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Article

Enzymes of Carbohydrate Metabolism in Cowpea (*Vigna unguiculata* L. Walp. cv. Caloona) Nodules

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The activities of enzymes of carbohydrate metabolism have been determinated in the host cytosolic and bacteroid fractions of cowpea (*Vigna unguiculata*) nodules formed with *B. japonicum* I 16 and in roots of nodulated cowpeas. The host cytosolic fraction of the nodules contained the enzymes of glycolytic pathway and the pentose phosphate pathway, whereas the bacteroids had only limited capacity for carbohydrate metabolism and appeared to be insufficient for the complete glycolytic pathway as well as starch synthesis and degradation. In a time-course study, using plants grown in a glasshouse, the acetylene-reducing activity (ARA) of the nodules increased in parallel with the total N content of plants and protein of the nodules until approximately 8 weeks after planting. Subsequently, the weight and size of the nodules and the weight of the plants continued to increase, but there was a sharp decrease in the ARA and the total N content of the plants.

Key words : *carbohydrate metabolism, glycolytic pathway, pentose phosphate pathway, acetylene-reducing activity, bacteroid.*

Cowpea (*V. unguiculata*) is a major legume crop which grows in many tropical regions of the world for human consumption and for feeding livestock. The plant is able to obtain a substantial proportion of its N symbiotically, but little is known of the physiology and biochemistry of its nodules.[1,2] Information on the morphology of cowpea nodules is also very limited, although it is known that they are determinate and have the presence of numerous significantly enlarged peroxisomes in the infected region of mature nodules.[3]

Symbiotic nitrogen fixation in leguminous root nodules is dependent on the supply of carbohydrates from the host plant. The main carbohydrate translocated into nodules is sucrose,[4] which is metabolized to provide nutrients for the bacteroids, energy and reductants for the nitrogenase reaction and carbon skeletons for the assimilation of fixed nitrogen. Recent studies have shown that all of the enzymes required to convert sucrose to pyruvate and to oxaloacetate via the glycolytic pathway are in the host cytosol of ureide-exporting nodules such as soybeans, and that the bacteroids in these nodules have only limited capacity for carbohydrate metabolism.[5,6] An alternative route for the breakdown of carbohydrates could be provided by the pentose phosphate pathway, which functioned mainly to generate NADPH and biosynthetic precursors such as ribose-5-phosphate and erythrose-4-phosphate.[7] Through a diversity of approaches, including ultrastructural, biochemical and immunocytochemical studies, the pathway of carbon metabolism has established extensively in soybean nodules.[8-11] Nevertheless, information on this aspect of cowpea nodule metabolism is very limited. In the present study, we have measured the activities of all the enzymes of glycolysis, as well as selected enzymes of the pentose phosphate pathway in the host cytosolic and bacteroid fractions of cowpea nodules. The activities were also determined in the roots of nodulated cowpeas for comparison.

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Abbreviations: ARA, acetylene-reducing activity; FW, fresh weight; Glc-6-P DH, glucose-6-phosphate dehydrogenase; 3-HB DH, 3-hydroxybutyrate dehydrogenase; PFK, phosphofructokinase; PFP, phosphofructophosphotransferase; 6-PG DH, 6-phosphogluconate dehydrogenase; PGM, phosphoglucomutase; PHI, phosphohexose isomerase; TPI, triose phosphate isomerase; UDPGP, UDP-glucose pyrophosphorylase

Materials and Methods

Materials. Cowpea (*Vigna unguiculata* L. Walp.) seeds were obtained from Hodder and Tolley Pty. Ltd. (Marayong, NSW) and perlite was from Australian Perlite Pty. Ltd.

(Sydney, NSW).

Growth of plants. Cowpea seeds were surface sterilized in 0.4% (w/v) sodium hypochlorite for 10 min, rinsed thoroughly with running tap water for 15 min, inoculated with *B. japonicum* I 16 and sown in moistened perlite in pots at a depth of approximately 2 cm and 3~4 cm apart. Plants were grown in glasshouse with average day and night temperatures of 25 and 19°C, respectively. N-free nutrient solution[12] was given to the plants every 3~4 days.

Preparation of extracts: Unless stated otherwise, all steps were carried out at 4°C. For the time-course studies, duplicate extracts were prepared from nodules that were harvested from one plant in each of 20 pots. The nodules were rinsed with distilled water and homogenized with a mortar and pestle in 3 volumes of 20 mM Hepes-KOH (pH 7.5), 1 mM EDTA, 5 mM $MgCl_2$, 20% (v/v) glycerol and 2 mM DTT. The homogenate was squeezed through a single layer of Miracloth (Calbiochem), centrifuged at 20,000 g for 20 min and protein content. Separated plants were harvested to determine total N content by the Kjeldahl method,[13] and ARA.

To study the distribution of enzymes between the host cytosolic and bacteroid fractions, nodules from 48- to 55-day-old plants were rinsed with distilled water and homogenized with a mortar and pestle in 10 ml of 50 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 1 mM EDTA, 5 mM 2-ME and 0.4 M mannitol (buffer A). The homogenate was centrifuged at 200 g for 5 min, and the supernatant was centrifuged at 4000 g for 10 min. The 4000 g pellet was washed twice in buffer A, resuspended in 5 ml of buffer A and a sample reserved for assays of non-sonicated bacteroid preparations. The remainder of the suspension was sonicated for four 15-s intervals (Will Scientific Biosonicator at 80% of maximum power), centrifuged at 20,000 g for 10 min and the supernatant was used for assays of sonicated bacteroid preparations. The 4000 g supernatant from the crude extract was centrifuged at 20,000 g for 10 min and the supernatant was used for assays of enzymes and protein content from the host cytosolic fraction of the nodules. Roots were rinsed with distilled water and homogenized with a mortar and pestile in 15 ml of buffer A. The homogenate was filtered through a single layer of miracloth, centrifuged at 200 g for 5 min and the supernatant was centrifuged at 20,000 g for 10 min. The supernatant was assayed for enzymes and protein content in cowpea roots.

Extracts were dialysed for 2 h against 1 L of the respective homogenizing buffer prior to the assay of all enzymes, except Fru-1,6-bisP aldolase, pyruvate kinase, PEP carboxylase and 3-hydrobutyrate dehydrogenase, which were assayed as soon as possible after extracts were prepared.

Assay of enzymes. Unless indicated otherwise, all enzyme assays were performed at 30°C in reaction mixtures which had a final volume of 1 ml. Assay mixtures were incubated for 5 min before intiating the reaction with one of the substrates. The amount of enzyme added to reaction mixtures was adjusted to give a linear reaction rate for 5~10 min, and a correction for non-specific reactions was made with blanks from which one of the substrates was ommitted from the respective reaction mixture. One unit of enzyme activity was defined as the amount of enzyme that catalysed the formation of 1 μmol product min^{-1}.

Conditions for the assay of the enzymes were optimized with respect to pH and the concentrations of all substrates, using the extract from the plant fraction of whole nodules. The composition of the optimized reaction mixtures were given. UDPGP (EC 2.7.7.9): 30 mM Tris-HCl, pH 8.5, 9 mM $MgCl_2$, 4 mM UDP-glucose, 5 mM PPi and 0.4 mg bovine serum albumin. Alkaline invertase, sucrose synthase and sucrose phosphorylase were assayed as described by Copeland *et al.* and Lee and Sturm.[14,15]

The remaining enzymes were assayed by monitoring the change in A at 340 nm in continuous assays in which activity was coupled to the reduction of $NAD(P)^+$ or oxidation of NADH. All reactions were initiated by the addition of substrate and were linear for at least 3 min. Hexokinase (EC 2.7.1.1): 50 mM Tris-HCl, pH 8.2, 50 mM KCl, 2 mM ATP, 3 mM MgCl2, 0.3 mM NAD^+, 2U Glc-6-P dehydrogenase (*Leuconostoc mesenteroides*) and 2 mM glucose. Fructokinase (EC 2.7.1.4): 50 mM Tris-HCl, pH 8.2, 50 mM KCl, 3 mM $MgCl_2$, 4U phosphoglucose isomerase (EC 5.3.1.9), 2U Glc-6-P dehydrogenase and 2 mM fructose. PGM (EC 2.7.5.1): 50 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, 0.5 mM $NADP^+$, 2.5 mM Glc-1-P, 75 μM Glc-1,6-bisP, 2U Glc-6-P DH and 2U 6-PG DH. PHI (EC 5.3.1.9): as for phosphoglucomutase, except that 2.5 mM Glc-1-P was replaced by 2.5 mM Fru-6-P and 75 μM Glc-1,6-bisP omitted. PFK (EC 2.7.1.11): 50 mM Tris-HCl, pH 8.5, 2 mM $MgCl_2$, 2 mM Fru-6-P, 1 mM ATP, 0.2 mM NADH, 0.4 BSA, 1U aldolase, 1U triose phosphate isomerase and 1U α-glycerol-P dehydrogenase.[16] PFP (EC 2.7.1.90): as for phosphofructokinase except that ATP was replaced by 2.5 mM PPi and 20 μM Fru-2,6-bisP was included. Fru-1,6-bisP aldolse (EC 4.1.2.13): 50 mM Hepes-KOH, pH 7, 1 mM Fru-1,6-bisP, 0.2 mM NADH and 2U triosephosphate isomerase and 2U α-glycerol-P dehydrogenase. TPI (EC 5.3.1.1): 50 mM Hepes-KOH, pH 7, 5 mM glyceraldehyde-3-phosphate, 0.2 mM NADH and 2U α-glycerol-3-P dehydrogenase. Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12): as described by Journet and Douce,[17] except that 50 mM Hepes-KOH, pH 7, was used. Phosphoglycerate kinase (EC 2.7.2.3): as described by Hong and Copeland,[18] except that 50 mM Hepes-KOH, pH 7, 1.5 mM $MgCl_2$ and 1 mM ATP were used. Phosphoglycerate mutase (EC 2.7.3.5), as in method I of Stitt and ap Rees,[19] except that 50 mM Tris-HCl, pH 8.5, 5 mM 3-phosphglycerate and 0.6

mM 2,3-diphosphoglycerate were used and AMP and adenylate kinase omitted. Enolase (EC 4.2.1.11): as described by ap Rees *et al.*,[20] except that 50 mM Tris-HCl, pH 8, 8 mM $MgCl_2$ and 5 mM 2-phosphoglycerate were used. Pyruvate kinase (EC 2.7.1.40): as described by Journet and Douce,[17] except that 50 mM Hepes-KOH, pH 7, and 2 mM PEP were used; the root extracts activity was also measured in reaction mixtures which contained 5 mM $NADP^+$, 5 mM glucose, 3U hexokinase, 4U Glc-6-P dehydrogenase and 0.3U 6-phosphogluconate dehydrogenase instead of NADH and lactate dehydrogenase. PEP carboxylase (EC 4.1.1.31): as described by Wong and Davies,[21] except that 50 mM Hepes-KOH, pH 7.5, was used. Glc-6-P DH (EC 1.1.1.49): 50 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, 0.5 mM $NADP^+$ or 1 mM NAD^+, 2.5 mM Glc-6-P and 0.3U 6-PG DH. 6-PG DH (EC 1.1.1.44) was assayed described for Glc-6-P DH except that Glc-6-P was replaced by 2.5 mM 6-PG. Transketolase (EC 2.2.1.1) and transaldolase (EC 2.2.1.2) were assayed as described by Simcox *et al.*,[22] 3-HB DH (EC 1.1.1.30) was assayed as described by Wong and Evans.[23] Protein content was determined by Coomassie Blue reagent (Bio-Rad) according to the manufacturer's instructions, using bovine serum albumin as a standard.

The ARA was measured by placing trimmed roots with nodules attached (approximately 3 g) into vials sealed with gas-tight stoppers. Acetylene was injected to a concentration of 10% (v/v), and the ethylene produced was measured gas chromatographically using a Porapak T column at 130°C, with N_2 as the carrier gas and a flame-ionization detector.

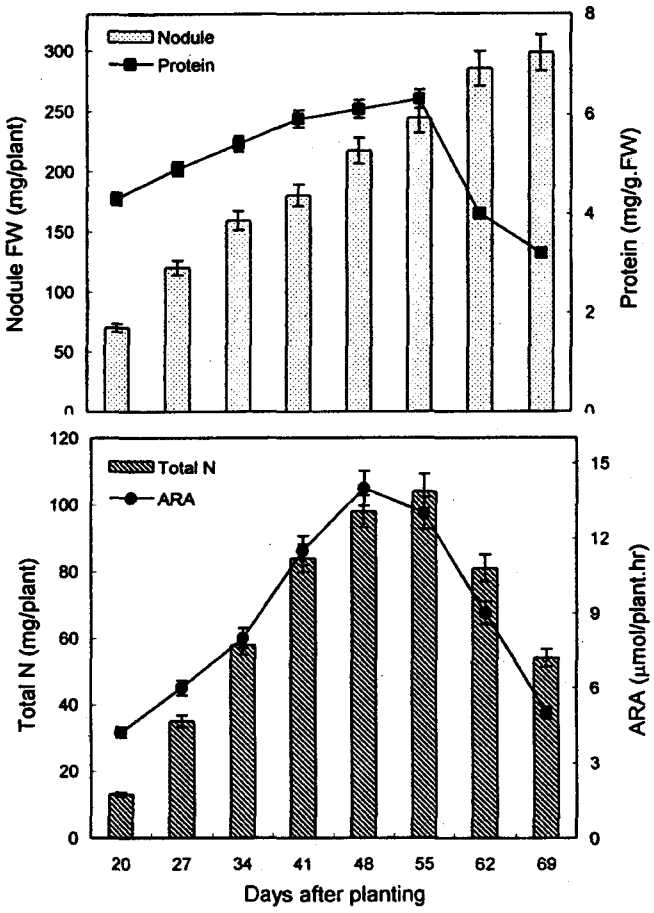

Fig. 1. Growth and N_2 fixation by cowpea nodules. ARA, total N content of the plants and protein content of the nodules were determined as described and are the means of at least 3 replicate measurements.

RESULTS

Cowpea nodules were first visible to the eye on the upper portion of the primary root of plants after approximately 2 weeks, and after 3 weeks additional nodules were observed on the lower parts of the primary root and on the upper portions of secondary roots. The young nodules were roughly spherical in shape with a central zone of pigmented tissue. Nitrogenase activity, as measured by the C_2H_2 reduction assay, was readily detectable by the 8th to 10th d. Accordingly, we sampled plants between 20 and 70 d after planting in order to encompass all phases of development and senescence. The ARA of the nodules increased to a maximum at approximately 8 weeks (Fig. 1). Subsequently, the fresh weight of the plants and nodules continued to increase, whereas the ARA [expressed per plant or FW of nodule], total N content of the plants determined after Kjeldahl digestion and protein content of the nodules decreased after 8 weeks (Fig. 1).

The cytosolic extracts from the host fraction of cowpea nodules contained all of the enzymes required to convert sucrose to pyruvate and to oxaloacetate via the glycolytic pathway. The specific activities, determined under the conditions that were optimized with respect to pH and substrate concentration, are shown in Table 1. The pentose phosphate pathway enzymes, Glc-6-P DH, 6-PG DH, transketolase and transaldolase, were also present in the cytosolic fraction of the nodules (Table 1). The absence of the bacteriod marker 3-HB DH from the host cytosolic extract indicated that it was not contaminated with bacteriod enzymes (Table 1). With the exception of hexokinase, all of the enzymes had higher specific activity in the host cytosolic extract of cowpea nodules than in the cowpea roots (Table 1).

To determine which enzymes were present inside the bacteroids, their activities were measured in bacteroid preparations before and after sonication. An increase in activity after sonication indicated that an enzyme was inside the bacteroids and not a contaminant from the cytosolic fraction. Thus, 3-HB DH was detected only in sonicated bacteroid preparations (Table 2). There was no increase following sonication in the activities of alkaline invertase, sucrose synthase, sucrose phosphorylase, fruc-

Table 1. Specific activities of enzymes of carbohydrate metabolism in the host cytosolic extract of cowpea nodules and cowpea roots.

Enzyme	nmol product formed/min·mg protein	
	Nodule cytosol	Roots
3-hydroxybutyrate DH	40±6	ND[a]
Alkaline invertase	15±1	21±1
Sucrose synthase	32±2	26±5
Sucrose phosphorylase	ND	ND
Hexokinase	7±1	15±2
Fructokinase	50±4	17±2
UDP-glucose-pyrophosphorylase	823±21	208±17
Phosphoglucomutase	1117±107	534±19
Phosphohexoisomerase	1864±61	1228±67
Phosphofructokinase	117±3	42±2
Phosphofructophosphotransferase	79±6	83±9
Fru-1,6-bisP aldolase	43±5	41±6
Triose phosphate isomerase	720±36	404±24
Glyceraldehyde 3-phosphate DH	252±12	122±18
Phosphoglycerate kinase	542±64	314±58
Phosphoglycerate mutase	362±24	175±23
Enolase	307±12	98±14
Pyruvate kinase	57±15	11±4
PEP carboxylase	114±13	27±9
Glucose-6-phosphate DH	125±21	47±7
6-phosphogluconate DH	143±19	38±5
Transketolase	131±35	72±9
Transaldolase	124±13	43±11

Nodules (5 g) and roots (5 g) of 48 to 55 days plants were extracted as described. The host cytosolic extract of the nodules contained 6.1±0.1 mg protein g^{-1} FW of nodules and the protein content of extracts activities from cowpea roots was 1.3±0.06 mg protein g^{-1} FW. Specific activities are in nmol product mg^{-1} protein min^{-1} and are the means±SE of at least 5 replicate extractions.

[a]ND--Not detected

Table 2. Activities of enzymes of carbohydrate metabolism in bacteriod fractions of cowpea nodules.

Enzyme	nmol product formed/min.g FW*	
	Before sonication	After sonication
3-hydroxybutyrate DH	ND	1579±56
Alkaline invertase	ND	ND
Sucrose synthase	ND	ND
Sucrose phosphorylase	ND	ND
Hexokinase	33±3	128±15
Fructokinase	ND	ND
UDP-glucose-pyrophosphorylase	85±7	175±10
Phosphoglucomutase	52±4	182±12
Phosphohexoisomerase	286±15	649±24
Phosphofructokinase	ND	ND
Phosphofructophosphotransferase	ND	ND
Fru-1,6-bisP aldolase	ND	ND
Triose phosphate isomerase	663±35	2459±129
Glyceraldehyde 3-phosphate DH	47±13	445±58
Phosphoglycerate kinase	201±28	1148±187
Phosphoglycerate mutase	157±30	620±21
Enolase	ND	684±49
Pyruvate kinase	ND	213±28
PEP carboxylase	ND	ND
Glucose-6-phosphate DH (NAD)	ND	94±23
Glucose-6-phosphate DH (NADP)	ND	43±15
6-phosphogluconate (NAD)	ND	104±31
6-phosphogluconate (NADP)	ND	35±13
Transketolase	ND	87±6
Transaldolase	ND	92±10

*Nodules (5 g) of 48 to 55 days plants were extracted as described. The values are the means±SE of at least 5 replicate extractions.

tokinase, PFK, PFP, Fru-1,6-bisP aldolase and PEP carboxylase, which indicates that these enzymes were absent from the bacteroids (Table 1). ADP-glucose pyrophosphorylase, phosphorylase and amylase activities were not detected in the bacteroids before or after sonication (not shown). On the other hand, substantial increases in the activities of hexokinase, UDPGP, PGM, PHI, enzymes involved in the conversion of triose phosphates to pyruvate and the pentose phosphate pathway enzymes occurred following sonication, indicating that these enzymes were present inside the bacteroids and not plant-derived enzymes that had become associated with the bacteroids during the isolation procedure. The specific activities of the enzymes in the bacteroids were considerably lower than those in the host cytosolic fraction of nodules.

DISCUSSION

Our study has shown that bacteroids isolated from cowpea nodules formed with *Bradyrhizobium japonicum* I 16 had limited capacity to utilize carbohydrates. No evidence was found in the bacteroids for the presence of the the enzymes of sucrose cleavage, invertase, sucrose synthase or sucrose phosphorylase. Sucrose synthase, which is one of the major soluble proteins of soybean nodules, was present to a small extent in an unwashed bacteroid preparation in the previous study.[11,24] It now appears that the activity was a result of comtamination from the plant enzyme. Traces of sucrose synthase activity have been reported in association with *B. japonicum* 61A76 bacteroids.[25]

The absence of PFK, PFP and Fru-1,6-bisP aldose indicated that *B. japonicum* I 16 bacteroids lacked a complete glycolytic pathway. A form of the pentose phosphate pathway, involving an NADP-dependent Glc-6-P DH, an NAD-dependent 6-PG DH, transaldolase and transketolase, may operate in the bacteroids, but in view of the low activities of these enzymes such a pathway is unlikely to play a quantitatively improtant role in the production of energy. Bacteroids isolated from soybean nodules formed with several strains of *B. japonicum* have been reported to contain small amounts of PFK, Fru-1,6-bisP aldolase, NADP-dependent Glc-6-P DH and NAD-dependent 6-PG DH, but the activities were considered too low to support nitrogen fixation.[25,26]

Most of the capacity for carbohydrate utilization was

in the cytosolic extract of cowpea nodules. This finding is consistent with the generally held view that organic acids, rather than sugars, are the main substrates taken up by the bacteroids from the cytosolic extract of the nodules.[27,28] It is interesting to note that the specific activities of many of the enzymes involved in the conversion of sucrose to organic acids via the glycolytic pathway were increased by a factor of two or more in the cytosolic extract of the nodules compared to the roots. This suggests that synthesis of enzymes in the pathway of sucrose breakdown may be enhanced in the nodules, either through the expression of enzyme forms unique to nodules (*i.e.* nodulins) or increased synthesis of enzyme forms normally present in roots or other parts of the plant. A nodule specific form of sucrose synthase has been shown to occur in soybeans.[29]

The catalytic potential of sucrose synthase in the cytosolic extract was approximately double that of alkaline invertase throughout the development of cowpea nodules suggesting that sucrose synthase plays a quantitatively important role in the breakdown of sucrose. In contrast, the specific activity of alkaline invertase was double that of sucrose synthase in chickpea and soybean nodules.[6,14] High level to a value comparable to that of alkaline invertase in the specific activity of sucrose synthase was noted during development of lupin nodules.[30] The cytosol of cowpea nodules as in chickpea, lupin and soybean nodules.[6,14,30] contained only a small amount of acid invertase activity, therefore, this enzyme is unlikely to have a significant role in the cleavage of sucrose.

Both PFK and PFP were in the cytosolic extract of nodules and in roots. The specific activity of PFP in extracts of roots was 2 times greater than that of PFK, whereas in the cytosolic extract of the nodules the catalytic potential of PFK was 1.5 times greater than that of PFP. This suggests that PFK plays a more important role than PFP in the pathway of sucrose utilization in cowpea nodules, but in roots PFP plays a more important role than PFK. It has been reported much higher activity of PFK activity than PFP in the cytosol of chickpea and soybean nodules.[14,25]

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