

The Biochemical and Physiological Genesis of Alliin in Garlic

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ABSTRACT

The health-giving properties of garlic are thought to be primarily derived from the presence and subsequent breakdown of the alk(en)ylcysteine sulphoxide (CSO), alliin and its subsequent breakdown to allicin. Two biosynthetic pathways have been proposed for CSOs, one proceeds from alkylation of glutathione through γ -glutamyl peptides to yield *S*-alkyl cysteine sulphoxides while the alternative is direct thioalkylation of serine followed by oxidation to the sulphoxide. Addition of allyl thiol to differentiating garlic tissue cultures resulted in the appearance of detectable levels of both *S*-allyl cysteine and alliin and also demonstrated that *S*-allyl-cysteine was oxidised stereospecifically to (+)-alliin by garlic tissue cultures, indicating the presence of a specific oxidase in the cells. Although these reports provide good evidence that *S*-allyl cysteine can be converted to alliin by garlic tissue cultures, it does not indicate whether γ -glutamyl-*S*-allyl cysteine or *S*-allyl cysteine is the substrate for oxidation *in vivo*. Garlic contains several cysteine synthases and at least one has the capacity to synthesise alliin. Studies on alliin distribution during bulb development are consistent with a process within which CSOs are synthesised primarily in leaves and translocated to garlic clove tissues during bulb development. Alliinase is the enzyme that initiates the conversion of alliin to allicin and its derivatives. Although multiple sequences can be identified within a single variety, the expression of the dominant isoform in both clove and leaf tissue does not vary significantly with stage of development, consistent with allinase genes being constitutively expressed.

Keywords: alk(en)ylcysteine sulphoxide, *Allium sativum*, alliin, allinase, flavour precursor, γ -glutamyl peptides, sulphur

Abbreviations: ATP, adenosine triphosphate; CSase, cysteine synthase; O-acetylserine (thiol) lyase; CSO, alk(en)ylcysteine sulphoxide; GST, glutathione-*S*-transferase family; OAS, O-acetylserine; SAT, serine acetyltransferase

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INTRODUCTION

The reputed health-giving properties of fresh garlic and commercial garlic extracts are thought to be derived from the presence and subsequent breakdown of the alk(en)ylcysteine sulphoxides (CSOs), alliin, isoalliin and methiin (Block 1992; Lawson 1996; Collin 2004) which act as flavour precursors in a range of *Allium* species. These odourless sulphur-containing cysteine derivatives are the final stable products of biosynthetic pathways and are stored in vesicles within the cytoplasm (Lawson 1996). However, when garlic tissue is damaged during culinary or manufacturing activities, the enzyme alliinase is released from the cell vacuoles and can cleave CSOs simultaneously liberated in the cytoplasm. The reaction products are pyruvate, ammonia and a volatile, low molecular weight thiosulphinates which rapidly undergoes a series of vapour-phase non-enzymic reactions to yield a complex mixture of sulphur products that change over time (Block 1992). In garlic, unlike most other *Alliums*, the major flavour precursor is alliin that yields a series of thiosulphinates, allyl sulphides, dithiines and ajoenes after the action of alliinase and subsequent

chemical decomposition. These are the source of the very characteristic garlic odour and also the proposed health-related properties. Other *Alliums* that contain alliin, such as *A. ursinum* (wild garlic, ransoms) also have a garlic-like odour. This dominates the odour sensation, masking the products of other CSOs. Garlic also contains trace levels of methiin (Horie and Yamashita 2006), which gives rise to odours described as 'cabbagy' or 'fresh onion' and intermediate concentrations of isoalliin which is the major CSO in onions and generates their characteristic smell (Whitaker 1976). The remaining sulphur compounds within the bulb, that together with the CSOs typically comprise 1-5% dry weight (Lancaster and Kelly 1983), are γ -glutamyl peptides such as γ -glutamyl allyl cysteine sulphoxide and γ -glutamyl isoallyl cysteine sulphoxide.

The path of synthesis of the flavour precursors is still speculative and it has been suggested that there are two routes to the major flavour precursor, alliin (Fig. 1). One possible route is from serine and an allyl source and the other is from glutathione and an allyl source (Lawson 1996). Central to this scheme is cysteine since it links carbon and nitrogen metabolism and is an essential precursor for all

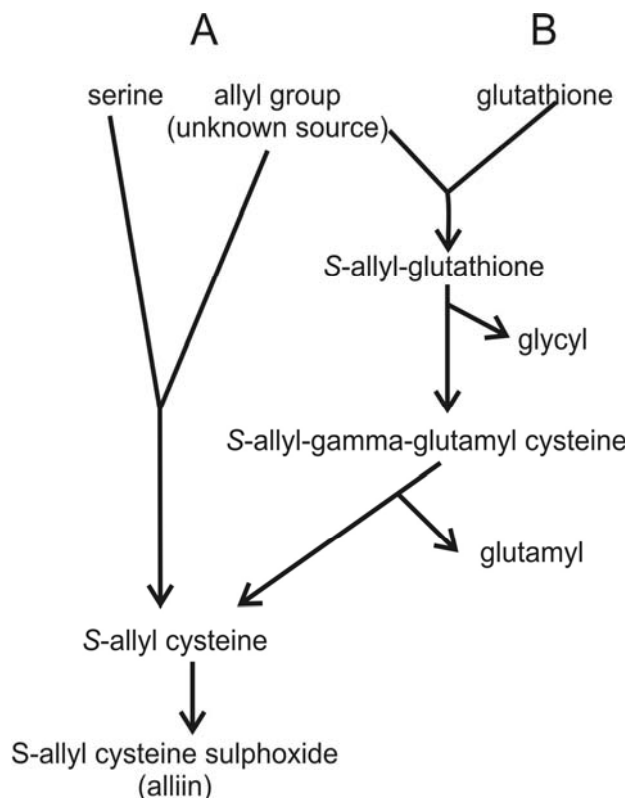


Fig. 1 Pathways proposed for the biosynthesis of alliin (A) the serine route (after Granroth 1970) and (B) the glutathione route (after Lancaster and Shaw 1989).

biological compounds that contain reduced sulphur including CSOs (Hofgen *et al.* 2001). Plants utilise inorganic sulphate as their source of sulphur and this is absorbed by the roots from soil and assimilated into cysteine after reduction to sulphide. The sulphate is activated to 5'-adenylsulphate by ATP sulphurylase and then reduced initially to sulphite by a glutathione-dependent reductase and then sulphide by a ferredoxin-dependent sulphite reductase (Hell 1997; Wirtz and Droux 2005). Cysteine synthesis occurs in green and non-green tissues (Madhavi *et al.* 1991; Barroso *et al.* 1998) in two sequential reactions. First, O-acetylserine (OAS), the carbon precursor of cysteine, is synthesized from L-serine through addition of an acetyl group from acetyl CoA by serine acetyltransferase (SAT) that is bound to cysteine synthase (O-acetylserine (thiol) lyase; OAS-TL; CSase) (Zhu *et al.* 1998). Bound CSase acts as a structural or regulatory subunit of SAT and is mostly inactive in amino-acid synthesis (Wirtz *et al.* 2001). Second, free CSase inserts sulfide into OAS. Thus for effective cysteine synthesis to occur, an excess of free CSase is required (Droux *et al.* 1998). It is thought that in plant cells cysteine is synthesized *in situ*. Compartment specific SATases and CSases have been localized in various plants (Urano *et al.* 2000).

The alliin and isoalliin that result from the activities of these enzymes are present throughout the plant but in Europe the only part of the garlic plant that is harvested are the dried cloves that form the basal bulb. Garlic bulbs are stored at low temperature for many months before use and there is good evidence that the content of sulphur compounds changes during this time (Hughes *et al.* 2006; Ichikawa *et al.* 2006) with a decline in γ -glutamyl peptides and increase in CSOs. There are also higher levels of flavour compounds in the outer, rather than inner, cloves within the bulb. The bulbs expand at a late stage of the development of the garlic plant but it is uncertain at which stage the accumulation of the alliin and isoalliin occurs in the bulb. Before the concentration of these flavour precursors can be manipulated it is important to understand the pattern of synthesis and accumulation during the growth of the plant.

INTERMEDIATES IN ALLIIN BIOSYNTHESIS

Two biosynthetic pathways (Fig. 1) have been proposed for CSOs, both supported by experimental evidence (Jones *et al.* 2004). One proceeds from alkylation of glutathione through γ -glutamyl peptides to yield S-alkyl cysteine sulphoxides (Lancaster and Shaw 1989) while the alternative is direct thioalkylation of serine followed by oxidation to the sulphoxide (Granroth 1970). In both cases the physiological source of the allyl group remains to be identified. *Allium* species contain substantial amounts of glutathione and γ -glutamyl derivatives of CSOs as well as CSOs (Whitaker 1976) so that determining the relationship between these compounds has proved problematical. It is possible that different pathways dominate in different tissues or at different stages in the life-cycle.

Tissue analysis, radiolabelling studies and more recently protein biochemistry and molecular biology have been used to try to resolve this question using several *Allium* species. One difficulty with analytical approaches is that damage to the tissues results in release of alliinase which rapidly converts CSOs to volatile sulphur compounds (see below, 'Alliinase'). Additionally, peptidases and γ -glutamyl transpeptidases present within the damaged plant tissues may initiate breakdown of the γ -glutamyl derivatives (Ceci *et al.* 1992; Hanum *et al.* 1995). To avoid these difficulties, some studies have analysed tissues disrupted after rapidly freezing or utilised intact plants or tissue cultures.

Tissue culture is particularly attractive because biosynthesis of secondary metabolites is frequently reduced or absent except after specific modifications to the growth medium thereby indicating a role for proposed biosynthetic intermediates (Collin 2001). Undifferentiated colourless garlic callus contains a very low level of methiin with only trace amounts of alliin, the reverse of the normal situation (Lancaster *et al.* 1989; Madhavi *et al.* 1991) indicating that the biosynthetic pathway is inhibited at some stage. Once the callus redifferentiates on a suitable growth medium, the levels of flavour precursors are comparable with normal vegetative tissue, indicating that secondary metabolism is not irreversibly blocked. The thin cuticle allows biosynthetic intermediates ready access to intact but unspecialised cells.

Addition of allyl thiol to differentiating garlic tissue cultures resulted in the appearance of detectable levels of both S-allyl cysteine and alliin within hours (Ohsumi *et al.* 1993) and also demonstrated that S-allyl-cysteine was oxidised stereospecifically to (+)-alliin by garlic tissue cultures, indicating the presence of a specific oxidase in the cells. Extending this approach to include onion callus, where the parental plant does not synthesise alliin, small amounts of alliin were formed in both undifferentiated garlic and onion callus following incubation with allyl cysteine and allyl thiol for two days (Hughes *et al.* 2005). Incubation with cysteine, glutathione or serine did not lead to alliin production. The amino acids serine and glutathione may be taken up by the garlic callus but could be restricted in their entry to the site of the secondary product pathway, ie that part of the pathway that leads from cysteine and glutathione to the synthesis of alliin and is specific for the *Alliums*, so that a recordable level of stimulation of alliin synthesis does not occur. In contrast allyl thiol and allyl cysteine must be incorporated into this pathway very readily leading to the production of alliin.

Although these reports provide good evidence that S-allyl cysteine can be converted to alliin by garlic tissue cultures, it does not indicate whether γ -glutamyl-S-allyl cysteine or S-allyl cysteine is the substrate for oxidation *in vivo*. The presence and activity of relevant genes or enzymes could be investigated to discover which pathway operates in garlic callus or plants and this approach will be discussed next.

GENES AND ENZYMES INVOLVED IN ALLIIN SYNTHESIS

The two proposed routes for alliin synthesis require rather different biosynthetic enzymes. The proposed glutathione route (**Fig. 1B**) involves allylation of the cysteine in glutathione, typically performed by members of the glutathione-*S*-transferase family (GST; Edwards *et al.* 2000), followed by transpeptidation to remove the glycyl group, oxidation of cysteine to a sulphoxide and cleavage of the glutamyl group to yield alliin (Lancaster and Shaw 1989; Lancaster *et al.* 1989; Randle *et al.* 1995). Studies utilising both garlic and onion have tested for the presence of relevant genes and enzyme activities for *de novo* CSO synthesis by this pathway.

After soluble proteins from garlic leaves were adsorbed onto a glutathione affinity matrix, a fraction with GST activity could be eluted with glutathione. One dimensional SDS protein electrophoresis showed the presence of only one major band of approximately 25 kDa, consistent with a GST. However, no enzyme activity was detected after incubation with a range of potential endogenous substrates and products, suggesting that it did not have a role in alliin biosynthesis (J Hughes, MC Wilkinson and MG Jones, pers. comm.). The recent report of a γ -glutamyl transpeptidase purified from onion (Shaw *et al.* 2005) also indicated that this activity was not involved in CSO synthesis. Although it may well have a role in transpeptidation in glutathione conjugate metabolism *in vivo*, the substrate specificity showed that it was unlikely to function as a peptidase in γ -glutamyl alk(en)yl cysteine sulphoxide hydrolysis.

The other biosynthetic route, operating through thioalk(en)ylation or alk(en)ylation of O-acetyl-serine or cysteine (**Fig. 1A**), currently has stronger experimental support for the presence of appropriate genes and enzymes. Evidence from the literature and tissue culture experiments suggests that CSase may be involved in the synthesis of alliin. Plants contain families of SAT and CSase, with cytosolic, chloroplastic and mitochondrial isoforms (Inoue *et al.* 1999; Warrilow and Hawkesford 2000; Wirtz *et al.* 2001). The mechanism of CSase, with an enzyme-bound intermediate, provides scope for the active site to evolve the capacity to insert substrates other than sulfide into OAS after a gene duplication event. Within the β -substituted alanine synthase family, of which the CSases forms a part, several are known to be involved in secondary, rather than primary, synthesis. For example, the secondary metabolites mimosine (*Mimosa* and *Leucaena*) and β -pyrazol-1-yl alanine (*Cucurbitaceae*) are both formed by CSases and the CSases from these and other plants are able to form CSOs, including alliin, *in vitro*, when provided with a suitable *S*-alkyl donor (Ikegami *et al.* 1988; Ikegami and Murakoshi 1994). Two forms of CSase have been isolated from leaves of *A. tuberosum* and both were capable of forming alliin *in vitro* when provided with OAS and allyl thiol (Ikegami and Murakoshi 1994). Garlic also contains several CSases and at least one has the capacity to synthesise alliin (Jones *et al.* 2004).

Current experimental evidence therefore supports a route for alliin biosynthesis proposed by Granroth (1970) via CSase, although definitive evidence is still needed. However, the inter-relationship and role of the large amounts of γ -glutamyl peptides present within garlic remains to be resolved. Investigation of the levels of CSOs and γ -glutamyl peptides throughout the garlic life-cycle and in different organs gives further insight into their function, and will be discussed next.

ALLIIN BIOSYNTHESIS AND PLANT DEVELOPMENT

The source of CSOs in garlic cloves is open to speculation. Studies on distribution of CSOs in *Alliums* show that they are found in a range of tissues, including leaf, bulb, root and flower scapes (Briggs *et al.* 2002) as well as in cell

culture and callus tissues (Lancaster 1991). Studies on garlic cloves in storage indicate that they have the capacity to synthesise CSOs from other organosulphur containing compounds present in the cloves (Hughes *et al.* 2006; Ichikawa *et al.* 2006). However, the origin of CSOs in the clove has to be considered in the context of the development of the whole plant.

Garlic has a lifecycle typical of species that form bulbs as organs for surviving through times of the year that are unsuitable for growth and for vegetative multiplication. It is possible to identify four stages of development beginning with the germinating garlic clove (L Trueman and B Thomas, pers. comm.). In stage one the plants grow slowly with little increase in dry or fresh weight, with the plants relying heavily on stored nutrients from the seed clove. In stage two the root and leaf tissue masses increase rapidly with root and leaf sulphur, nitrogen, carbon, protein and CSO content rising to near maximal values. While concentrations of CSOs in roots are high compared to other parts of the plant, root CSOs represent only a minor proportion of the total CSOs in developing garlic. In stage three, the bulb initiates and develops. During this stage, sulphur, nitrogen, carbon, protein and CSOs are lost from the root, which is correlated with root senescence and death at the end of this period while levels of CSOs, sulphur, nitrogen, carbon, and protein decline in the leaf and increase in the bulb. Finally in stage four the bulb is mature, the aerial parts of the plant became dehydrated, leaves senesce and leaf sulphur, nitrogen, carbon and protein levels fall, concurrent with an equivalent rise in bulb levels. These findings are consistent with a process within which alliin and other CSOs are synthesised primarily in leaves and translocated to garlic clove tissues during bulb development. (Bloem *et al.* 2004) also found that decreases in alliin in garlic leaves during the bulb development phase was accompanied by a corresponding increase in the bulb and interpreted this as a translocation from leaves to bulb.

ALLIINASE

Alliinase is the enzyme that initiates the conversion of the alkyl cysteine sulphoxide flavour precursor (alliin) to allicin and its derivatives. Alliinase is extremely abundant in garlic tissue consisting of at least 10% of the total clove protein (Van Damme *et al.* 1992). The enzyme from bulb tissues is a glycoprotein containing 6% carbohydrate and exists as a dimer of two subunits of MW 51.5 kDa each (Rabinkov *et al.* 1994) and the crystal structures of both the apo- and reaction intermediate-bound *holo* enzyme have recently been reported (Shimon *et al.* 2007). Expression studies by Northern and Western analyses showed high expression in bulb tissues while leaves had a lower level of expression. In the same study, roots exhibited abundant alliinase enzyme activity but it was not detected by the antibodies to the bulb protein or in Northern analysis using the bulb cDNA sequence. This suggests that roots have an alliinase isozyme with very low homology to the bulb enzyme. There is biochemical evidence that there are both alliin/isoalliin and methiin specific-enzymes in *Allium* tissues (Lawson 1996). Thus understanding the number, expression characteristics and role of different alliinase genes in garlic could be crucial in maximising the production of the breakdown products of the CSOs. Alliinase is located in the vacuoles of vascular bundle sheath cells which are located around the phloem (Ellmore and Feldberg 1994), rather than the abundant storage mesophyll cells that contain the CSOs (Lawson 1996) which may be linked to the dynamic re-mobilization of CSOs during development. This contrasts with onion where both alliinase and CSOs are present in all cells, respectively in the cell vacuoles and cytoplasmic vesicles (Lancaster *et al.* 1989).

PCR screening of the bulb and leaf cDNA libraries indicated that at least five different alliinase sequences were present in the variety Messidrome (L Trueman pers. comm.). Alliinase expression in the leaf was restricted to one isoform.

Bulb-derived sequences fell in to four groups but sequences identical to the major sequence in the leaf were most prominent suggesting that this sequence may encode the dominant alliinase for the whole plant. Collectively the expression of this isoform in both clove and leaf tissue does not vary significantly with stage of development consistent with the alliinase genes being constitutively expressed.

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