Phytochemistry, Vol. 25, No. 5, pp. 997–1001, 1986.
Printed in Great Britain

0031 9422/86 $3.00 + 0.00

THE 4-AMINOBUTYRATE SHUNT IN *SOLANUM TUBEROSUM*

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(*Revised received* 30 *October* 1985)

Key Word Index—*Solanum tuberosum*; Solanaceae; potato tubers; metabolism; 4-aminobutyrate; shunt pathway; enzymes.

Abstract—In potato tubers, 4-aminobutyrate was formed from L-glutamate and was converted to succinate by the operation of the 4-aminobutyrate shunt. The three enzymes of the shunt pathway were detected in the tubers. Glutamate decarboxylase was found exclusively in the cytoplasm, whereas 4-aminobutyrate transaminase and succinate semialdehyde dehydrogenase were present both in mitochondria and cytoplasm in a 1:3 ratio. 4-Aminobutyrate transaminase gave good activity with pyruvate and 2-oxoglutarate.

INTRODUCTION

4-Aminobutyrate (4-AB) is a non-protein amino acid widely distributed in nature. In particular, many higher plant tissues contain considerable amounts of this compound in their free amino acid pool [1, 2]. Accumulation and metabolism of 4-AB in edible plant tissues such as potato tubers assumes significance as 4-AB is a well known inhibitory neurotransmitter in the human brain [3] and it also has other roles in mammals [4]. Although the formation of 4-AB from L-glutamate by α-decarboxylation catalysed by the enzyme glutamate decarboxylase GDC, EC 4.1.1.15) is well known in higher plants [5, 6], there are only few reports on the further metabolism of 4-AB. Limited data available from few plant species [7, 8], mainly based on [^{14}C]4-AB tracer studies, suggest that 4-AB is metabolized to succinate via SSA (succinate semialdehyde) by the operation of the 4-AB shunt or bypass pathway (Fig. 1) as in mammalian brain [9]. However, the presence of all the three enzymes of the 4-AB shunt, GDC, 4-AB transaminase (4-AB-T, EC 2.6.1.19) and succinic semialdehyde dehydrogenase (SSADH, EC 1.2.1.24) has been convincingly demonstrated in only two higher plant species to data, namely radish leaves [10] and wheat embryo [11].

As a part of our detailed investigations on the accumulation, metabolism and role of 4-AB in potato tubers which is known to contain one of the of the largest amounts of 4-AB among higher plants [1, 12, 13], we report here the operation of the 4-AB shunt in potato tubers.

RESULTS AND DISCUSSION

The 4-AB content of potato tubers was determined by a specific and sensitive enzymatic method using commercial 'Gabase'. The 4-AB content of freshly harvested potatoes was found to be in the range of 3.2–4.1 μmol/g fr. wt of the tissue. This finding confirmed the earlier reports on the high 4-AB content of potatoes [1, 12, 13].

The source of 4-AB in potatoes is L-glutamate and the active conversion of glutamate to 4-AB by decarboxylation occurred in potato tissue. This was evident from the incorporation of label from L-[U-^{14}C]glutamate predominantly to 4-AB. On feeding radiolabelled glutamate into potatoes, 5,10 and 25% of label appeared in 4-AB at the end of 4, 24 and 96 hr of incubation, respectively. However, appreciable incorporation was also observed into glutamine, aspartate, asparagine and succinate with longer incubation periods (96 hr) and the greatest incorporation was in glutamine, accounting for 14% of the label.

In order to determine the metabolism of 4-AB in potato tuber, they were fed with [U-^{14}C]4-AB and the distribution into various cellular substances at different time intervals was examined. The results shown in Table 1 showed that 85–90% of the total radioactivity from labelled 4-AB fed to potato tuber was recovered from the respired CO_2 plus 80% ethanol extract. The ethanol extract on further analysis showed that incorporation was mainly in the amino and organic acids fractions. The ethanol insoluble and neutral sugar fractions contained only negligible radioactivity and they were not analysed further. 4-AB was actively metabolized in potatoes and 50% of the infiltrated 4-AB was transformed into other compounds in 20 hr and as much as 16% of ^{14}C from 4-AB appeared in the respired CO_2 in the same period. The appearance of label from [^{14}C]4-AB in succinate and malate initially and in aspartate, glutamate and their amides thereafter suggested that 4-AB was first converted to succinate by the operation of the 4-AB shunt (Fig. 1). Succinate entered the tricarboxylic acid cycle and was further metabolized, thereby accounting for the appearance of label in amino acids derived from the tricarboxylic acid cycle intermediates. The labelling pattern observed in keto acids was also consistent with this contention.

The intermediate in the conversion of 4-AB to succinate is succinic semialdehyde (SSA) [14], so an attempt was made to measure SSA levels in potato tuber. Failure to detect SSA in potato by an enzymatic method which could detect as little as 8 nmol/g fr. wt of tissue indicated that free SSA did not accumulate in the cells, possibly due to its rapid conversion to succinate or some other intermediate.

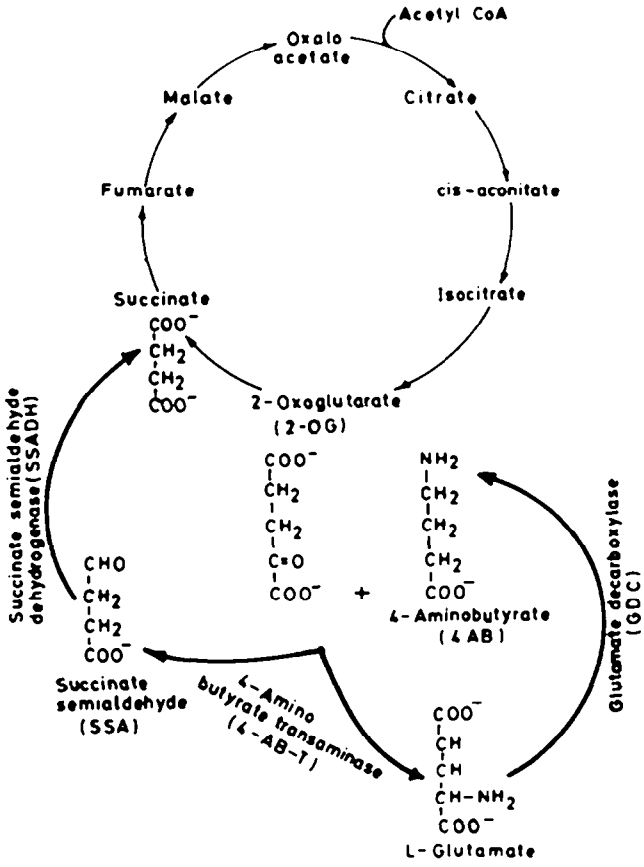

Fig. 1

Table 1. Distribution of ^{14}C from [U-^{14}C]4-AB in various compounds in potato tubers fed with 8×10^6 cpm of radioactive 4-AB

Fraction	Time after feeding [^{14}C]4-AB in hr 4	24	96
	(cpm $\times 10^{-6}$)		
CO_2	0.07	0.76	1.52
Ethanol-soluble fraction	7.0	6.5	5.5
Amino acid fraction	6.6	4.2	3.04
4-AB	4.9	2.0	0.3
Glutamate	trace	0.05	0.6
Glutamine	trace	0.4	1.0
Aspartate	0.2	0.7	1.2
Organic acid fraction	0.34	1.55	2.0
Succinate	0.15	0.9	0.65
Malate	0.09	0.54	0.52
Citrate	0.06	0.41	0.33
Neutral sugars fraction	0.035	0.02	0.028
Keto acids			
Oxalo acetate	0.05	0.34	0.45
α-Ketoglutarate	0.034	0.05	0.08

Values mean of two independent determinations.

The earlier reports showed that this is true in the case of vertebrate brain [14] and radish leaves [15].

The above experiments suggested that in potatoes, 4-AB is formed from glutamate and its further metabolism takes place via the 4-AB shunt pathway giving succinate via SSA. In this case, the existence of the individual enzymes of this pathway, viz. GDC, 4-AB-T and SSADH, need to be demonstrated in potato tubers. Since the presence of active enzymes of this pathway has not been convincingly established in potato tubers previously [2, 7, 12, 16], preparation of cell free extracts was done under a wide range of conditions to optimize the maximum extractability of each of the enzymes. The variations included are: the use of different buffers, buffers at different pH; the presence or absence of chelating agent, metal ions, detergents and phenolic binders; as well as different methods for efficient extraction of the tissue, such as grinding in a mortar with pestle, homogenization in a Waring blendor, acetone powder preparation and freezing and thawing of the tissue. Out of these trials, the media that yielded maximal activity were chosen for the study of individual enzymes.

By employing the procedures described in the Experimental; the activities of all three enzymes of the 4-AB shunt pathway have been demonstrated in potato tubers and a typical activity profile is shown in Table 2. The expected reaction products were confirmed on thin layer chromatograms of the respective reaction mixtures. Only barely detectable activity of GDC [2, 12] and 4-AB-T [16] were reported earlier from potato tubers and SSADH activity has not been previously demonstrated in this tissue. Specific activities of 4-AB shunt enzymes reported in this work compare favourably with reported values for other higher plants [5, 6, 10, 17]. There was considerable excess of GDC activity relative to 4-AB-T activity (GDC/4-AB-T ratio being 23) in potato tubers. This was true in the case of radish [10] and soybean [17] enzymes also. Although the exact significance of this finding is not yet understood, it could be inferred that a rapid decarboxylation of L-glutamate followed by a relatively slow turnover of 4-AB might be one of the reasons for the high-AB content of potato tubers. However, potato 4-AB-T, unlike the enzyme from other plant sources [7, 10, 17] which were only weakly active or inactive with 2-α-oxoglutarate (2-OG) as compared to pyruvate as the amino group acceptor, gave an appreciable activity with both keto acids. The pyruvate-2-OG efficiency ratio for potato 4-AB-T was 1.8 whereas the reported values were as high as 8–19 from other plant sources [7, 10, 17].

An assay of 4-AB shunt enzymes in different regions of the potato tubers namely bud, cortex, pith and vascular regions revealed that they are uniformly distributed throughout the tuber (data not shown). The intracellular distribution of the enzymes determined by the differential centrifugation of the potato homogenates is given in Table 3. GDC activity was not associated with any particulate fraction and was exclusively present in the soluble phase. In contrast, 4-AB-T and SSADH enzymes were distributed in the mitochondria and soluble phase in 1:3 ratio. A recovery of greater than 90% fumarase activity of the crude homogenate in the mitochondrial fraction ruled out the possibility that 4-AB-T and SSADH activity found in the cytoplasm could be due to the rupture of mitochondria during isolation. Our work on the intracellular distribution of 4-AB shunt enzymes confirms and further extends the earlier work [10, 17] on the dual localization of GAB-T in higher plants.

Table 2. Activity of 4-AB shunt enzymes in potato tubers

Enzyme	Activity pkat/g fr. wt	Activity pkat/mg protein
Glutamate decarboxylase	2920	486
4-AB-pyruvate transaminase	125	23
4-AB-2-oxo-oglutarate transaminase	69	12.2
Succinate semialdehyde dehydrogenase	1100	166

Table 3. Intracellular distribution of 4-AB shunt enzymes

Fraction	Glutamate decarboxylase (pkat)	Succinate 4-AB-pyruvate transaminase (pkat)	Semialdehyde dehydrogenase (pkat)	Catalase absorbance change/min	Fumarase absorbance change/min	Protein (mg)
100 *g* supernatant	2340	108	617	1.02	0.42	4.95
10000 *g* pellet (mitochondria)	44	22	173	0.18	0.375	0.46
105000 *g* pellet (microsomes)	25	11	17.3	0.076	0.008	0.25
105000 *g* supernatant (soluble phase)	2200	77	427	0.77	0.04	4.25

All activities and protein content correspond to 1 g fr. wt of tubers.

The operation of the 4-AB shunt provides an alternate route for glutamate to enter the tricarboxylic acid cycle apart from its normal conversion to 2-OG. In addition, this shunt assumes greater significance in tissues under conditions where normal conversion of 2-OG to succinate is restricted. We are currently working on these lines to understand the role of 4-AB in potato tuber physiology.

EXPERIMENTAL

Plant material. Potato tubers (*Solanum tuberosum*, Kufri Chandramukhi cultivar) were obtained from the local market within one month after harvest, were stored at room temp. during the studies and were used within a month to avoid complications due to sprouting.

Chemicals. L-[U-^{14}C]Glutamate (130 mCi/mmol) and [U-^{14}C]4-AB (213 mCi/mmol) were obtained from the isotope group, BARC (Bombay). Hyamine hydroxide was the product of New England Nuclear. Gabase and fine chemicals were from Sigma. All other reagents were of highest analytical purity grade.

Determination of 4-AB. Sliced potato tuber tissue (100 g) was extracted with ice cold 0.6 M $HClO_4$ in a Waring blender (3 × 200 ml). After removal of the acid insoluble material by centrifugation (15 000 *g*, 20 min, 0–2°), the amino acids in the acid extract were separated by pH adjustment to 7 with 1 M KOH, removal of $KClO_4$ by centrifugation and ion exchange chromatography on Dowex-50 (H^+ form). 4-AB present in Dowex-50 eluates, after removal of alkali and concn, was determined enzymatically using a commercial 'Gabase' preparation [18].

Determination of SSA. SSA amount in buffered extracts of potatoes was determined enzymatically using a purified SSADH preparation from potato tubers (unpublished data).

Labelled experiments. L-[U-^{14}C]Glutamate or [U-^{14}C]4-AB containing 1 μCi in 10 μl soln was placed in a 2 mm slot made by a cork borer in each of the potatoes (4 numbers in each expt) and the radioactive soln was allowed to imbibe (about 15 min.). The potatoes were then transferred to a desiccator which was made air tight. CO_2 free and moisturized air was passed through the desiccator by an aeration pump at a constant flow and the expired air was collected in 50 ml 0.3 M KOH contained in a Pettern–Koffer tube. At each time interval, an aliquot of the alkali soln was mixed with 10 ml of Bray's fluid and was counted for radioactivity in a Beckman LS 100 counter with an efficiency of 94% for ^{14}C. The potato samples at each time interval were extracted with boiling 80% EtOH, EtOH was evapd at 30° *in vacuo* and the concd samples were put through tandem columns (1 × 9 cm) of Dowex 50 (H^+) and Dowex-1 ($HCOO^-$). Amino acids from Dowex-50 were eluted with 2 M NH_4OH, separated by TLC on cellulose with MeOH–pyridine–H_2O (20:1:5) as the solvent system [19], and the amino acid spots were visualized by spray with 0.5% ninhydrin in 5% AcOH in Me_2CO. Organic acids from Dowex-1 were eluted with 4 M HCOONa, separated by TLC on cellulose with Et_2O–HCOOH–H_2O (20:2:1) as solvent [20] and spots were detected by spray with bromocresol green in 96% EtOH just made alkaline with 0.1 M NaOH. Duplicate spots not sprayed with detection reagents were scraped from the plate, eluted with 75% EtOH and counted for radioactivity.

Incorporation of [^{14}C]4-AB label into various ketoacids was determined in a separate experiment. Potatoes incubated with [^{14}C]4-AB for different time intervals were extracted with ice cold 0.6 M H_3PO_3 and keto acid hydrazones were prepared and purified according to the method of ref. [21]. Individual keto acid hydrazones were separated by TLC on cellulose with *n*-BuOH–EtOH–0.5 M NH_3 (7:1:2) as solvent system.

Preparation of cell-free extracts. All operations were at 0–2°. Sliced potato tissue (50 g) was ground in a mortar with the grinding medium (2 × 100 ml), filtered through 8 layers of cheese cloth and the filtrate was centrifuged (30 000 *g*, 30 min). The clear pale yellow supernatant was used as the enzyme source. Extraction media used were: GDC; 50 mM NaPi buffer pH 5.8 containing 14 mM 2-mercaptoethanol (2-ME) and 40 μM pyridoxal-5'-phosphate (PLP); 4-AB-T; 100 mM NaPi buffer pH 7.3 containing 14 mM 2-ME, 1 mM EDTA, 50 μM PLP and 0.2% Triton-X 100 and SSADH; 10 mM NaPPi buffer pH 7, containing 14 mM 2-ME, 1 mM EDTA, 0.1% Triton X 100 and 0.5% polyclar-AT.

Sub-cellular fractionation. Sub-cellular fractionation of potato tissue was done according to the method of ref. [22]. 20 g sliced tissue was macerated in 30 ml of semi-frozen grinding medium containing 0.3 M mannitol, 1 mM EDTA, 20 mM NaPPi buffer pH 7.5, 0.1% BSA and 4 mM L-cysteine in a Waring blender at low speed (3 × 5 sec), the homogenate was filtered and the filtrate was subjected to differential centrifugation. The pellet fractions were resuspended in the extraction medium without mannitol but containing 0.5% Triton-X 100. Fractions prepared with medium containing no BSA were used to determine protein content. Marker enzymes, fumarase [23] and catalase [24] were assayed spectrophotometrically.

Enzyme assays. GAD was assayed radiometrically [25] by measuring the $^{14}CO_2$ liberated from L-[U-^{14}C] glutamate using Conway micro diffusion units. The outer well contained in a total vol. of 2.5 ml; 50 mM NaPi buffer pH 5.8, 0.2 mM PLP and 3–4 mg enzyme protein. The inner well contained one ml of hyamine hydroxide. The reaction was initiated by the addition of L-[U-^{14}C] glutamate (24 mM, 156 μCi/mmol) and the units were immediately made air tight. The units were incubated at 37° for 1 hr with occasional agitation, the reaction was stopped by the addition of 20% TCA and an aliquot of the hyamine base was measured for radioactivity. Units with boiled enzyme (15 min at 100°) served as controls and the values never exceeded 5–7% of the experimental values. 4-AB-T was assayed by the method of ref. [10]. A 3 ml assay mixture contained 100 mM borate buffer pH 9, 5 mM 2-ME, 0.3 mM PLP, 10 mM [U-^{14}C]4-AB containing 1 μCi, Na salt of the keto acid (pyruvate or 2-OG) and 3–4 mg of enzyme protein. The reaction mixtures were incubated at 37° for 1 hr, 1 ml of 20% TCA was added and the ppt was removed by centrifugation. The clear supernatants were applied on Dowex 50 (H^+) and the radioactivity in the column effluents was taken as a measure of enzyme activity. The complete reaction mixture minus keto acid served as the control. SSADH was assayed spectrophotometrically at 340 nm at room temp. In 3 ml, the reaction mixture contained 100 mM NaPPi buffer pH 9, 14 mM 2ME, 0.5 mM NAD^+ and enough enzyme to give an A change of 0.05–0.1/min. Reaction was initiated by the addition of 100 μM SSA to the experimental cuvette.

Protein estimation. Protein content was determined by Lowry's method [26].

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