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Spectrophotometric Assay of Allicin in Iranian Garlic Products

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Allicin (diallyl thiosulfinate) is considered the most important biologically active organosulfur compound found in crushed garlic (*Allium sativum* Linn.). The amount of allicin in Iranian garlics (grown in North) and some commercially available garlic products has been determined by a UV spectroscopic method. This simple spectrophotometric method has the advantage of not requiring an allicin standard for quantitative determination of allicin. It was found that the amount of total thiosulfinates and allicin is slightly different in various garlic extracts and is significantly higher than its quantity reported by others using garlic from different parts of the world. Variations in the amount of thiosulfinates may be related to climate conditions where garlic is grown.

Key Words: Allicin, Thiosulfinates, Garlic products, Spectrophotometric determination, *Allium sativum* Linn.

INTRODUCTION

Garlic (*Allium sativum* Linn) has been widely used as vegetable, spice and for seasoning since ancient times. It has also been known as a medicinal plant applied as a medication for lowering blood pressure, reduction of serum cholesterol and triglycerides and inhibition of platelet formation. Up to now, no satisfactory explanation has been given that how the active biochemical mechanisms in garlic might cause a physiological response in human beings. Allicin (diallyl thiosulfinate), one of the major active compounds of freshly crushed garlic, has a variety of antimicrobial activities[1–4]. It is an inhibitor of sulfydryl-containing enzymes and an unstable chemical compound. Allicin may be degraded in the presence of heat or organic solvents to form a variety of degradation compounds during processing of garlic for the manufacture of some garlic products. All of the thiosulfinates are sulfides and include diallyl mono-, di- and oligosulfides, vinyldithiins and ajoenes[5–7]. The beneficial effects of allicin on lipid profile in hyperlipidemic rabbits and inhibition of cholestrol biosynthesis by allicin in rat hepatocyte cells have been demonstrated[8–10]. Abramovitz *et al.*[11] have shown that allicin reduces the formation of fatty acid streaks (atherosclerosis) in hyperlipidemic mice. The other major beneficial effect of garlic is due to its antithrombotic

actions. This field of garlic research has been extensively studied. Garlic is about 13 times more potent than onion in inhibiting platelet aggregation in raw form[12]. Allicin is the most potent antiplatelet constituents of garlic because of its *in-vitro* effects. Epidemiological studies have suggested that garlic plays a significant role in the reduction of deaths caused by malignant diseases. This has led many investigators to examine garlic and garlic consitituents for their antitumour and cytotoxic actions both *in-vitro* and in laboratory animals. The data from these investigations suggest that garlic contains several potentially important agents that possess antitumour and anticarcinogenic properties[13].

It has been reported that intact garlic cloves contain a group of flavour precursors such as alliin (S-allylcysteine sulfoxide), S-(E)-1-propenylcystein sulfoxide and S-methylcystein sulfoxide. It is well known that the enzyme alliinase, which is activated when the cellular tissue of garlic is disrupted, converts these alk(en)ylcysteine sulfoxides into alk(en)yl thiosulfinates; the pungent principles of raw garlic[14]. Allicin is the major thiosulfinate and represents the main marker for the evaluation of garlic[15].

The quantity of allicin and other thiosulfinates in garlic has been measured using HPLC, TLC and GC by some investigators[16–19]. All of these methods require an allicin internal or external standard or even both for quantitative determination of allicin and other thiosulfinates in garlic. On the other hand, allicin is very unstable and, therefore, must be synthesized just before use as a standard. To avoid this time consuming production of allicin, we have used a special spectrophotometric assay that does not need allicin as standard. This spectrophotometric assay of allicin is a modification of the method used by Han *et al.*[14], and is based on the fact that one molecule of allicin reacts with two molecules of cysteine to form two molecules of S-allyl mercaptocysteine (S-AMC). The unreacted cystein can then be quantified using Ellman's reagent [5,5′-dithio-bis-(2-nitrobenzoic acid), DTNB]. The method can also be used to measure the total concentration of thiosulfinates in garlic extracts and garlic products. In contrast to GC and HPLC, the method has the advantage of not requiring an allicin standard for quantitative determination of allicin or total thiosulfinates.

The purpose of this study was to measure the total concentration of thiosulfinates and therefore, allicin in various Iranian garlic products in order to explain the beneficial effects of garlic as a natural antibiotic.

EXPERIMENTAL

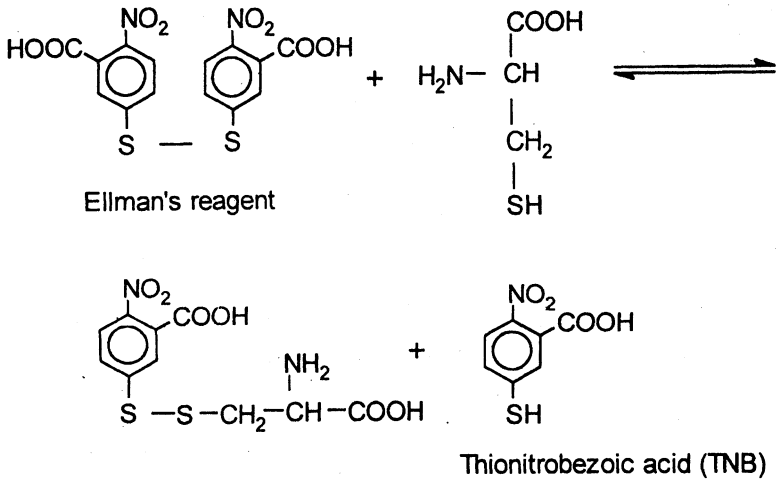
The following chemical compounds were used as supplied by the manufacturer without further purificaiton:

1. L-cystein from Merck Chemical Company.
2. 5,5′-Dithio-bis-(2-nitrobenzoic acid) (DTNB) from Merck.
3. Tris (hydroxymethyl) aminomethane (tris) from Merck.
4. Garlic and garlic powder from the local grocery shop.
5. Garlic tablets from the local pharmacy.

The following solutions were freshly prepared in the laboratory:

1. Tris buffer solution, 1 M tris, pH 8.0
2. 20 mM cystein solution, freshly prepared in this buffer
3. DTNB solution, made by dissolving 50 mM NaOAc and 2 mM DTNB in distilled water.
4. Tris buffer solution, 1 M tris, pH 8.0.
5. Garlic powder, prepared from thin (2–3 mm thick) slices of garlic dried overnight in oven (50°C) and ground into a fine powder.
6. Garlic solution, 1 g of prepared or commercially garlic powder and garlic tablets in 30 mL distilled water.

The method was based on the determination of unreacted cystein (by Ellman's reagent, **Scheme-I**) after its reaction with total thiosulfinates in garlic extracts. A known amount of cystein was added to some garlic extract and the reaction allowed to proceed. The remaining cystein in the reaction mixture was determined using Ellman's reagent. The amount of cysteine used depends on the total concentration of sulfinates. It has been found that the amount of allicin is 60–80% of total garlic thiosulfinates[16]. Therefore, multiplying the value obtained for the total thiosulfinates by 0.7 would estimate the amount of allicin present.



Scheme-I

The reaction of cystein with Ellman's reagent [5,5′-dithio-bis-(2-nitrobenzoic acid), DTNB]

In practice, 0.5 mL of solution 1 was mixed with 0.5 mL of solution 5 and the mixture incubated at room temperature for 15 min (garlic solution). The other reagents (according to Table-1) were added and the reaction was completed after all the contents were mixed. The amount of 2-nitro-5-thiobenzoate (NTB) was determined by measuring the absorbance of the reaction mixture at 412 nm in a 1 cm UV quartz cell. The molar extinction coefficient (1 cm light path) was 14,150 for the liberated NTB anion[20]. As it is shown in Table-1, four concentrations of each garlic sample were used in order to assure the consistency of the results.

The factor of dilution was then considered in calculatin of allicin concentration and the results in Table-2 are the mean value in each case.

TABLE-1
REACTION OF CYSTEIN WITH GARLIC EXTRACTS

Sample	Blank	1	2	3	4
Garlic solution	0	15	20	25	30
DTNB	50	50	50	50	50
Tris	100	100	100	100	100
Water	850	835	830	825	820

TABLE-2
RELATIONSHIP BETWEEN THE AMOUNT OF ALLICIN (mM) IN GARLIC AND THE EXTRACTION CONDITION

Conditions* / Sample†	1	2	3	4	5	6	7
Garlic	5.482	5.498	5.462	5.462	5.468	5.471	5.483
Garlic powder	5.470	5.490	5.450	5.453	5.464	5.450	5.467
Garlic tablets	5.466	5.493	5.454	5.458	5.470	5.454	5.472

*The extraction conditions are as follows:
1. Distilled water only
2. 1 M hydrochloric acid 20 mM
3. 20 mM hydrochloric acid
4. 80°C for 1 h
5. Boiling water for 30 min
6. Boiling water
7. 8 M urea

†The samples were prepared by dissolving 1 g garlic product in 30 mL water.

RESULTS AND DISCUSSION

Each mole of the unreacted cystein remained in the sample producing one mole of NTB on reaction with Ellman's reagent **(Scheme-I)**. The concentration of liberated NTB in samples (in mM) was calculated from their absorbances at 412 nm, using molar absorption coefficient of 14,150 for NTB anion[20]. The amount of NTB (mM) is equal to the concentration of unreacted cystein in each sample (*a* mM). The quantity of allicin in each sample extracted in various conditions is presented in Table-2. It can be seen in this table that the amount of allicin extracted depends on the type of garlic product used.

Calculations

Sample absorbance (A_s) = total volume/sample volume × total absorbance (A_t).
Sample concentration (C_s) = A_s/14150 mM.
Concentration of unreacted cystein = C_s mM.
Concentration of cystein reacted with total thiosulfinates = 20 mM – C_s mM.
Concentration of total thiosulfinates in samples = C_s/2 mM.
Concentration of allicin = C_s/2 × 0.7 mM.

The mechanism of the cystein reaction with allicin is not well known. It is suggested that two molecules of allyl sulfonic acid dimerizes to form a molecule of allicin that then reacts with another molecule of cystein[21].

To examine the presence of sulfydryl groups in the garlic extracts that may react with DTNB solution and interfere with our assay, the garlic extract was added to DTNB solution (in the absence of cystein). There was no increase in the absorbance of the sample at 412 nm which proves that no NTB is formed, *i.e.*, sulfydryl groups are not present in the garlic extract. The spectrophotometric method used here is sensitive enough to measure the concentration of allicin in the micromolar range. Using 4,4′-dithiodipyridine (DTDP) instead of DTNB for the reaction with cystein, produces 4-thiopyridinone. This reduced product has a molar absorbance of 19,800 at 324 nm and the method is shown to be more sensitive than when using DTNB[22].

The concentration of allicin in various Iranian garlic and garlic products (Table-2) was found to be slightly higher than the value obtained for garlic from other parts of the world[14, 16]. We have also examined fresh garlic extracts from various parts of Northern Iran and the results are similar, *i.e.*, higher than some reported values for allicin contents. It is expected that the value may be even higher in the garlic grown in other parts of Iran and countries with similar climate conditions (hot and exotic). The nature of garlic from Northern Iran is more moderate due to the more moderate temperature and rainy weather in most seasons of the year. However, even this garlic is stronger (in terms of allicin and other thiosulfinate contents) than Western species. The results in Table-2 also show that the concentration of allicin, *i.e.*, thiosulfinates is slightly higher in garlic powder prepared in the laboratory compared to the garlic powder and garlic tablets obtained from commercial sources. This reduction may be due to the storage and processing time that affects the quality of the product and we have found that this value is slightly higher when the powder is prepared from fresh garlic. It may also be due to the volatility of thiosulfinates which causes their loss during storage.

According to our results, the amount of allicin does not depend on the extraction conditions, *i.e.*, none of the denaturing agents used would inhibit the alliinase activity. This finding is in contrast to the results obtained by Lawson[23] who has claimed that HCl is a potent inhibitor of the enzyme. We have also tried 8 M solution of urea and no deactivation of allinase was noticed and the amount of allicin is almost the same in every case (Table-2). We suggest that allinase is able to retain its biological activity in extreme denaturing conditions due to its compact shape. It may also be possible that some chemical species such as heavy metals are present in the garlic grown in this part of the world that protects enzyme allinase from being denatured. The nature of these agents is not yet known and we are researching to find out the possible types of chemicals.

The concentration of other garlic thiosulfinates (20–40% of total value) can also be determined using this simple and precise UV method. It has been found that all garlic thiosulfinates are highly sulfidryl reactive[16] and any other thiosulfinate can also react with two molecules of cystein.

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