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Identification of the protein components displaying immunomodulatory activity in aged garlic extract

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ABSTRACT

Ethnopharmacological relevance: Traditionally, garlic (*Allium sativum* L.; Alliaceae) has been known to boost the immune system. Aged garlic has more potent immunomodulatory effects than raw garlic. These effects have been attributed to the transformed organosulfur compounds; the identity of the immunomodulatory proteins in aged garlic extract (AGE) is not known.
Aim of the study: The major aims are to examine the changes occurring in the protein fraction during ageing of garlic and to identify the immunomodulatory proteins.
Materials and methods: Changes occurring in garlic during ageing have been examined by protein quantitation and gel electrophoresis. Purification and identification of the immunomodulatory proteins have been achieved by Q-Sepharose chromatography and mitogenic activity.
Results: Only two major proteins (12–14 kDa range by SDS-PAGE) are observed in AGE. The purified protein components QA-1, QA-2, and QA-3 display immunomodulatory and mannose-binding activity; QA-2 shows the highest mitogenic activity. The identity of QA-2 and QA-1 proteins with the garlic lectins ASA I and ASA II, respectively, has been confirmed by hemagglutination analysis. QA-3 exhibits mitogenic activity, but no hemagglutination activity.
Conclusions: The immunomodulatory activity of AGE is also contributed by immunomodulatory proteins. The major immunomodulatory proteins have been identified as the well-known garlic lectins.

1. Introduction

Many health-beneficial effects of garlic, *Allium sativum* L. (Alliaceae), have been demonstrated based on its diverse biological and pharmacologic effects observed experimentally and clinically (Koch and Lawson, 1996; Amagase et al., 2001; Amagase, 2006). Garlic and its organosulfur compounds have been shown to reduce risk factors for cardiovascular diseases (Lau et al., 1991; Campbell et al., 2001), to suppress cancer cell growth in vitro (Sigounas et al., 1997) and in vivo (Katsuki et al., 2006; Ishikawa et al., 2006). Furthermore, garlic has been shown to be a possible immune response modifier (Lau et al., 1991).

Aged garlic extract (AGE) is an odorless product prepared by prolonged aqueous extraction of fresh garlic for approximately 20 months; it has been reported to have an array of pharmacologic effects, including immunomodulation (Kyo et al., 2001; Yeh and Liu, 2001; Arunkumar et al., 2005; Biren et al., 2006). AGE contains stable, water-soluble organosulfur compounds that have been thought to be the bioactive principles for numerous health benefits (Yeh and Liu, 2001; Yuncu et al., 2006; Gardner et al., 2007). The strong tumor inhibiting properties of S-allyl mercaptocysteine (SAMC) for prostate, colon and breast cancers were discovered only after its isolation from aged garlic extract (Sigounas et al., 1997).

The majority of the immunomodulatory actions of garlic have been well studied using purified organosulfur compounds. However, garlic's immunomodulatory effects exerted by protein(s) have been reported rarely. It is believed that a low mol. wt. protein (~14 kDa) is responsible for garlic's immunomodulatory effect (Ghazanfari et al., 2002; Hassan et al., 2003). However, it is not clear whether the protein immunomodulatory effect is due to a single protein or a mixture of proteins (Colic and Savic, 2000; Colic et al., 2002), or due to their association with organosulfur compounds. Hirao et al. (1987) reported that the F-4 protein fraction isolated from AGE stimulated the activities of macrophages and spleen cells. Additional reports on protein immunomodulators in garlic indicated the presence of a low mol. wt. glycoprotein (11 or 14 kDa) (Morioka et al., 1993; Ghazanfari et al., 2002; Hassan et al., 2003). Although these studies have described the detection of immunomodulatory proteins varying from 8 to 14.5 kDa, their isolation, identity and characterization have not been described so far. There are claims that they are glycoproteins devoid of agglutinin activity, indicating that the proteins with immunomodulatory

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property appear to be different from the well-known lectins present in garlic (Van Damme et al., 1992; Smeets et al., 1997).

Only a few studies have described the presence of immunomodulatory protein components isolated from the protein fraction of AGE. However, these immunomodulatory proteins have not been characterized in detail, and their identities are unknown. In the present study, the changes occurring in the protein fraction of garlic during the ageing process and the isolation of the protein components from AGE are described. The purified protein components have been evaluated for their immunomodulatory activities on murine thymocytes and splenocytes.

2. Materials and methods

2.1. Chemicals and reagents

Ovalbumin (OVA), bovine serum albumin (BSA), avidin, lysozyme (LSZ), insulin, and avidin–alkaline phosphatase (ALP) were purchased from Bangalore Genei, Bangalore, India. Concanavalin A (Con A), Q-Sepharose FF anion-exchange resin (bead size: 24–44 μm), phytohemagglutinin (PHA), Ficoll-hypaque, and RPMI-1640 medium were products of Sigma–Aldrich Co., St. Louis, MO, USA. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide A.R.] was a product of HiMedia Laboratories Ltd., Mumbai, India. Fetal calf serum (FCS) was obtained from Sera-Lab (Sussex, England). Flat-bottom 96-well microtiter plates (MICROLON) were purchased from Grenier Bio-One GmbH, Frickenhausen, Germany. Tissue culture plates (24-well, gamma-sterilized) were procured from Tarsons Products Ltd., Kolkata, India. All other chemicals and reagents used were of analytical grade. Garlic used in this study was procured from the local grocery.

2.2. Experimental animals

Thymus was obtained from 8- to 10-week-old BALB/c mice (25 ± 2 g), and spleen was obtained from 12- to 14-week-old CFT Wistar rats (275 ± 2 g) for the isolation of thymocytes and splenocytes, respectively. 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.

2.3. Preparation of aged garlic extract (AGE)

AGE was prepared by extracting fresh garlic in 25% ethanol for a period of 20 months as described earlier by Hirao et al. (1987). Garlic bulbs (500 g) were separated into cloves, cleansed and the skin was peeled off. The peeled cloves (~400 g) were cut into small pieces and soaked in 500 mL of 25% ethanol in a closed glass jar, and aged naturally at ambient temperature (25 °C). Aged garlic extract was then decanted using muslin cloth. Decanted extract was filtered through Whatman No.1 filter paper and the clear solution was stored at −20 °C for further studies. Protein quantitation of aged garlic extract or chromatographic pools was performed using the dye-binding method of Bradford (1976), using BSA as standard.

2.4. Re-extraction of aged garlic

After preparing AGE by incubating garlic pieces for 10 months, the retained garlic pieces were air dried for 3–4 days, and then incubated in 500 mL of 25% ethanol in a closed glass jar. Aliquots of re-extracted solution were taken out at different time intervals (up to a period of 4 months) for protein quantitation as well as analysis by SDS-PAGE.

2.5. SDS-PAGE analysis

Weekly aged, fortnightly aged, and monthly aged garlic extracts have been used for the analysis of changes in protein pattern occurring during ageing process. SDS-PAGE (18%, reducing) was performed as per Laemmli (1970). Gels were visualized by staining with Coomassie brilliant blue R-250. Protein profiles of garlic extract aged for different times were then compared with the protein profile of raw garlic extract (RGE).

2.6. Isolation of proteins from aged garlic extract

Aged garlic extract prepared as described above was either stored as such at −20 °C or in a lyophilized state. In order to obtain only the protein components free from the organosulfur garlic oil compounds, 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, Beford, MA, USA), and the retentate was dialyzed using 3.5 kDa cut-off dialysis membranes. Separation of different proteins present in the retentate fraction of AGE was carried out by anion-exchange chromatography.

2.7. Anion-exchange chromatography on Q-Sepharose

One hundred milliliters of aged garlic extract (derived from ~120 g raw garlic) was concentrated by ultrafiltration using 3 kDa cut-off membrane disc filter, followed by ammonium sulfate precipitation (at 90% saturation) and lyophilization. AGE (lyophilized material) was subjected to Q-Sepharose FF (0.8 cm × 8 cm) chromatography. The column had been pre-equilibrated with 10 mM Tris–HCl buffer, pH 8.0. After adsorption of the sample and washing with 150 mL of the above buffer, the bound proteins were eluted by step-wise elution using the same buffer containing different concentrations of NaCl (0.1–2 M). The presence of protein in different step-eluate fractions was monitored by absorbance at 280 nm. The major components were then pooled, concentrated, dialyzed and lyophilized.

2.8. Hemagglutination (HA) assay

HA assay was performed as described by Burger (1974). Five milliliters of rabbit blood was collected and put into 5 mL of Alsever's solution (20.59 g glucose, 8 g tri-sodium citrate dihydrate, 4.29 g NaCl and 0.55 g citric acid dissolved and made up to 200 mL using distilled water; working dilution—1:5), swirled and mixed thoroughly. After centrifugation at 1000 × *g* in the cold for 10 min, the supernatant was removed and the pelleted erythrocytes was washed 3–4 times with saline (0.9% NaCl), and finally resuspended in PBS, pH 7.4. After adjusting the erythrocyte suspension to 2% erythrocytes concentration in a Klett–Summerson photoelectric colorimeter with 660 nm filter (model 8003, Arthur H. Thomas Co., Philadelphia, PA, USA; a reading of 400 represents 2% erythrocytes concentration), 1% crude trypsin was added and incubated at 37 °C for 1 h. After incubation, the cells were centrifuged at 1000 × *g* at 4 °C for 10 min, washed with saline and readjusted to 2% concentration for use in the hemagglutination assay.

HA activity of the purified proteins from AGE was carried out as follows: briefly, a 2% suspension of rabbit erythrocytes (0.2 mL) was added to a serially diluted protein solution in a hemagglutination plate, gently mixed and incubated at 37 °C for 1 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 hemagglutination unit (HAU) is equivalent to concentration of protein in the last well that completely agglutinates an equal volume of standardized RBC suspension. Hemagglutination 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.

2.9. Glycoprotein-binding assay of purified aged garlic extract proteins

Microtiter wells were coated with 10 μg of purified aged garlic extract proteins (QA-1, QA-2, and QA-3) in 0.1 M carbonate-bicarbonate buffer, pH 9.6 at 4 °C overnight. After the blocking step using 2% gelatin in PBS, the microtiter wells were incubated with avidin–ALP 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 substrate (*p*-nitrophenyl phosphate), the absorbance was measured after 30 min at 405 nm. Inhibition experiments were performed using D-mannose (final concentration, 200 mM) incubated with avidin–ALP at 37 °C for 1 h separately, prior to transferring to the microtiter plate.

2.10. Isolation of thymocytes and splenocytes

Thymocytes and splenocytes were isolated as described by Colic et al. (2002). Since weaning and younger rats (<4-week-old) were unavailable for isolation of thymus, younger mice (<4-week-old) was used for isolation of thymocytes. Thymus and spleen was collected under aseptic conditions from BALB/c mice and Wistar rats, respectively. After centrifugation (380 × *g* at 4 °C for 10 min), the pelleted cells were washed three times with PBS (400 × *g* at 4 °C for 10 min), and finally resuspended in complete RPMI-1640 medium.

The isolated murine thymocytes and splenocytes were counted using trypan blue (0.2%) stain. After mixing the cell suspension with Trypan blue (10 μL each) and diluting with 250 μL of diluent buffer (PBS with 1% BSA), the cells were observed and counted in the outer four chambers of an improved Neubauer (0.1 mm deep) hemocytometer. Percentage viability of thymocytes and splenocytes in the isolated cell suspension was checked by Trypan blue exclusion method. Cells, which are dead or partially damaged, appear as dark blue against a light blue background; viable cells appear clear without any stain against the light blue background.

2.11. MTT assay for cell proliferation

The mitogenic activity of protein components was followed by MTT assay (Mosmann, 1983). The assay was performed in 96-well tissue culture plates; the wells received 100 μL of RPMI-1640 complete medium containing the purified proteins (1–5 μg), followed by 100 μL cell suspension (thymocytes: 1×10^5 cells/mL or splenocytes: 1×10^6 cells/mL). The culture plates were incubated in a CO_2 incubator (5% CO_2) at 37 °C for 72 h. Thymocytes or splenocytes in the absence of proteins represent control, and blank was carried out with complete medium only. After incubation, 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 aspirated to an Eppendorf tube, and centrifuged at 750 × *g* 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 positive control (PHA and Con A).

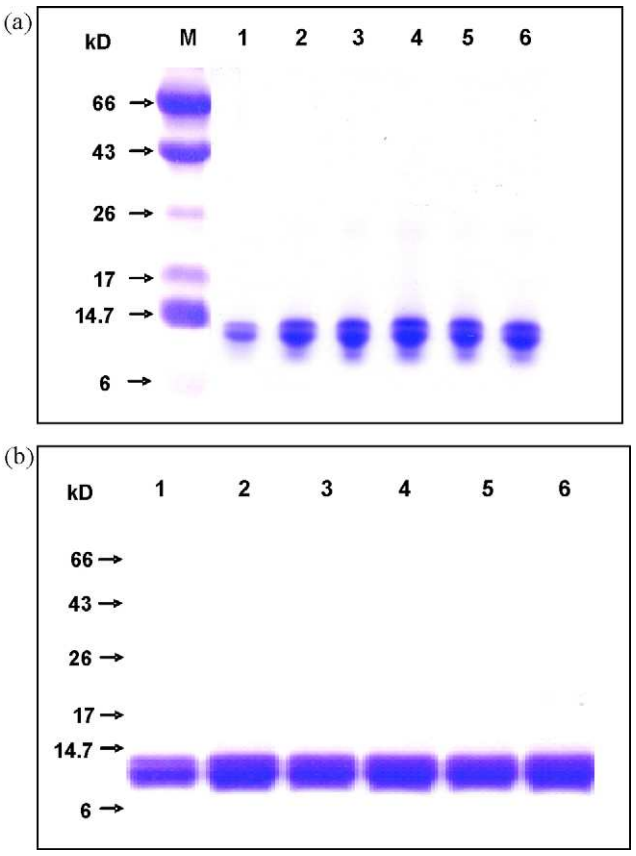

Fig. 1. SDS-PAGE (18%, reducing) analysis of garlic incubated in 25% ethanol for various periods. Staining: Coomassie blue. Protein load: 10 μg. (a) Analysis of 1–6-week-old garlic extracts. Lane M refers to mol. wt. markers; lanes 1–6: 1-week to 6-week-old aged garlic extracts, respectively. (b) Analysis of 15–90-days-old garlic extracts. The numbers (in kD) on the left represent mol. wt. markers; lanes 1–6 represent 15, 30, 45, 60, 75, and 90 d-old garlic extracts, respectively.

2.12. Statistical analysis

All assays were performed in triplicate. Results are expressed as mean ± standard deviation (S.D.). Data were analyzed by one-way analysis of variance (ANOVA) and Student's *t*-test to determine the statistical significance. A *p*-value of <0.05 was considered to be statistically significant. The statistical analysis was performed using SPSS Software Package 10.0 version (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Changes in protein amounts and fractions during ageing process

The protein content of aqueous garlic extract incubated for various periods from 1 to 4 weeks was found to be 1.1 (1 week), 1.2 (2 weeks), 1.9 (3 weeks), and 2.0 (4 weeks) mg/mL. The detection of proteins in aqueous garlic extracts was seen as early as 1 week during the ageing process, and remained fairly constant after 4 weeks up to a period of 10 weeks (data not shown). The SDS-PAGE analysis (reducing) of garlic extracts prepared at weekly intervals for 6 weeks is shown in Fig. 1(a). It is very clear that only one intense band (as a doublet) having a molecular mass of ~12–13 kDa is seen in all the samples analyzed. Further, aqueous garlic extracts incubated

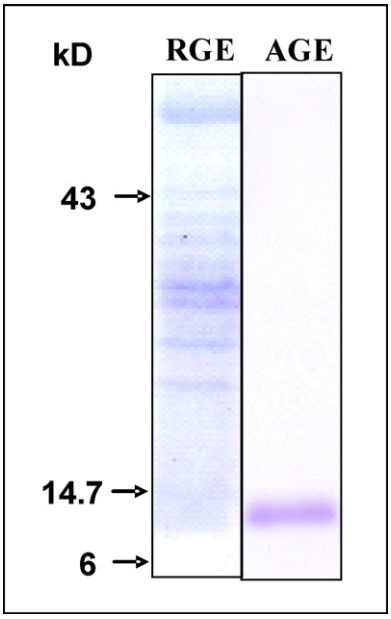

Fig. 2. Analysis of aged and raw garlic extracts by SDS-PAGE (18%, reducing). Protein load: 10 μg. The numbers (in kD) on the left represent mol. wt. markers; RGE: raw garlic extract (fresh); AGE: aged garlic extract (1-week-old).

for various periods from 15 to 90 days were analyzed by SDS-PAGE. The gel pattern is shown in Fig. 1(b). In all the samples, an intense doublet around ~12–13 kDa is seen, and the protein amounts are constant in all samples excepting the 15-day sample. Aqueous garlic extracts incubated for periods beyond 3 months, and up to 20 months showed similar SDS-PAGE patterns (data not shown). Typically, ~250 mg protein is obtained per 100 g of starting material (raw garlic).

The protein content of re-extracted aged garlic was analyzed from 1 to 4 months, and the results were found to be as follows: 0.22 (1 month), 0.32 (1½ months), 0.46 (2 months), and 0.46 (4 months) mg/mL. SDS-PAGE analysis (reducing) of re-extracted aged garlic showed a single clear band of ~13 kDa molecular mass in all the samples analyzed similar to that seen in the case of aged garlic extract (data not shown). Thus, only 25% of protein amount are present in the re-extracted aged garlic at 2 months compared to the protein amount of aged garlic extract at 3–4 weeks.

3.2. Comparison of aged garlic extract with raw garlic extract (RGE)

In order to compare the SDS-PAGE profile of aged garlic extract with fresh garlic extract, a sample of raw garlic extract (RGE) was prepared using distilled water (without an incubation period or incubation in 25% ethanol). The SDS-PAGE patterns (reducing) are shown in Fig. 2, wherein it can be seen that there is only a doublet in AGE whereas 6–8 protein bands in the molecular mass range of 12–70 kDa are seen in RGE. Only the 12–13 kDa doublet is common between the two extracts. The presence of protein bands in the 45–70 kDa molecular mass range may be attributed to the presence of the subunits of alliinase and the high molecular weight mannose-specific agglutinin (*Allium sativum* agglutinin of 110 kDa or ASA_{110}) reported earlier (Rabinkov et al., 1994; Gupta and Sandhu, 1997).

3.3. Separation of proteins present in AGE by anion-exchange chromatography

Aged garlic extract was subjected to ultrafiltration using 3 kDa membrane disc filter followed by dialysis of the retentate using

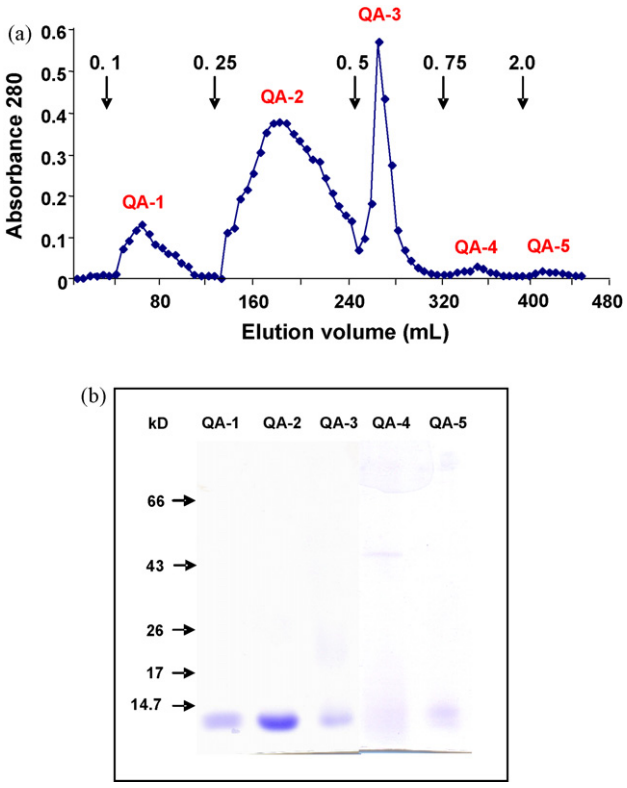

Fig. 3. Q-Sepharose chromatography of AGE. (a) Q-Sepharose FF (0.8 cm × 8 cm) chromatography of AGE by step-wise elution using different concentrations of NaCl in 10 mM Tris–HCl buffer, pH 8. Arrows indicate the point of application of buffer containing the indicated NaCl concentration (M): QA-1, 0.1 M; QA-2, 0.25 M; QA-3, 0.5 M; QA-4, 0.75 M; QA-5, 2 M. Protein load: 55 mg. Absorbance at 280 nm indicates protein detection. (b) SDS-PAGE (18%, reducing) of AGE and its Q-Sepharose components. Stain: Coomassie blue. Protein load: 7.5 μg. Lanes QA-1 to QA-5 represents the respective components of Q-Sepharose step-elution.

3.5 kDa cut-off dialysis membranes. Anion-exchange chromatography was carried out on Q-Sepharose at pH 8. Fig. 3(a) represents the elution profile of AGE using step-wise NaCl elutions in 10 mM Tris–HCl buffer, pH 8. Three major peaks labeled QA-1, QA-2, and QA-3 were observed at step-elutions of 0.1, 0.25, and 0.5 M NaCl concentrations, respectively. Two very minor peaks (QA-4 and QA-5) were observed with 0.75 and 2 M NaCl elutions, respectively. The magnitude of QA-3 peak as monitored by A_{280} varied somewhat between chromatographic runs. The fractions representing QA-3 were found to be slightly yellowish; this may be due to the coelution of an acidic organosulfur compound. SDS-PAGE analysis of these protein components are shown in Fig. 3(b). All the protein components appeared as a single band or doublet around ~12–13 kDa molecular mass.

Components QA-1 and QA-2 were found to be active by hemagglutination (HA) assay; however, the other 3 components (QA-3, QA-4 and QA-5) were devoid of hemagglutination activity. The specific HA activity of the various components of Q-Sepharose chromatography is shown in Table 1. It can be seen from this data that the specific HA activity of QA-1 component is ~50% of that of QA-2.

3.4. Glycoprotein-binding assay for purified proteins from AGE

In order to confirm the binding of purified proteins from AGE to the mannose residues of *N*-linked glycoproteins, we used avidin–ALP conjugate as a source of glycan (avidin contains 13% glycans while ALP is a non-glycoprotein). Con A was used as reference lectin having glucose/mannose specificity. Remarkable binding of avidin–ALP was seen in the case of all the three purified proteins

Table 1
Hemagglutination (HA) activity of aged garlic extract fractionated by Q-Sepharose FF chromatography.

Sample	Starting protein concn. (μg/mL)	HA	HA titer (μg/mL)	Specific HA activity (units/mg)
QA-1[a]	25.0	+	0.780	1280
QA-2	25.0	+	0.390	2560
QA-3	62.5	–	0	0
QA-4	62.5	–	0	0
QA-5	62.5	–	0	0

[a] QA-1 to QA-5 represent the protein components in order of elution from Q-Sepharose chromatography of aged garlic extract as shown in Fig. 3(a).

from AGE (Table 2). Approximately 42–45% inhibition in binding was seen in the case of incubation with 200 mM D-mannose for QA-1, QA-2, and QA-3. Con A showed a similar magnitude of binding and inhibition with mannose.

3.5. Immunomodulatory activity

The mitogenic activity of Q-Sepharose fractionated aged garlic extract (QA-1 to QA-5 pools) towards murine thymocytes was studied in vitro, and the results are shown in Fig. 4(a). Phytohemagglutinin (PHA) and Con A were used as positive controls at both suboptimal (1 μg/mL) and optimal (4 μg/mL) concentrations. QA-2 pool showed the maximum mitogenic activity followed by QA-1/QA-4, QA-5, and QA-3 components. It should be noted here that though the protein components representing QA-3, QA-4, and QA-5 did not possess hemagglutination activity, these components exhibited considerable mitogenic activity. In comparison to Con A, the proliferation activity of QA-2 and QA-1 were 2.9 and 1.7, respectively.

The proliferative effect of Q-Sepharose components on rat splenocytes is shown in Fig. 4(b). Here also, all the five components of AGE induced proliferation of splenocytes. QA-2 component showed the highest mitogenic activity followed by QA-5, QA-3/QA-4, and QA-1. In comparison to Con A, the proliferation activity of QA-2 showed a 1.8-fold increase, while that of QA-1 was equivalent to that of the positive control Con A.

4. Discussion

Many preclinical and clinical studies have shown that AGE possesses superior diverse biological effects through immune enhancement and chemopreventive activities, in comparison to fresh garlic extract (Kyo et al., 2001; Biren et al., 2006). Many of these effects have been attributed to its antioxidant activity. AGE is known to contain fructans (fructose polymers), stable water-soluble sulfydryl compounds such as SAMC and S-allyl cysteine (SAC), certain proteins and free amino acids and their derivatives, most notably fructosyl-arginine (Koch and Lawson, 1996). Other garlic supplements, or even raw garlic, do not contain all of these compounds, which only become available during the ageing process (Koch and Lawson, 1996; Sigounas et al., 1997; Amagase et al., 2001; Kyo et al., 2001; Biren et al., 2006).

Table 2
Glycoprotein-binding assay for purified proteins from aged garlic extract and its inhibition by mannose.

Protein sample[a]	Absorbance at 405 nm[b] (without mannose)	Absorbance at 405 nm[b] (with 200 mM mannose)
QA-1	0.351	0.192
QA-2	0.300	0.173
QA-3	0.359	0.200
Con A	0.279	0.156

[a] Amount of protein coated: 10 μg.
[b] Mean of triplicate values; avidin–ALP dilution, 1:2000; absorbance value for reagent control = 0.108.

In the present study, changes occurring in the protein fraction of garlic subjected to ageing process have been investigated. Proteins appeared as early as 1 week during the ageing process. After 4 weeks of ageing in ethanolic solution, the amount of protein remained fairly constant. SDS-PAGE analysis (reducing) showed only the presence of a doublet around 13 kDa. Compared with raw garlic extract (RGE), high mol. wt. agglutinins like ASA_{110} and alliinase, and other protein components were conspicuously absent in AGE. Alliinase (110 kDa) is a two-subunit glycoprotein of 55 kDa subunits each (Rabinkov et al., 1994). ASA_{110} is a high molecular weight glycoprotein agglutinin of two identical subunits of 47 kDa (Gupta and Sandhu, 1997). It is not clear why these two abundant proteins are not released during the ageing process.

At least two proteins have been separated from aged garlic extract by anion-exchange chromatography. Based on the demonstration of hemagglutination activity in QA-1 and QA-2 protein

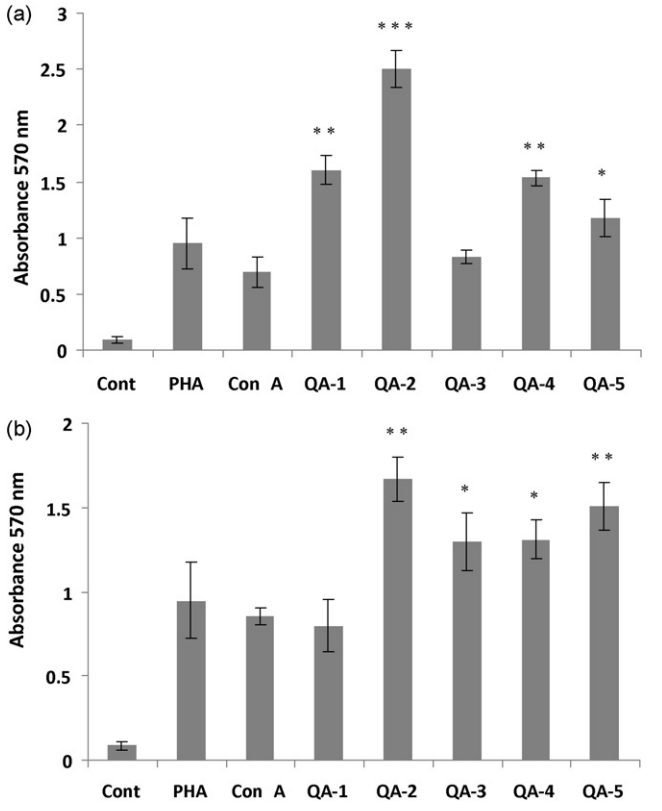

Fig. 4. Mitogenic activity of protein components isolated from AGE by MTT assay. (a) Proliferation of BALB/c mouse thymocytes (1×10^5 cells/mL) by Q-Sepharose components of AGE. Protein amount: 1 μg. Positive controls: concanavalin A (Con A) and phytohemagglutinin (PHA). Cont represents control. The proliferation activity was measured as the absorbance at 570 nm; values are mean ± S.D. ($n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. positive control Con A. (b) Proliferation of rat splenocytes (1×10^6 cells/mL) by Q-Sepharose components of AGE. Protein amount: 4 μg. Positive controls: concanavalin A (Con A) and phytohemagglutinin (PHA). Cont represents control. The proliferation activity was measured as the absorbance at 570 nm; values are mean ± S.D. ($n = 3$). * $p < 0.05$, ** $p < 0.01$ vs. positive control Con A.

components, we conclude that these proteins represent ASA II and ASA I (*Allium sativum* agglutinins II and I), respectively. These two agglutinins have molecular masses of 25 kDa (known as ASA_{25}); ASA II is a homodimer of two 12.5 kDa subunits, while ASA I is a heterodimer of 11.5 and 12.5 kDa subunits. Both ASA I and ASA II are known to have strict specificity for mannose residues in glycoproteins (Dam et al., 1998; Bachhawat et al., 2001). Further, it was confirmed that QA-1 and QA-2 are able to bind to mannose-containing glycans of a glycoprotein such as avidin–ALP; this glycan binding could be inhibited using mannose. QA-3 also shows similar results seen in the case of QA-1 and QA-2. It is very likely that QA-3 may represent subunits of either ASA I/ASA II or both, since QA-3 definitely lacks hemagglutination activity.

Although these garlic agglutinins have been cloned, sequenced, expressed, and their carbohydrate specificities defined, very little is known about their immunomodulatory activities. From this study, it is clear that these two agglutinins demonstrated mitogenic activity similar to those shown by the mitogenic lectins, Con A and PHA. It should be noted here that the high molecular weight agglutinin (ASA_{110}) at concentrations of 5–15 μg/mL stimulated guinea pig and human peripheral blood lymphocytes (Gupta and Sandhu, 1996).

The presence of an immunomodulatory protein of ~13 kDa was first reported by Hirao et al. (1987); this protein did not exhibit hemagglutination activity. The presence of lectin or agglutinin activity in garlic was first described by Van Damme et al. (1992) in the early 1990s. Although many researchers have reported the presence of immunomodulatory proteins in aged garlic extract prior to the detection of agglutinins in garlic, their identities with agglutinins have escaped the attention of several investigators so far. This situation probably arose due to the detection of immunomodulatory proteins much before the detection of agglutinins in garlic. Much of the research work have shown the potential immunomodulatory and immunotherapeutic potentials of AGE as a whole, in various conditions like cardiovascular diseases, infectious diseases, cancer, autoimmune diseases and allergy (Amagase et al., 2001; Kyo et al., 2001; Biren et al., 2006).

This study describes the isolation of different components of aged garlic extract having immunomodulatory activity, and confirms their identities with the well-studied low molecular weight garlic agglutinins, ASA_{25}. Among the two ASA_{25} agglutinins, ASA I is twice as potent as ASA II in terms of hemagglutination and mitogenic activities. Thus, it appears that the potent immunomodulatory effects of AGE is contributed by ASA_{25} agglutinins in addition to the transformed organosulfur compounds which have been investigated in detail earlier (Horie et al., 1992; Takeyama et al., 1993; Sigounas et al., 1997; Salman et al., 1999). The presence of very small amounts of other immunomodulatory proteins devoid of hemagglutination activity may actually represent subunit forms of ASA_{25}. With the availability of commercially pure organosulfur compounds, it will be interesting to investigate the synergistic effect of the two agglutinins (ASA I and ASA II) and the individual organosulfur compounds of AGE on the immunomodulatory potential in vitro.

5. Conclusions

During the preparation of aged garlic extract, only low molecular weight proteins of 12–14 kDa (reducing electrophoresis conditions) are released. AGE is a complex mixture of many components—the transformed organosulfur compounds, the low molecular weight immunomodulatory proteins, fructans, and several amino acids and their derivatives. The potent immunomodulatory activity of AGE appears to arise from the presence of different classes of substances in a highly concentrated form. The identity of the two major immunomodulatory proteins has been confirmed as garlic agglutinins ASA I and II based on specific hemagglutination and mannose-binding activities. Our work further illustrates the presence of minute amounts of other immunomodulatory proteins which are devoid of hemagglutination activity. A simple procedure for the separation of the immunomodulatory proteins ASA I and II from AGE has been developed. Modulation of immune functions by garlic may contribute to the treatment and prevention of certain diseases caused by immune dysfunction.

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