These lectins were further analyzed for their ability to enhance IgG antibody response against a co-administered poor antigen OVA to assess their adjuvanticity.

Con A, the prototype lectin having glc/man specificity, and RGE containing all the components of whole garlic were also used for comparing the in vivo effects of garlic lectins by mucosal route. In the test group where OVA was administered with garlic lectins in the dose, ASA I group shows enhanced anti-OVA IgG response as compared to the response in the group where OVA alone was administered. The IgG response seen in OVA+ASA I group was significantly higher as compared to OVA+ASA II group. The OVA+ASA I group shows ~2 fold higher (significant at $p \leq 0.005$) anti-OVA IgG response compared to OVA group (Fig. 3A). However, OVA+ASA II group shows only a marginal increase which is not significant at $p \leq 0.005$ as compared to OVA group. The OVA+con A group shows similar results as that of OVA+ASA I group. The OVA+RGE group shows maximum anti-OVA response, and is slightly more compared to ASA I and con A groups (Figs. 3A).

The OVA-specific IgG response in control and treated groups was measured for its titre by serial dilution of serum. The results are shown in Fig. 3B. The antibody titre from the sera collected on day 50 after sacrificing the animals of both control and treated groups were measured. It was observed that the anti-OVA IgG response was stronger in OVA+ASA I and OVA+con A groups as compared to OVA and OVA+ASA II groups. Detectable antibody titre was observed at 1:10000 serum dilution only for OVA+ASA I, OVA+con A, and OVA+RGE groups. For OVA and OVA+ASA II, groups, significant antibody detection was seen only at 1: 100 serum dilution. These observations clearly indicate that ASA I, Con A and RGE enhance the antibody production against co-administered antigens and can act as an useful adjuvant to boost the immune response against the weak immunogenic antigen, namely, OVA.

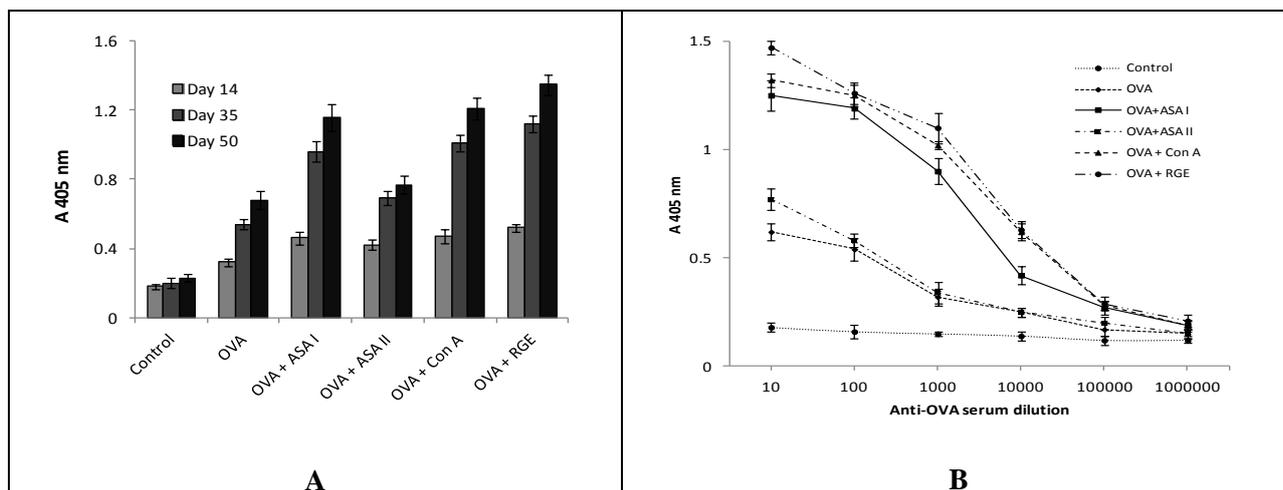


Fig. 3: A. Anti-ovalbumin IgG response after ovalbumin was co-administered with two garlic lectins by intranasal immunization. A. Anti-OVA IgG response against OVA alone, and OVA with garlic components or con A at different times of dose administration. Coating antigen: 10 μ g (100 μ L of 0.1 mg/mL concentration); serum dilution, 1:10. B. The anti-OVA antibody titre (ELISA value) for control and treated groups (OVA+ASA I, OVA+ASA II, OVA+Con A, OVA+RGE). The initial dilution started with 1:10 dilution, and was subsequently serially diluted by 10-fold using the coating buffer

4. Conclusion

Dietary lectins can enter into systemic circulation and stimulate or inhibit the activation of immune cells depending on their lectin-cellular interactions. Dietary lectins can also make a passage for the co-existent poorly antigenic dietary proteins, and can act as adjuvants in boosting the specific immune response against co-administered antigens. Garlic lectins, in particular ASA I, exhibit strong systemic responses by both intradermal and intranasal administration. Hence, it can be logically stated that ASA I is a strong dietary immunogen from garlic and also possesses mucosal adjuvant effect on co-administered food antigens. From the present observations, it can be clearly inferred that garlic lectins, upon mucosal administration, can trigger both systemic and mucosal immune responses. In conclusion, it appears that garlic lectins, in particular ASA I, have the potential for use as adjuvants in boosting the immune response of co-administered antigens. To enhance the efficacy of new generation of (generally) poorly immunogenic recombinant ‘subunit’ vaccines, there is an increase demand for adjuvants and delivery systems that are effective when administered by mucosal routes [7] and [8]. To date, the most effective mucosal adjuvants are the lectin subunits of A-B toxins from *Vibrio cholerae* (cholera toxin; CT) and *Escherichia coli* (heat-labile enterotoxin; LT) and their derivatives [10]. A nontoxic dietary lectin ASA I, promise to be a potential future adjuvant for IgG antibody response against poorly immunogenic antigens.

5. References

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