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Short Communication

Subcellular Localization of Asparaginase and Asparagine Aminotransferase in *Pisum sativum* Leaves[1]

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ROBERT J. IRELAND AND KENNETH W. JOY
Biology Department, Carleton University, Ottawa, Ontario K1S 5B6 Canada

ABSTRACT

Protoplasts isolated from young and mature pea leaves (*Pisum sativum* L.) were broken and their contents fractionated by differential centrifugation or on sucrose-density gradients. Asparaginase was found only in the cytosol of young leaves. Asparagine aminotransferase was found in both young and mature leaves and was localized exclusively in the peroxisome. This corroborates the observation that asparagine transamination is catalyzed by the serine:glyoxylate aminotransferase.

Expanding legume leaves receive most of their nitrogen in the form of asparagine (10). Pea leaves possess two enzymes capable of metabolizing asparagine (4): asparaginase (EC.3.5.1.1), which produces NH_3 and aspartate, and an aminotransferase (EC.2.6.1.14) which produces 2-oxo-succinamate and either glycine or alanine (5). Asparaginase activity is high in young leaves but decreases rapidly as the leaf expands; aminotransferase activity, however, remains high throughout leaf development (4). Feeding studies have shown that when the leaf is half expanded, and the two activities are approximately equal, most of the asparagine is metabolized by the aminotransferase. The presence of two metabolic routes for asparagine in the same tissue suggests that regulatory mechanisms must be in operation. Asparagine aminotransferase, like aminotransferases in general, is not highly regulated, and the properties of pea leaf asparagines have not been examined. One important method of regulation in plant metabolism is subcellular compartmentation, and it seemed possible that this could be involved in asparagine metabolism.

This report describes the subcellular location of asparagine transamination and deamidation in pea leaves.

MATERIALS AND METHODS

Plant Material. *Pisum sativum* L. (cv Little Marvel; McKenzie, Steele, Briggs Seeds, Brandon, Manitoba, Canada) were grown hydroponically as described previously (5).

Isolation of Protoplasts from Mature Leaves. Fully expanded leaves (4–12 g) were removed from 3-week-old plants (leaves three and four from five-leaf plants), and protoplasts were isolated by a procedure modified from that of Wallsgrove *et al.* (18). The lower epidermis was removed with forceps and the leaves floated on 0.4 M sorbitol containing 1 mM KH_2PO_4 and 5 mM $MgCl_2$ (pH 5.5) in a Petri dish. After 5 min, the leaves were transferred to another dish containing 1% (w/v) Cellulysin (Calbiochem-Behring Corp.) in the same medium. The dishes were placed in a controlled environment (dark) at 30°C, and were gently swirled every 30 min. After 3 h, the mixture was filtered through a coarse nylon net: the leaves were washed with buffered sorbitol (as above), and the washings also poured through the nylon net. The filtrate was centrifuged at 200*g* for 2 min, and the pellet was resuspended in 15% (v/v) Percoll (in 50 mM Tricine, pH 7.5, containing 5 mM $MgCl_2$, 1% BSA, and 0.5 M sucrose). This was placed in a centrifuge tube and overlayered with an equal volume of 10% (v/v) Percoll in the same buffer, followed by an equal volume of 0.5 M sorbitol in 50 mM Tricine (pH 7.5) containing 5 mM $MgCl_2$. After centrifugation at 200*g* for 10 min, intact protoplasts were removed from the upper interface with a pasteur pipette.

Isolation of Protoplasts from Young Leaves. All solutions involved in the preparation of protoplasts from young leaves contained 50 mM KCl. Young leaves (1 d after emergence from stipule) were harvested from 3-week-old plants, and abraded on both surfaces with carborundum, and floated on buffered sorbitol as above. After 5 min, they were transferred to the Celluysin medium and sliced into 2-mm strips with a razor blade. They were then subjected to vacuum infiltration for a few min, incubated for 3 h, and their protoplasts harvested and purified as above.

Subcellular Fractionation. BSA was added to the protoplasts to give 0.1% (w/v), which were then broken by five passages through a 20-μm nylon mesh attached to the tip of a 20-ml syringe. Subcellular organelles were then fractionated by differential centrifugation or on sucrose-density gradients. Differential centrifugation consisted of a 2,200*g* spin for 2 min followed by a 20,000*g* spin for 10 min. Sucrose density gradients consisted of 5 ml 60% (w/w) sucrose in 50 mM Tricine (pH 7.5, containing 0.1% BSA) overlayered with 25 ml of a linear 60 to 25% (w/w) gradient of sucrose in the same buffer, over which the broken protoplasts (6–8 ml) were directly layered. After centrifugation at 25,000*g* for 2.5 h in a Beckman SW 27.1 rotor, the tubes were pierced with a syringe needle and 1.6-ml fractions collected.

Enzyme Assays. Asparaginase was determined by measuring aspartate formation, and asparagine aminotransferase by measuring alanine formations from pyruvate or by spectrophotometric determination of 2-oxosuccinamic acid production as described previously (4). Fumarase was determined by the method of Racker (13), catalase by the method of Luck (8), and Chl by the method of Wintermans and De Mots (19). All assay mixtures contained 0.1% (v/v) Triton X-100.

RESULTS AND DISCUSSION

We have previously shown that, although young leaves contain high levels of asparaginase, mature leaves contain very little (4). Inasmuch as the aminotransferase levels are similar at all stages of development, it would have been desirable to use young leaves

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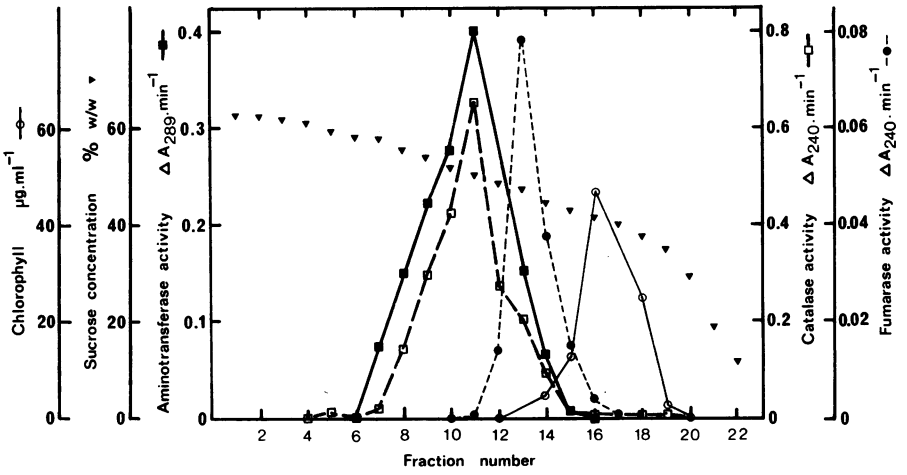

FIG. 1. Distribution of enzyme activities in sucrose density gradients of ruptured protoplasts isolated from mature pea leaves. Chl (○), sucrose (▼), asparagine aminotransferase (■), catalase (□), fumarase (●).

Table I. *Distribution of Enzyme Activities in Organelle Fractions Separated by Differential Centrifugation of Broken Pea Leaf Protoplasts*

Protoplast Source	Fraction	Enzyme Activity				
		Catalase	Fumarase	Chl	Asparaginase	Asparagine aminotransferase
		% of total recovered				
Young leaf	2,200*g* pellet	7	2	94	ND[a]	7
	20,000*g* pellet	87	95	4	ND	91
	Supernatant	6	3	1	100	2
Mature leaf	2,200*g* pellet	7	ND	98	ND	8
	20,000*g* pellet	90	97	2	ND	91
	Supernatant	3	3	ND	ND	1

[a] Not detectable.

as a protoplast source. However, although protoplasts were readily prepared from mature leaves, preparations from young leaves proved far more difficult. It was not possible to remove either epidermis by peeling and thus the procedure of abrading/chopping/infiltration was adopted. This produced poor yields of protoplasts and so, where possible, mature leaves were used as the protoplast source. Pea leaf asparaginase showed complete dependency on K^+ for activity, as described by Sodek *et al.* (15). It was therefore necessary to add KCl to all solutions used in preparing young leaf protoplasts.

Differential centrifugation provided a convenient method for the separation of broken protoplasts into three discrete fractions: a chloroplast-enriched 2200*g* pellet, a mitochondrial and peroxisomal-enriched 20,000*g* pellet, and a cytosolic (supernatant) fraction. Table I shows that only slight breakage of the organelles occurred, very little of the Chl or marker enzymes being found in the supernatant. The aminotransferase from both young and mature leaves was localized in the 20,000*g* pellet, whereas the asparaginase was found in the supernatant, and thus appears to be cytosolic. Sucrose density gradients of broken protoplasts from mature leaves revealed asparagine aminotransferase to peak with catalase, and thus localized exclusively in the peroxisome. This was as expected, as we have recently shown (5) that asparagine aminotransferase is the same enzyme as the serine:glyoxylate aminotransferase, which resides in the peroxisome (14) and plays a key role in photorespiration (17).

Asparagine is thus metabolized in two distinct subcellular compartments in pea leaves. In young leaves, when both enzymes are active, asparaginase will produce aspartate and ammonia in the cytosol, whereas peroxisomal transamination will produce 2-oxosuccinamate and either glycine or alanine. In mature leaves, asparagine metabolism is restricted to the peroxisome. The products of asparaginase activity can be dealt with by well established routes, but the location and nature of 2-oxosuccinamate metabolism is not clear. The product of 2-oxosuccinamate reduction, 2-hydroxysuccinamate, has been detected in high levels in pea leaves (7), but we have also detected 2-oxosuccinamate deamidase activity (unpublished).

Although asparagine aminotransferase has been well documented in animal systems (*e.g.* 3,9), there are few reports of its occurrence in plants (6, 16). Cincerova (2) described an asparagine aminotransferase from wheat which used α-ketoglutarate preferentially to pyruvate as an amino acceptor; and Nahler and Ruis (12) reported an asparagine aminotransferase from germinating castor bean that was associated with glyoxysomes and mitochondria and also was active with α-ketoglutarate. We have been unable to find asparagine:α-ketoglutarate aminotransferase activity in peas, and it is possible that this activity may be due to aspartate contamination of the asparagine used in the earlier experiments.

Inasmuch as asparagine is found in high levels in leaf tissue (11), it is interesting to speculate on the possible role of asparagine in photorespiration. Serine, glutamate (17), and alanine (1) have all been implicated in the animation of glyoxylate, and it now appears that asparagine should be added to this list.

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