

RESEARCH ARTICLE

Identification and expression analysis of *S-alk(en)yl-L-cysteine sulfoxide lyase* isoform genes and determination of allicin contents in *Allium* species

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Abstract

Alliinase is the key enzyme in allicin biosynthesis pathway. In the current study, the identification and sequencing of alliinase genes along with determination of allicin contents were reported for *Allium* species with a novel report for Iranian endemic species. The presence of different isoforms in the *Allium* being discovered for the first time. In bulbs tissue, the highest allicin concentration was in *Allium sativum*, *A. umblicatum*, and *A. fistolosum* (1.185%, 0.367%, and 0.34%, respectively), followed by *A. spittatum* (0.072%), *A. lenkoranicum* (0.055%), *A. atroviolaseum* (0.36%), *A. rubellum* (0.041%), and *A. stamineum* (0.007%). The highest allicin content in the leaves and roots were in *A. sativum* (0.13%), and *A. stamineum* (0.195%), respectively. The ORFs length ranged from 1416 in *A. sativum* (*iso-alliinase2; ISA2*) to 1523 bp in *A. sativum* (*alliinase*); the identity with *A. sativum* (*alliinase*) varies from 95% to 68% for *A. ampeloprasum*, and *A. sativum* (*iso-alliinase1, ISA1*) respectively. These data suggested that both *ISA1* and *ISA2* had a high expression in the roots and bulbs compared to *A. sativum* as the control in all species. Note that *ISA1* and *ISA2* were not expressed in the leaves. The results showed that isoforms expression patterns among different tissues in *Allium* species were variable. The presence of various isoforms is a possible explanation for the difference between the species in terms of obtained results, especially the amount of allicin.

Introduction

Allium L. is one of the largest genera in the family of the Amaryllidaceae, encompassing over 900 species [1,2]. The main center of its diversity is central Asia, including the territory of Iran and the Mediterranean, while the second distribution center for garlic and many *Allium* is western North America [3–6]. *Allium* species are primarily found in temperate, semi-arid, and arid regions of the northern hemisphere [7]. The results of recent classifications propose 15 subgenera and 56 sections for *Allium* [5], from which more than 30 species, including several

endemics, grow in Iran [8]. The flavor of freshly chopped *Allium* species, such as garlic, is due to the alliin lyase enzyme activity (also known as alliinase or S-alk(en)yl-L-cysteine sulfoxide lyase). The major organosulfur components in *Allium* species are alliin and isoalliin [9]. In an intact cell, alliin exists in the cytoplasm and lies there while being physically separated from alliinase which is stored in the vacuoles [10,11]. In garlic, alliinase was first described by Stoll and Seebeck in 1947 [12]. Alliin is a natural substrate of alliinase (EC 4.4.1.4). The enzyme hydrolyzes it to pyruvate, ammonia, and allicin [13]. Allicin is unstable and it quickly decomposes into other compounds, such as diallyl disulfide [10,12,14]. It is an organosulfur compound which plays some roles in the prevention and treatment of diseases [15]. It has also a variety of antimicrobial activities and medical roles, such as reducing cholesterol, triglyceride, lowering blood pressure, stimulating the immune system, plus antifungal, anticancer, antioxidant, and anti-inflammatory effects [16–18]. On the other hand, alliinase is the key enzyme in allicin biosynthesis pathway. Alliinase is an important enzyme which is a part of the plant defense against pathogenic microbes and herbivores. It is a protein that exists in all tissues of garlic [19]. The domestic *Allium* (such as onion, garlic, chives, and leek) contains high concentrations of organic sulfur compounds especially in their bulbs and leaves [9, 13–14]. In the parenchymatous bundle sheaths of garlic, alliinase comprises up to 10% of the soluble clove protein material [11]. Alliinase characterization has been studied from some *Alliums*, such as *A. sativum*, *A. cepa*, *A. tuberosum*, and *A. ascalonicum* [20–24]. Less work has been devoted to the phylogenetic analysis and the protection of wild *Allium* species as garlic relatives [25–27]. It has been reported that some *Allium* extracts contain allicin, with many studies reporting that the amounts of allicin in *Allium* extracts vary in different regions [10–13,28]. Information obtained using sequencing as the base for the phylogenetic analysis, allows scholars to identify changes in genes. It can enrich our understanding of how genes and species evolve. Enriching our understanding of *Allium* genetics and biochemical characterization as information resources are important to survey the diversity of gene, studies on plant biology and genetic studies as well as *Allium* breeding. In the current report, alliinase genes have been identified and sequenced, and the allicin contents have been determined for *Allium* species with a novel report for Iranian endemic species. Finally, the presence of different isoforms in the *Allium* has been discovered for the first time.

Results and discussion

Identification and characterization of alliinase isoforms

A fragment of approximately 1500 bp of the alliinase gene was successfully amplified with the new primers, using cDNA as template. The sequences of fragments were deposited in the NCBI GenBank Database (Table 1; Fig 1).

In this study, alliinase gene was scrutinized and two *iso-alliinase* genes (*ISA1* and *ISA2*) were investigated. The ORF length of gene sequences ranged from 1416 in *A. sativum* (*ISA2*) to 1523 bp in *A. sativum* (*Alliinase*). The G+C content of the analyzed alliinase genes ranged from 39.3% (in *A. sativum*—*ISA2*) to 43.5% (in *A. rubellum*). The nucleotide sequences from all *Allium* species were aligned and compared to alliinase amino acid sequences of *A. sativum* (control). Identity with *A. sativum* (*Alliinase*) was 95% for *A. ampeloprasum*, 92% for *A. umbellatum* and *A. lenkoranikum*, 91% for *A. ascalonicum*, *A. chinensis*, *A. fistulosum* and *A. rubellum*, 85% for *A. tuberosum*, 70% for *A. macrostemon*, 69% and 68% for *ISA2* and *ISA1* (in *A. sativum*) respectively (Table 1). There is an EGF-like domain in the N-terminal part of the alliinase structure; these domains are small disulfide-rich structures in a conserved form (a polypeptide with ~ 50 amino-acid residues long) [29–31]. Frequently, EGF-like domain constitutes modules for binding to other proteins and they are often unusual in plant proteins

Table 1. Information of the identified alliinase gene from the different species.

Species		Accession number	ORF length (bp)	G+C content (%)	Identity ^a (%)
<i>A. sativum</i>	Alliinase	FJ786257	1523	43.3	100
	ISA1	MH492261	1494	41.4	68
	ISA2	MH492262	1416	39.3	69
<i>A. fistulosum</i>		MF972076	1443	42.7	91
<i>A. umbilicatum</i>		MH021987	1440	42.7	92
<i>A. lenkoranicum</i>		MH021989	1440	42.5	92
<i>A. rubellum</i>		MH021988	1440	43.5	91
<i>A. ampeloprasum</i>		MG742366	1449	43.1	95
<i>A. ascalonicum</i>		MG742367	1440	41.2	91
<i>A. chinensis</i>		MG742368	1440	41.4	91
<i>A. macrostemon</i>		MG742369	1428	42.2	70
<i>A. tuberosum</i>		MG742370	1434	41.1	85

^a with *A. sativum* (alliinase) as a control

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found in the secreted proteins [30–32]. Among plant enzymes, alliinase is an example of a catalytic domain fused to an EGF-like domain. Note that the sequence alignment of complete alliinase sequences from different species shows a strictly conserved pattern (C-x18-19-C-x-C-x2-C-x5-C-x6-C, Fig 2).

This pattern has been known in different species and is reported by some researchers in *Allium* species [30–34]. The functional role of EGF-like domain in alliinases is unclear. Its possible role is associated with the vacuolar localization of alliinase, where one may speculate that it may act as a binding site for other proteins or a hypothetical alliinase receptor [34]. A phylogenetic tree of alliinase was constructed from different plants using MEGA7.0 based on CLUSTALW2 alignments. The results revealed that alliinase from *A. tuberosum*, *A. chinensis*, *A. fistulosum*, *A. ascalonicum*, *A. cepa*, *A. umbellicatum*, *A. umpeloperasum*, *A. sativum* (alliinase), *A. rubellum*, *A. lenkoranicum* was grouped into one cluster, while *A. macrostemon*, *A. sativum* (ISA1), and *A. sativum* (ISA2) were classified into another cluster (Fig 3).

Alliin contents

The alliin concentration of eight *Allium* species was analyzed using HPLC. The experiment was carried out via a completely randomized design (CRD) with three replications. The results

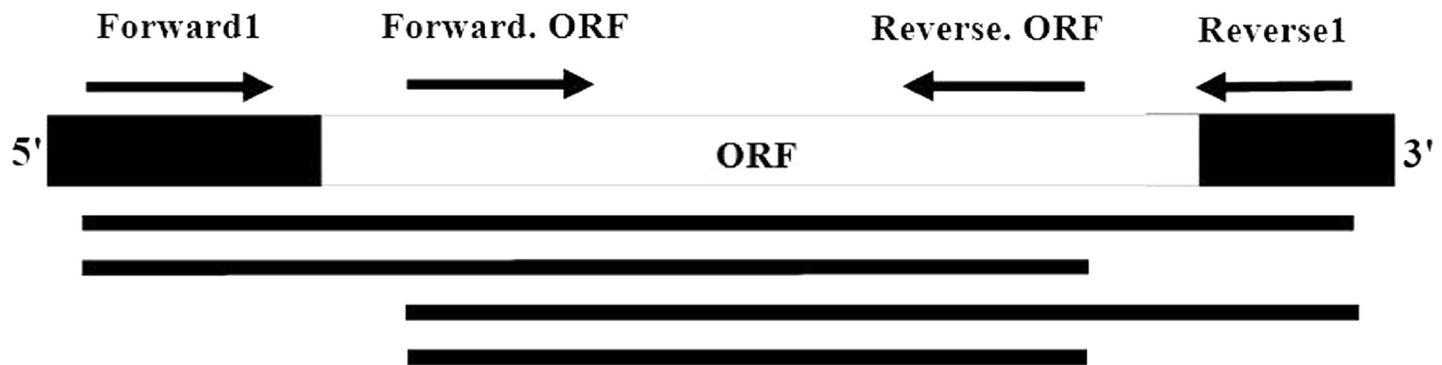


Fig 1. Positions of nested-PCR primers, and the expected lengths of PCR products, containing *Alliinase* gene. Arrows and lines represent primers and PCR products of each primer pairs, respectively.

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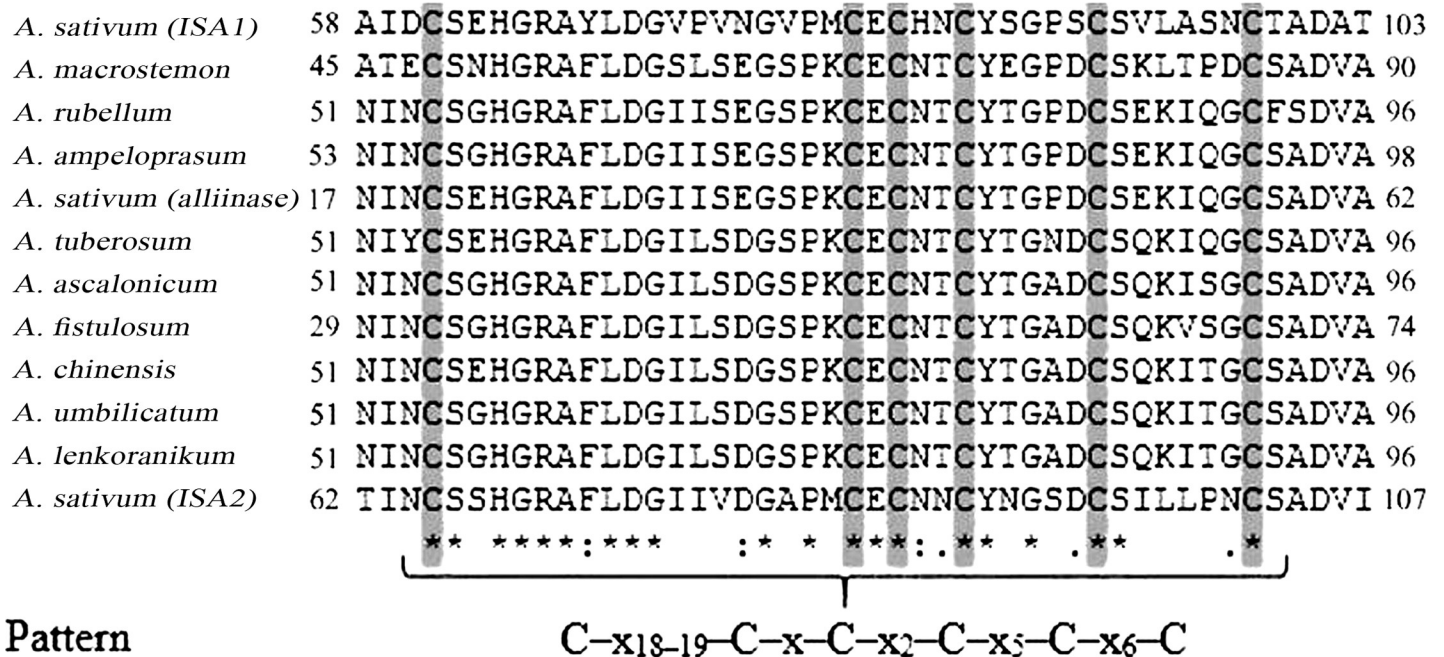


Fig 2. Sequence alignment of the N-terminal segments of iso-alliinases (ISA) protein sequences. Black letters on gray background, residues of conserved cysteine residues in EGF-like domain.

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from the ANOVA indicate significant differences among the species (Table 2). The results for each tissue (bulbs, leaves, and roots) are shown in Fig 4.

All activities were done quickly because alliin was extremely unstable and instantly decomposed into organosulfur compounds such as diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DAT), dithiins, and ajoene [35]. In bulb tissue, the highest alliin concentration was in *Allium sativum* (control), *A. umbilicatum*, and *A. fistulosum* (1.185%, 0.367%, and 0.34%, respectively), followed by *A. stipitatum* (0.072%), *A. lenkoranicum* (0.055%), *A. atroviolaceum* (0.36%), *A. rubellum* (0.041%), and *A. stamineum* (0.007%; Fig 4). Further, alliin content (%) in the leaves and roots for *A. sativum*, *A. stipitatum*, *A. atroviolaceum*, *A. fistulosum*, *A. umbilicatum*, *A. lenkoranicum*, *A. stamineum*, and *A. rubellum* was 0.13, 0.075, 0.053, 0.035, 0.047, 0.088, 0.057, and 0 for leaves, and 0.052, 0.148, 0.037, 0.028, 0.076, 0.077, 0.195, and 0.001 for roots, respectively (Fig 4). For the first time, we report that the extracts from the whole Iranian endemic *Allium* contain alliin. Large variations in alliin contents were found across different parts (Fig 4).

The presence of the alliin precursor and its derivative products in green garlic extracts has also been reported [28,36–39]. In a study, in the quantification of the total thiosulfinate of different *Allium* spp. by HPLC analysis, *A. sativum* showed higher amounts of total thiosulfinate compared to the other species [40]. Alliin contents in the dry weight of garlic ranging from 1 to 4 mg g⁻¹ have been reported by many researchers [39,41–42]. Wang *et al.* (2014) reported that the amounts of alliin ranged from 0.81 to 3.01% [42]. According to British Pharmacopoeia, the minimum alliin content, in order to ensure pharmaceutical and economic viability of garlic powder, is 4.5 mg g⁻¹ [28,43]. It has been shown that the alliin content in *Allium* extracts varies considerably across different regions [28,44,45]. It is well known that the alliin content is reasonably variable, and based on the amount of alliin determined for *Allium* in this study (Fig 4), it may provide pharmacological effects in some *Allium* species.

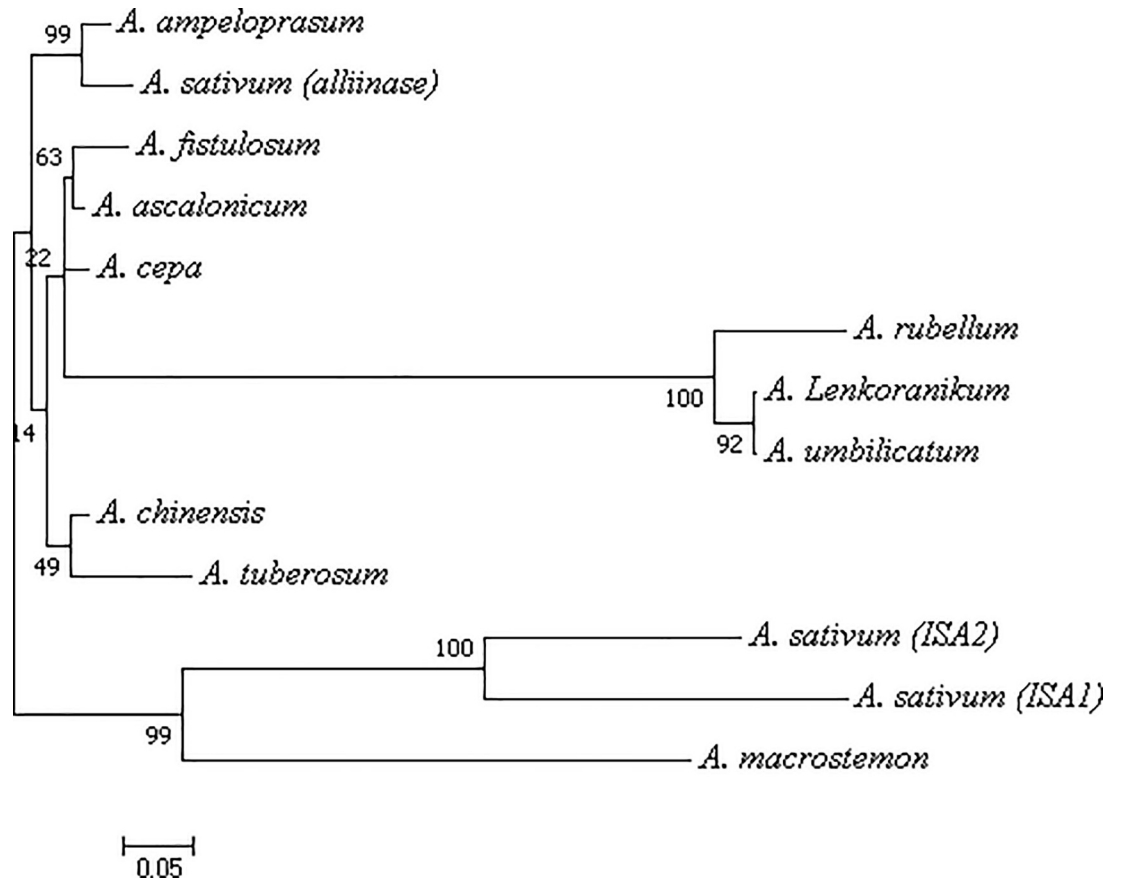


Fig 3. Phylogenetic analysis of identified *Allium. iso-alliinase* (ISA) and other alliinase proteins from various species, using Mega7.InK software, through the maximum likelihood method. Protein accession numbers are as: *A. cepa*: AAA32639.1 and *A. sativum* (alliinase): ACN78838.1. Numbers on the branches represented bootstrap support for 1000 replicates.

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Relative expression analysis of alliinase genes

The qPCR technique was applied to determine the relationship between the alliin content and the gene expression pattern of alliinase isoforms influencing the alliin content. The expression of alliinase genes from bulbs, leaves, and roots in eight species was also examined: *Allium lenkoranicum*, *A. atroviolaceum*, *A. fistulosum*, *A. stipitatum*, *A. sativum* (control), *A. rubellum*, *A. stamineum*, and *A. umbellicatum*. The qPCR was carried out with three biological replications for each sample and three technical replications for each biological sample.

Table 2. ANOVA for alliin contents in *Allium* species.

S.O.V	df	Ms		
		roots	bulbs	leaves
Species	7	0.011**	0.461**	0.004**
Error	16	0.0001	0.0012	0.0001
Total	23			
	CV%	0.013	0.084	0.007

Significances are indicated

** $p < 0.01$

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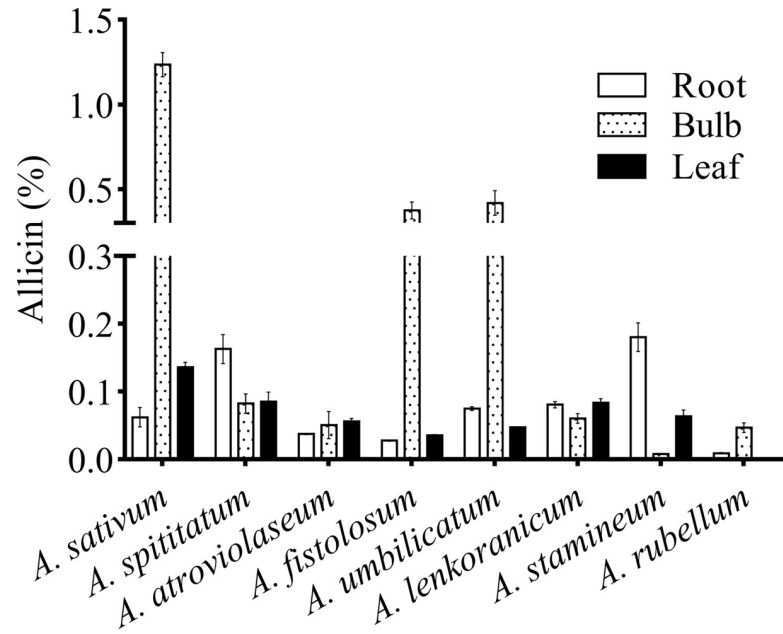


Fig 4. Alliin content (%) in eight *Allium* species, including *A. sativum*, *A. stipitatum*, *A. atroviolaceum*, *A. fistulosum*, *A. umbellicatum*, *A. lenkoranicum*, *A. stamineum*, and *A. rubellum*. Error bars represent SE (n = 3).

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ANOVA showed significant differences between species and among different tissues (bulb, root and leaf; $p < 0.01$) for the genes expressions levels (Table 3).

The maximum levels of gene expressions of the *alliinase* gene in bulbs were detected in *A. umbellicatum* and *A. fistulosum* (~ 1.6 and 1.5-fold, respectively; Fig 5). However, *A. sativum* with a high content of alliin (1.185%) had a low gene expression level compared to *A. umbellicatum* and *A. fistulosum*. Furthermore, this condition occurred in the leaves and roots, where *A. umbellicatum*, *A. fistulosum*, and *A. lenkoranicum* with a low alliin content (0.047, 0.035, and 0.088% for leaves; 0.076, 0.028, and 0.077% for roots, respectively) had a higher *Alliinase* gene expression (~ 6.7, 7, and 4.6 fold for leaves; 11.3, 5.2, and 2.3 fold for roots, respectively) compared to *A. sativum* -*alliinase*- (Fig 5). The relative expression of *alliinase* gene in the leaf of *A. rubellum* was low (0.15-fold), while no alliin content was detected. Note that the *alliinase* expression varies among the bulbs, leaves, and roots of garlic. It has also been suggested that garlic root tissue expresses a distinct *alliinase* isozyme with very low homology to the bulb enzyme [21].

Table 3. ANOVA for three isoforms expression levels of *alliinase* involved in alliin biosynthetic pathway in *Allium* species.

S.O.V	Df	Ms						
		<i>Alliinase</i> (roots)	<i>Alliinase</i> (bulbs)	<i>Alliinase</i> (leaves)	<i>ISA1</i> (roots)	<i>ISA1</i> (bulbs)	<i>ISA2</i> (roots)	<i>ISA2</i> (bulbs)
species	7	40.28**	1.037**	25.58**	3.63**	4.56**	2.83**	37.26**
Error	16	0.28	0.006	0.13	0.011	0.022	0.009	3.79
Total	23							
CV%		0.79	0.12	0.63	0.45	0.26	0.21	4.65

Significances are indicated

** $p < 0.01$

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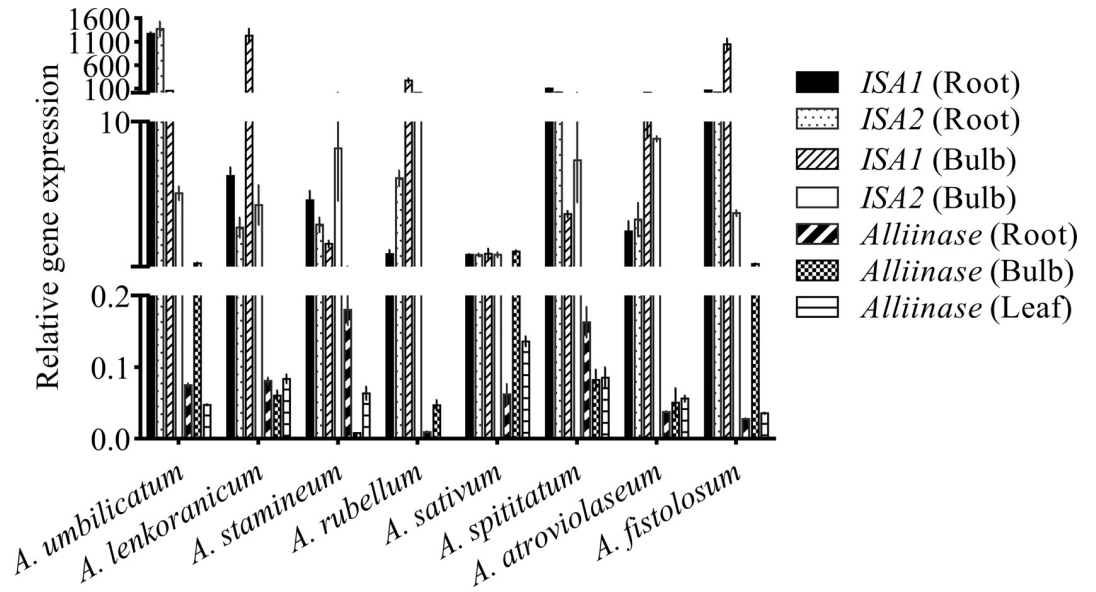


Fig 5. Relative expression of iso-alliinase gene (alliinase, ISA1 and, ISA2) in eight *Allium* species, including *A. sativum*, *A. stipitatum*, *A. atroviolaceum*, *A. fistulosum*, *A. umbilicatum*, *A. lenkoranicum*, *A. stamineum*, and *A. rubellum*. Error bars represent SE (n = 3).

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Table 4. Information and sequences of used primers in real time PCR and alliinase identification.

Use	Gene	Primer	Sequence (5' → 3')
Nested-PCR	<i>Alliinase</i> (Outside of ORF)	Forward1	GGGAGATTATAAGGAAGTGGAAC
		Reverse1	TTAAATGAATGGACGGCGG
	<i>Alliinase</i> (Inside of ORF)	Forward. ORF	GTGACATGGAGTTTGAAGGC
		Reverse. ORF	CGCTTCAACCATATCCTTCAAATAG
	<i>Alliinase</i> (Out /In.)	Forward1	GGGAGATTATAAGGAAGTGGAAC
		Reverse ORF	CGCTTCAACCATATCCTTCAAATAG
	<i>Alliinase</i> (In. / Out)	Forward ORF	GTGACATGGAGTTTGAAGGC
		Reverse1	TTAAATGAATGGACGGCGG
	<i>Iso-Alliinase 1</i> (Outside of ORF)	Forward1	CAACAACACTACAAACGCAC
		Reverse1	CGACATATTCTTCACTCATCCC
	<i>Iso-Alliinase 1</i> (Inside of ORF)	Forward. ORF	TCCCTTCTCAGACTATCC
		Reverse. ORF	ATGAAAGGACGCTCGATTG
	<i>Iso-Alliinase 2</i> (Outside of ORF)	Forward1	GACTAAACAAAGCGAAATAGAGG
		Reverse1	GTAGAGAACACACAGGTACAA
	<i>Iso-Alliinase 2</i> (Inside of ORF)	Forward. ORF	TAATCAGAGGTGGGAAACT
		Reverse. ORF	TAATAAGGCTCAACCGTGC
qRT-PCR	<i>β-Actin</i>	Forward	TTGCTGGTGATGATGCTCCAAG
		Reverse	CCATGTCATCCCAGTTGCTCAC
	<i>Alliinase</i> (Inside of ORF)	Forward	ATGGGTGAAGTGTGAATGGGA
		Reverse	AGTCTTCTCTTCGCTTCAACC
	<i>Iso-Alliinase 1</i>	Forward	CAATCGAGCGTCTTTCATC
		Reverse	CGACATATTCTTCACTCATCCC
	<i>Iso-Alliinase 2</i>	Forward	TGAAGAGGTACTIONTAGAAATGGAC
		Reverse	GTAGAGAACACACAGGTACAA

<https://doi.org/10.1371/journal.pone.0228747.t004>

In the current study, the presence of *iso-alliinase* genes was identified. Accordingly, three isoforms of the enzymes were identified as *Alliinase*, *ISA1*, and *ISA2*. Primary data analysis for roots indicated that the gene expression level did not match and it was not consistent with alliin contents across all species. Other findings revealed that the previously designed primers for *Alliinase* gene were not suitable for amplified *Alliinase* gene. To solve this failure, the design of primers was carried out according to the differences, specifically for each gene (Table 4). Expression studies using qPCR indicated that the highest level of *ISA1* gene in bulbs was detected in *A. lenkoranicum* and *A. fistulosum* (~1229.9 and 1040.9 fold, respectively), followed by *A. rubellum* (271.79 fold), *A. umbilicatum* (55.7 fold), *A. atrovioleaceum* (11.14 fold), *A. stipitatum* (3.08 fold), and *A. stamineum* (1.73 fold). These expression levels for *ISA2* were 4.37, 3.81, 12.35, 5.15, 8.85, 7.4, and 8.19 folds respectively (Fig 5). In the roots for *ISA1* and *ISA2* in *A. umbilicatum*, *A. fistulosum*, *A. stipitatum*, *A. lenkoranicum*, *A. stamineum*, *A. atrovioleaceum*, and *A. rubellum*, the values were 1271.42 and 1405.45; 60.05 and 17.38; 101.84 and 21.06; 6.32 and 2.85; 4.7, and 3.03; 2.67 and 3.38; 1.01, and 6.17 fold, respectively (Fig 5). These data suggested that both *ISA1* and *ISA2* had a high expression in the roots and bulbs compared to *A. sativum* as the control. This situation existed in all species except for *A. rubellum*, such that, the expression amounts were lower relatively in bulb compared to the roots in all species. Note that *ISA1* and *ISA2* were not expressed in the leaves (Ct values > 35 or not detectable; S1 Fig and S2 Fig).

Rabinkov *et al.* (1994) described that alliinase cloned in *A. sativum* tissues was expressed in the bulbs and leaves with a high alliinase activity, but not in the roots [21]. This demonstrated the presence of a nonhomologous alliinase gene in *A. sativum* roots. The proteins of alliinase isoform from *A. sativum* (*ISA1* and *ISA2*) were coded by a cDNA with low sequence identity to other *Allium* alliinases. Regarding the roots of *A. sativum*, Rabinkov *et al.* (1994) reported a protein with low sequence homology to *A. sativum* alliinase cDNA, but with an alliinase activity [21]. Alliinase in *A. cepa* is active as a trimer and tetramer [45–47]. Van Damme *et al.* (1997) reported that in some *Allium* spp., alliinase has been shown to aggregate with low molecular-mass lectins into stable active complexes [48], while Lancaster *et al.* (2000) found that multimeric forms did not aggregate in the onion root [47]. In some *Alliums*, it has been reported that the root alliinase has a wider C-S lyase activity. Expression studies using RNA and northern analysis showed that *A. cepa* root alliinase cDNA was expressed to a far greater extent in roots than in leaves and bulbs confirming that the alliinase cDNA of leaves was not expressed in roots [47]. Note that the expression is inconsistent at different growth stages of plant. Low alliinase activity in the seeds of *A. cepa* (cv. Rijnsburger) was reported by Freeman (1975) which was less than 2% of that in bulbs, and increased rapidly during the seedling development to reach a stable maximum, 15 to 20 days post-germination [48].

Conclusion and discussion

This study aimed to investigate the *Allium* species, especially some Iranian Endemic *Allium* species in terms of alliin contents, and to identify as well as analyze the expression of *alliinase* isoform genes. The results showed that alliin contents and *alliinase* gene expression levels in the *Alliums* were highly variable (Figs 4 and 5). Numerous factors affect the production of secondary metabolites in plants such as genotype, plant genetic, plant physiology, environmental and ecological conditions [49–51,52,53–55]. To eliminate the environmental effects, the plants were propagated under the same conditions. Eliminating environmental effects via plant propagation under the same conditions have been noted by others studies [42,52,56]. In the current study, the presence of three different isoforms in the *Allium* was discovered for the first time. Note that in comparison with simple pathways, the complex metabolic pathways were affected

by more regulatory elements. In other words, fewer variable factors such as lower number of genes affect the end product in a simple network. The complexity of the pathway affects the metabolite production rate. In this regard, alliinase hydrolyzes alliin to alliin [13]. The biosynthetic pathway to alliin is still not clear [57]. Alliinase is the key enzyme in alliin biosynthesis pathway. In addition to the complexity of the biosynthetic route, genes expression levels and enzyme activities also affect the alliin contents. Most studies have shown a close relationship between metabolome and genes expression levels [51,52,55,57,58,59,60]. The results showed that the expression patterns of isoforms were variable among different tissues in *Allium* species (Fig 5). The presence of various isoforms is a possible explanation for the difference between the species in terms of obtained results, especially the amount of alliin.

It has been suggested that two different alliinase isoforms are present in *A. sativum*, one of which is specific for 1-PECSO (Trans-(+)-S-(1-propenyl)-L-cysteine sulphoxide) and alliin, while the other is specific for MCSO ((+)-S-Methyl-L-cysteine sulphoxide) [41]. It has been reported that the enzyme isoform was inactive in some *Allium* species during *in situ* test for alliinase activity in the IEF gel [47]. Lancaster *et al.* (2000) reported that two isoforms of alliinase existed in the onion root, where isoform 1 had pI = 9.3, while isoform 2 had pI = 7.6, 7.9, 8.1, and 8.3 [47]. They stated that both alliinase isoforms (I and II) showed similar enzymatic activity across the range of substrates. In contrast, only C-S lyases with Cys sulfoxide lyase activity have been reported for *Alliums* [61]. Lancaster *et al.* (2000) declared that the isoform I could not be sequenced. On the other hand, the identity between *Alliinase*, *ISA1* and *ISA2* with the isoform II of onion root *alliinase* was 54.30, 81.1, and 64.85 respectively. In the sequence from other *Allium* alliinases, *A. cepa* root alliinase has wider C-S lyase activity. *A. cepa* root alliinase may have a function in sulfur assimilation and remobilization in roots [47].

Further studies such as enzyme assay for measuring the enzymatic activity across the different species, studying x-ray crystallography and the molecular dynamics of proteins can also be beneficial to obtain more details. Indeed, although this study intended to enhance our knowledge about the *Allium* species, further studies are still required to clarify the details. More studies contribute to a better understanding of the gene expression pattern, e.g. examining different growth stages (i.e., initial stage, vegetative growth, reproductive phase and, maturation stage) or under growth conditions (i.e., changes in temperature, soil composition and light). According to our research, the amino acid sequences of alliinases described here displayed a substantial similarity with other known alliinases. These findings are in accordance with other results from different papers suggesting that the alliinase gene displays a high variability among different species. Thus, it cannot be used as a phylogenetic marker; however, it can easily discriminate between closely related species [62].

Materials and methods

Allium lenkoranicum, *A. atrovioleaceum*, *A. fistulosum*, *A. stipitatum*, *A. sativum*, *A. rubellum*, *A. stamineum*, and *A. umbellicatum* were collected from the natural habitats across various geographic locations of Iran (Table 5). To eliminate the environmental effects, the collected bulbs were maintained and propagated under the same conditions as in Iranian Biological Resource Center (IBRC), Tehran, Iran. The sampled plants at the immature stage were frozen in liquid nitrogen and then stored at -80°C until further analysis.

Determining the amount of alliin

HPLC system and chromatographic conditions. The HPLC system consisted of Agilent 1100 HPLC Series system (Agilent, Santa Clara, CA, USA). Its analyses were performed on a C18 column (250 mm \times 4.6 mm) where the alliin was detected at 254 nm wavelength by a

Table 5. Local information of studied *Allium* species.

Species	IBRC ^a No	Local collection sites	Latitude (N) Longitude (E)	Altitude (m)
<i>A. sativum</i>	-	Bahar, Hamadan, Iran	34° 55' 48" 26'	1722
<i>A. fistulosum</i>	-	Ghalea Now-e Ghar, Tehran, Iran	35° 50' 51" 50'	1189
<i>A. stipitatum</i>	P1010429	Mian Mil, Kermanshah, Iran	34° 36' 47" 46'	2526
<i>A. umbilicatum</i>	P1009439	Kangelu, Alborz, Iran	35° 50' 51" 03'	1340
<i>A. lenkoranikum</i>	P1008766	Chashm, Semnan, Iran	35° 57' 53" 08'	1570
<i>A. rubellum</i>	P1009972	Qutor, Western Azerbaijan, Iran	38° 36' 44" 40'	1524
<i>A. ampeloprasum</i>	P1009877	Payam, East Azerbaijan, Iran	38° 19' 45" 47'	2220
<i>A. ascalonicum</i>	-	Changsha, Hunan, China	28° 13' 112" 56'	48
<i>A. chinensis</i>	-	Changsha, Hunan, China	28° 13' 112" 56'	48
<i>A. macrostemon</i>	-	Changsha, Hunan, China	28° 13' 112" 56'	48
<i>A. tuberosum</i>	-	Changsha, Hunan, China	28° 13' 112" 56'	48

^a Iranian biological resource center

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UV-visible detector. Methanol and water (50:50%, v/v) were used as mobile phase with a flow rate of 0.7 ml min⁻¹ at ambient temperature. The final injection volume was 20 µl. The allicin content was evaluated according to the method described in British pharmacopoeia with some modifications [28,43].

Preparation of the *Allium* extracts and internal standard. Butylparahydroxybenzoate was used as the internal standard (IS) for the quantification of allicin which was prepared in the mobile phase (20 mg in 100 ml) [28,43]. Half of the materials collected from five plants, were directly frozen in liquid nitrogen and powdered, using mortar and pestles. Further, 0.8 g powder was homogenized with 20 ml distilled water and sonicated for 5 min continuously at 100% amplitude, using an ultrasonicator (Elmasonic s30, Germany) in an ice container, and then incubated for 30 min at 25°C. The obtained mash was poured through a five-layer cheese-cloth and allow to drain, then transferred into 50 ml falcon tube. The extracts and cell debris were separated by centrifugation (6000 g) for 20 min at 4°C. The supernatant was transferred into a new sterile 50 ml falcon tube. Also, 10 ml supernatant was diluted to 25 ml by adding mixture A (solution of anhydrous formic acid (1%; v/v): methanol (HPLC grade), 40:60), and centrifuged at 6000 g for 5 min at 4°C. Further, 0.5 ml of IS was diluted to 10 ml with a supernatant of the second centrifugation in a volumetric flask. Note that allicin is unstable at high temperatures and the assay must be carried out as quickly as possible. Thus, the sample solutions were stored at -70°C, before injection [14].

Determination of allicin. The following equation was used to calculate the amount of allicin in the samples [28,43].

$$\text{Allicin (\%)} = S_1 m_2 \times 22.75 S_2 m_1 \text{ where;}$$

m_1 = mass of the garlic powder (0.8 g),

m_2 = mass of butyl parahydroxybenzoate (0.02 g) in IS solution,

S_1 = area of the peak corresponding to allicin,

S_2 = area of the peak corresponding to internal standard.

Assembly of reads and identification of alliinase gene

The plants were separated into bulbs, leaves, and roots. Half of each tissue (bulb, leaf, and root) was cut and mixed for RNA extraction and expression analyses, while the other half was

considered for alliin measurement. Alliinase gene for *A. ascalonicum*, *A. chinensis*, *A. macrostemon*, and *A. tuberosum* was predicted through the NCBI Sequence Read Archive (SRA, <http://www.ncbi.nlm.nih.gov/sra>). SRA accession numbers were SRX1560692, SRX1560673, SRX1560658, and SRX1560563, respectively. Read sequences for desired *Allium* were downloaded from several RNAseq projects (<http://www.ncbi.nlm.nih.gov/sra/>). Then, more than 400 individual reads for each *Allium* with substantially similar reads were selected, using the Offline BLAST Software v.2.7.0 [63]. After assembling the read sequences, using Codon Code Aligner v. 5.0.1. Program, the consensus sequences for assembled reads were created. Next, an ORF was found for each chosen consensus sequence via ORF finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). Finally, gene-specific PCR primer pairs were designed for PCR-amplification of alliinase genes, based on the complete ORF cDNA sequences (Table 4). The primers were confirmed by the Oligo Analyzer v.3.1 (eu.idtdna.com/calc/analyzer) and NCBI/Primer-blast (www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). For predicting the alliinase isoforms, different contigs resulting from assembling *Allium sativum* reads sequences (SRA accession number: SRX305368) were used for further analysis after which the primers were designed based on the obtained desired contigs (Table 4).

RNA extraction and cDNA Synthesis

Total RNA was extracted using Ribospin™ Plant kit (GeneAll Biotechnology Co., Ltd., Songpa-gu, South Korea) according to the manufacturer's instructions. The RNA samples were treated with Qiagen RNase-Free DNase (Qiagen, 79254, Qiagen Inc., Midland, ON, Canada) for 30 min at 37°C to remove the genomic DNA. Following the manufacturer's protocol, 1 µg of total RNA was used to make cDNA at a volume of 20 µl, using Thermo Scientific Revert-Aid™ First-Strand cDNA Synthesis Kit (Fermentas, K1622, Thermo Fisher Scientific, Hudson, NH, USA).

Nested PCR detection and phylogenetic construction

The nested primers for the amplification of alliinase genes were designed based on different positions on consensus sequences to amplify the untranslated region (UTR) and coding region of alliinase gene fragments overlapping with each other to confirm the characterized ORFs (Table 4 and Fig 1). The expected length of alliinase was amplified by RT-PCR from first-strand cDNA from leaves, using Hyperscript™ RT PCR master mix (GeneAll Biotechnology Co. Ltd, Fig 1). PCR was carried out with newly specific primers for *alliinase* gene in three repeats. The PCR program was as follows: 95°C for 3 min, followed by 34 cycles at 95°C for 5 min, 55°C for 30 S, 72°C for 1.30 min, with the final elongation of 10 min at 72°C. PCR products were separated in 1% (w/v) agarose TAE gel. The amplified PCR products of long fragments were cleanup by gel recover kit (Top Gel Recovery Kit, TOPAZ GENE RESEARCH., Cat. No.: TGK1006, Iran), and subjected to direct sequencing by an automatic sequence and dye-termination sequencing system (Macrogen Co., Seoul, South Korea). The sequences were edited and assembled by employing SeqMan (DNASTar) [64]. Also, the identification of open reading frames (ORFs) and conserved domains, as well as translated protein sequences were done using the BLASTN, BLASTP, ORF finder, available at <http://www.ncbi.nlm.nih.gov/>, and Pfam, available at <http://pfam.xfam.org/>. After a BLASTP search on the NCBI database, alliinase protein sequences were selected from different species with more than 50% identity with the coding region of the consensus sequences. To determine the relationship between the identified alliinase and the protein downloaded from the BLASTP search, multiple alignments were run using web-based Clustal Omega program (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). The maximum likelihood method in MEGA7 was done for phylogenetic tree construction and 1000 iterations were applied for calculating the bootstrap value [65].

Real-time PCR assay

The designed primer pairs were specific for the amplification of β -actin used as reference genes (Table 4). The qPCR was performed using specific primers (Table 4) on a BioRad Mini-Opticon real-time PCR detection system (Applied Biosystems, Foster City, CA, USA) with the fluorescent dye SYBR® Green Master Mix 2X (Ampliqon, A323402, Denmark) in accordance with the manufacturer's instructions. Specifically, 1 μ l of the first strand cDNA was used as a template in 20 μ l reactions, including 10 μ l SYBR® Green PCR Master Mix and 0.3 pmol of each primer. The qRT-PCR was run at 95°C (15 min), 35 cycles at 95°C (20 s), 59°C (30 s), and 72°C (30 s), followed by gradient: 60–95°C (5 s). The dissociation stage was accomplished to determine the PCR product size and to detect possible primer dimers. Triplets of all samples were run, and the negative control of the Master Mix in addition to primers was performed in all qPCR runs. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method [66,67].

Supporting information

S1 Data. Certification and track changes's files for english edit.
(ZIP)

S1 Fig. qPCR amplification curve for ISA1 in the leaves of eight *Allium* species, including *A. sativum*, *A. atrovioleaceum*, *A. fistulosum*, *A. umbellicatum*, *A. lenkoranicum*, *A. stamineum*, and *A. rubellum* (S1-S8; Ct > 35 or not detectable).
(TIF)

S2 Fig. qPCR amplification curve for ISA2 in the leaves of eight *Allium* species, including *A. sativum*, *A. atrovioleaceum*, *A. fistulosum*, *A. umbellicatum*, *A. lenkoranicum*, *A. stamineum*, and *A. rubellum* (S1-S8; Ct > 35 or not detectable).
(TIF)

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