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INTRA-CELLULAR LOCALIZATION OF THE BIOSYNTHETIC PATHWAY TO FLAVOUR PRECURSORS IN ONION

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Key Word Index—*Allium cepa*; Alliaceae; onion; protoplasts; cellular localization; enzymes; *S*-alk(en)yl-L-cysteine sulphoxides; γ-glutamyl peptides.

Abstract—The contents of protoplasts from leaves of sprouted onions (unlabelled and labelled with ^{35}S) were separated into chloroplasts, mitochondria and cytoplasm or sucrose density gradients. ^{35}S labelled γ-glutamyl cysteine and glutathione were found in the chloroplasts, and labelled glutathione, γ-glutamyl peptides and flavour precursors were found in the cytoplasm. γ-Glutamyl cysteine synthethase was shown to be localized in the chloroplasts, whilst γ-glutamyl transpeptidase was found in the cytoplasm. The data suggest that the biosynthesis of glutathione occurs in the chloroplasts and subsequent metabolism from glutathione, via the γ-glutamyl peptides to flavour precursors is cytoplasmic.

INTRODUCTION

Allium species characteristically contain a high concentration of non-protein sulphur amino acids (between 1 and 5% dry wt). One class of these secondary metabolites, the *S*-alk(en)yl-L-cysteine sulphoxides gives rise to the characteristic aroma and flavour of *Allium* species. In the intact cell the sulphoxides (flavour precursors) are compartmentalized in the cytoplasm and the hydrolytic enzyme alliinase in the vacuole [1]. Disruption of cellular compartmentalization results in release of alliinase, and the subsequent hydrolysis of the sulphoxides to volatile sulphides.

Onions contain *S*-propenyl, *S*-propyl and *S*-methyl cysteine sulphoxides. Also present in the cell are other non-protein sulphur amino acids, the γ-glutamyl peptides. The sulphoxides (or flavour precursors) are synthesized from glutathione via S-2-carboxypropyl glutathione and γ-glutamyl peptides [2]. Several pieces of evidence indicate that chloroplasts may play a key role in the biosynthetic pathway to flavour precursors: (i) the reduction of SO_4 and assimilation of S into cysteine is known to occur within the chloroplasts [3]. Glutathione synthesis has been shown to be localized in the chloroplasts of tobacco and peas [4]. (ii) Flavour precursors are thought to be synthesized in the green leaves of intact onion plants [5]. (iii) Flavour precursor are synthesized in photomixotrophic callus, but not in heterotrophic callus [6].

The purpose of this work was to identify the intracellular localization of key enzyme activities and intermediates in the biosynthetic pathway to flavour precursors and, in particular, to determine which part of the pathway is localized in the chloroplasts.

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RESULTS

Intracellular localization of enzymes

Intact chloroplasts and mitochondria released from isolated leaf mesophyll protoplasts were located on gradients at 42% (w/w) sucrose and 32% (w/w) sucrose respectively, by measuring chlorophyll and cytochrome oxidase activity. The distribution of these markers (Fig. 1) showed no detectable chlorophyll at the top of the gradient, indicating little or no plastid breakage, and no cross-contamination between peak mitochondrial and chloroplast fractions on the gradients. The measured position of mitochondria and chloroplasts was similar to that found with pea leaf protoplasts [7].

γ-Glutamyl cysteine synthetase, which catalyses the formation of γ-glutamyl cysteine from glutamate and cysteine, is an enzyme in the glutathione cycle [4] and was located very predominantly in the chloroplast fraction (Fig. 1). γ-Glutamyl transpeptidase, considered to be the final enzyme in the biosynthetic pathway to flavour precursors [2], was found in the soluble fraction at the top of the gradient (80% of total activity). Some of this enzyme activity (*ca* 20%) was also seen at 35% (w/w) sucrose (Fig. 1), which could indicate some peroxisomal localization [7]. However, catalase activity was not measured as a peroxisomal standard in these experiments.

Intracellular localization of γ-glutamyl peptides and flavour precursors

Radioactivity was present throughout the sucrose gradient, but was greatest in the soluble fraction at the top of the gradient (75% of total radioactivity on the gradient), with a smaller peak of radioactivity in the chloroplast fraction (4%). Radioactivity within the chloroplast fraction was attributable mainly to sulpholipids (81.6%), whereas soluble fractions contained lesser amounts of sulpholipids (33.5%).

The sulphur compounds soluble in methanol–water were separated on Dowex-1 ion exchange columns. Flavour precursors, cysteine and methionine (0.1 M HOAc eluant) were further separated by electrophoresis. γ-Glutamyl peptides in 1.0 and 2.0 M HOAc eluants were separated on TLC.

Most of the radioactivity in the soluble fraction was located in free flavour precursors, cysteine and methionine (0.1 M HOAc) (Table 1). Separation of 0.1 M HOAc eluant by electrophoresis showed that cysteine/methionine accounted for about 10% of the radioactivity and flavour precursors *ca* 90%. The soluble fraction also contained 10 γ-glutamyl peptides (5 in 1.0 M HOAc, 5 in 2.0 M HOAc) including γ-glutamyl propyl/propenyl cysteine sulphoxides, *S*-2-carboxypropyl glutathione and glutathione. γ-Glutamyl cysteine was not detectable in the soluble fraction.

Neither free flavour precursors (0.1 M HOAc) nor γ-glutamyl peptides eluting in 1.0 M HOAc were detectable in the chloroplast fraction (Table 1). Although radioactivity was present in the 2.0 M HOAc eluate it was due almost entirely to glutathione, with a barely detectable amount corresponding to γ-glutamyl cysteine.

DISCUSSION

In *Allium* spp. the biosynthetic pathway from cysteine to flavour precursors has been shown to proceed via γ-glutamyl cysteine, glutathione, *S*-2-carboxypropyl glutathione and γ-glutamyl peptides [2]. The results above suggest that in onions the biosynthetic pathway from SO_4 to cysteine and then to the flavour precursors is localized partly within the chloroplasts and partly within the cytoplasm.

γ-Glutamyl cysteine synthetase and its product γ-glutamyl cysteine were localized in the chloroplasts. This is the first reported direct evidence of a chloroplastic localization for this enzyme. Glutathione was also found within the chloroplasts, but not the sulphur peptides found in subsequent steps of the biosynthetic pathway.

Glutathione and the γ-glutamyl peptides which are intermediates in the biosynthetic pathway to flavour precursors were located at the top of the sucrose density gradients and are thus presumably cytoplasmic. Glutathione has also been found in the cytoplasm and vacuoles of tobacco [8] and has been shown to move freely from the chloroplasts into the cytoplasm [9]. In onion cells it is likely that the cytoplasmic glutathione, which gives rise to the flavour precursors, is transported from the chloroplasts.

The location of the flavour precursors in the cytoplasm was also confirmed by direct experience during the experiment. As the protoplasts lysed the vacuoles burst (seen under the light microscope) and a strong onion odour was liberated. The fact that this occurred in the presence of intact chloroplasts (Fig. 1), provides further support for the cytoplasmic localization of the flavour precursors.

γ-Glutamyl transpeptidase, the final enzyme in the pathway, along with its substrate γ-glutamyl propyl–propenyl cysteine sulphoxide and its products, the free cysteine sulphoxides were also located within the cytoplasm. A smaller amount of γ-glutamyl transpeptidase activity was found on the sucrose density gradient at a position which indicated a possible peroxisomal localization. In their work with tobacco cell suspension cultures, Steinkamp and Rennenberg [10] found 77% of γ-glutamyl transpeptidase activity was localized in the soluble cytoplasmic fraction and 23% was associated in the membranes, but not with mitochondria or chloroplasts. Their localization used differential centrifugation and this method would not have preserved peroxisomes intact. It is possible that the membrane-associated γ-glutamyl transpeptidase identified in their work is, in fact, peroxisomal membrane material. A role for γ-glutamyl transpeptidase in peroxisomes is not immediately apparent and further work on the non-cytoplasmic localization of this enzyme is proceeding.

Although the biosynthetic pathway from glutathione to flavour precursors is localized in the cytoplasm, the presence of chloroplasts does seem to be necessary for flavour precursor synthesis. Lancaster *et al.* [6] have shown that light-grown photomixotrophic callus produces flavour precursors, whereas light-grown bleached callus does not. Shigeoka *et al.* [11] found that a low

Table 1. Amount of radioactivity (^{35}S) in sulphur compounds (separated on Dowex-1 ion-exchange column, electrophoresis and TLC) in cellular organelles (cpm × 10^4/lysed protoplasts)

Ion-exchange eluate (M HOAc)	Fraction from sucrose density gradient: Soluble	Chloroplast
0.1	32.1	...
1.0	1.2	...
2.0	1.2	0.5

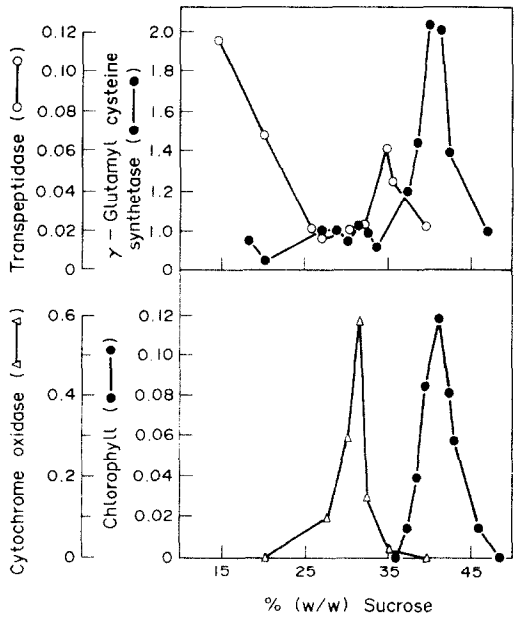

Fig. 1. Distribution of enzymes in sucrose density gradients of whole homogenates of ruptured *Allium* leaf protoplasts. All units given are arbitrary.

level of glutathione synthesis occurred with dark grown *Euglena* cells, and that high levels of glutathione were induced by blue light in green cells, but not in bleached cells. In *Allium* spp. chloroplasts may be necessary to provide the large amount of glutathione necessary for the large amount of flavour precursor synthesis.

Alternatively chloroplasts could be providing a developmental signal necessary for the expression of the biosynthetic pathway in the cytoplasm and independent of their photosynthetic role. The findings of Shigeoka *et al.* [11] that the photoinduction of glutathione was not dependent on photosynthesis *per se* supports this concept.

Turnbull *et al.* observed the presence of vesicles in regenerating callus, and correlated these organelles with the development of flavour precursors in the roots and shoots [12]. It is feasible that such organelles are associated with flavour precursor synthesis and/or storage.

EXPERIMENTAL

Plant The epidermal layer was removed from green leaves of sprouted onion bulbs (*Allium cepa cv.* Pukekohe Long Keeper), with a pair of forceps, and the exposed (or underlying) mesophyll tissue (1 g) was placed downwards on 10 ml 2% cellulase (Onozuka R10), 0.5% macerozyme in 0.6 M sorbitol, 3 mM $CaCl_2$, 3 mM MES and left overnight, in the dark, without shaking. Protoplasts were released from the cell wall debris by squirting this mixture through a wide mouthed plastic pipette and were then pelleted by centrifugation at 100 *g* for 3 min before being washed × 4 with 10 ml 0.6 M sorbitol, 10 mM MES pH 5.5.

Lysing protoplasts. Washed protoplasts were pelleted, lysed by the addition of 0.45 M sorbitol, 25 mM Tris HCl, 10 mM mercaptoethanol pH 7.5 followed by three passages under pressure through 20 μM mesh. The volume of lysed protoplasts was brought up to 2 ml with 0.45 M sorbitol and BSA added to 10 mM. This soln was left to stand for 30 min to allow any cellular debris and intact protoplasts to settle to the bottom of the tube.

Sucrose density gradients. Lysed protoplasts (2 ml) were layered on a 30 ml linear gradient of 25% (w/v) to 60% (w/v) sucrose in 50 mM Tris–HCl pH 8.0, 10 mM 2-mercaptoethanol over a 5.0 ml bottom layer of 70% (w/v) sucrose. The gradients were centrifuged in a Beckman model L8-70 centrifuge with an SW28 rotor for 5 min at 2110 *g* (4000 rpm), followed by 10 min at 33 800 *g* (16 000 rpm). The gradient was collected in 1.2 ml fractions by upwards displacement using an Isco gradient fractionator and the density of the fractions [%(w/w)sucrose] determined using a Bausch–Lomb refractometer.

Enzyme assays. For assay of γ-glutamyl cysteine synthetase the enzyme reaction contained in 100 mM Tris–HCl, pH 8.0: glutamate (15 mM), cysteine (15 mM), KCl (40 mM), $MgSO_4$(10 mM), ATP (5 mM), phosphoenolpyruvate (10 mM) and pyruvate kinase (20 units) in a final vol. of 0.5 ml. The reaction was started by the addition of enzyme and incubated 15 min at 25°. The reaction was stopped by the addition of 50 μl of 3 M $HClO_4$ and the mixture neutralized with 50 μl of 4 M KOH. Precipitated protein and insoluble $KClO_4$ was removed by centrifugation and 500 μl of the supernatant used to determine γ-glutamyl cysteine [13], after reduction in $NaBH_4$. A γ-L-glutamyl-L-cysteine standard was prepared according to ref. [14].

γ-Glutamyl transpeptidase was assayed at 25°, according to ref. [15]. Liberated *p*-nitroaniline was diazotized and measured at 530–550 nm as described by ref. [16]. Chlorophyll was determined by measuring absorbance of gradient fraction aliquots at 654 nm in 95% EtOH [17] and cytochrome oxidase according to ref. [18].

^{35}S Labelled protoplasts. Labelled $Na_2{}^{35}SO_4$ (1078 Ci/mol = 2.04 mCi/ml) from Radiochemical Centre, Amersham, U.K. was fed continuously for 24 hr to leaves of sprouted onion bulbs which had been cut. This was done to fully label the biosynthetic pathway to flavour precursors. Protoplasts were prepared and washed × 4 as described above. Prior to the lysis of the protoplasts 200 μl 0.1 M hydroxylamine HCl, pH 7.0, was added (to give a final concentration of 0.01 M hydroxylamine) to inhibit alliinase [19] and maintain the flavour precursors intact. The lysed protoplasts were layered on to sucrose gradients, run and collected as described above.

Isolation of ^{35}S labelled compounds. Radioactivity was counted in aliquots of the fractions from the gradients using a triton X-100 scintillant [20], and a ^{14}C window and external standard technique [21]. Peaks of radioactivity were found in fractions corresponding to cytoplasm (top of the gradient), and chloroplasts. Sulphur compounds including flavour precursors and γ-glutamyl peptides were extracted from these fractions and separated on ion-exchange columns and TLC as described in detail in ref. [2].

Labelled compounds in the 1.0 and 2.0 M HOAc ion exchange eluates were separated using TLC, whilst flavour precursors, cysteine, and methionine in the 0.1 M HOAc eluate were separated by cellulose electrophoresis. The plates were dipped in a commercial fluor, dried and exposed to Kodak X-Omat AR film at −70° for 24 hr [22]. The X-ray film was developed, its image intensity determined by a scanning densitometer, and then calibrated against images of known radioactivity. Radioactivity from individual compounds was summed to provide a total for each HOAc eluate.

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