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# CARBOHYDRATE STATUS AND SUCROSE METABOLISM IN MUNGBEAN ROOTS AND NODULES

JYOTI CHOPRA, NARINDER KAUR and ANIL K. GUPTA*

Department of Biochemistry, Punjab Agricultural University, Ludhiana 141004, India

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**Key Word Index**—*Vigna radiata*; Leguminosae; mungbean; nodule; root; sucrose metabolism; sucrose synthase; invertase.

**Abstract**—The activities of acid invertase, alkaline invertase and sucrose synthase (cleavage) were measured in roots and nodules of mungbean at different stages of development. High activities of these enzymes were observed at 30 and 50 days after sowing in nodules. Compared with other sucrose metabolising enzymes, alkaline invertase activity was highest in nodules at all stages of development. Activity of sucrose metabolizing enzymes in roots was significantly less than that observed in the nodules. Most of the sucrose appears to be transported as such through the roots to the nodules. Sucrose was the dominant free sugar and the concentration of glucose was higher than that of fructose at all stages of nodule development. Alkaline invertase from nodules was purified to electrophoretic homogeneity by ammonium sulphate fractionation, DEAE cellulose chromatography and Sephadex G-150 chromatography. The enzyme showed a sharp pH optimum at pH 7.0–7.5, optimum temperature of 40°, $K_m$ of 3–4 mM for sucrose, non-competitive inhibition by $HgCl_2$ ($K_i$, 40 $\mu$M) and had an $M_r$ of approximately 355 000. Compared with raffinose and stachyose, sucrose was the better substrate for alkaline invertase. Energy of activation of alkaline invertase with different substrates was in the order, stachyose > raffinose > sucrose. The enzyme was unable to hydrolyse maltose and *p*-nitrophenyl-α-D-glucopyranoside, showing its true β-fructosidase nature. Because of its low $K_m$, low energy of activation with near saturated substrate environment in nodules, alkaline invertase appeared better placed physiologically for sucrose cleavage. 

## INTRODUCTION

Symbiotic nitrogen fixation in leguminous root nodules is dependent on the supply of carbohydrates by the host plant to the rhizobium bacteria in the nodules. Photosynthate, predominantly in the form of sucrose, is translocated from the shoots to the root nodules [1, 2] providing nutrients for the bacteroids, energy and reductants for nitrogenase and carbon skeletons for the assimilation of fixed ammonia [3]. Available evidence suggests that sucrose after entering the legume nodule is cleaved by cytosolic alkaline invertase and/or sucrose synthase [4–6] and bacteroids had only limited capacity for carbohydrate metabolism [7, 8]. However, the information about the major enzymes involved in sucrose cleavage in the cytosol of nodules in legume crops is controversial and probably dependent upon the nature of the crop. In nodules of soybean, a group of workers suggested alkaline invertase to be the main enzyme of sucrose breakdown, whereas both alkaline invertase and sucrose synthase might be involved in the cleavage of sucrose in mature nodules [4, 8]. Kouchi *et al.* and Anthon and Emerich, on the basis of estimated higher activities of sucrose synthase as compared with alkaline invertase, suggested that this enzyme might be mainly responsible for sucrose breakdown in soybean nodules [7, 9]. However, in chickpea nodules, during active growth (until flowering), activity of alkaline invertase was 4–5-fold higher than that of sucrose synthase [10]. High activities of sucrose synthase and alkaline invertase in the period of highest nitrogen fixation in pea nodules testified to their direct participation in the process of molecular nitrogen fixation [11].

Information in the literature on carbohydrate metabolism comes mainly from soybean and chickpea and there is little information on mungbean, which is an important economic pulse crop. The present study was therefore aimed at differentiating the activity pattern of sucrose synthase and alkaline

*Author to whom correspondence should be addressed.

Table 1. Changes in the carbohydrate contents (mg $g^{-1}$ fr. wt) of the nodules and roots

| Days after sowing | Nodules | | | Roots | | |
|---|---|---|---|---|---|---|
| | sucrose | glucose | fructose | sucrose | glucose | fructose |
| 20 | 21.3 ± 2.6 (178.2) | 2.6 ± 0.30 (22.2) | 0.03 ± 0.00 (0.3) | 5.5 ± 0.5 (28.0) | 2.3 ± 0.02 (11.7) | 2.8 ± 0.20 (13.9) |
| 30 | 11.4 ± 1.9 (83.8) | 1.34 ± 0.02 (9.9) | 0.10 ± 0.00 (0.7) | 7.7 ± 0.1 (37.2) | 2.3 ± 0.04 (10.8) | 1.7 ± 0.03 (8.4) |
| 40 | 13.3 ± 0.8 (113.4) | 1.31 ± 0.40 (11.3) | 0.41 ± 0.01 (3.5) | 15.8 ± 1.5 (63.8) | 3.2 ± 0.07 (12.9) | 2.3 ± 0.04 (9.2) |
| 50 | 10.4 ± 0.5 (92.8) | 1.21 ± 0.02 (10.8) | 0.97 ± 0.10 (8.6) | 12.5 ± 0.8 (42.9) | 1.5 ± 0.01 (5.1) | 3.8 ± 0.20 (12.9) |

invertase in nodules and roots, along with carbohydrate status of these organs and to purify and characterize the major sucrose catabolizing enzyme from the nodules of mungbean.

## RESULTS

### *Carbohydrate composition of nodules*

Sucrose was the dominant sugar out of glucose, fructose and sucrose at all stages of nodule development. Though the fructose content in nodules increased with development, it remained lower than that of glucose (Table 1). Sucrose was also the dominant sugar in roots. The content of sucrose in roots increased from 5.5 mg $g^{-1}$ fr. wt at 20 days after sowing (DAS) to 15.8 mg $g^{-1}$ fr. wt at 40 DAS and then declined (Table 1).

### *Changes in activity of sucrose metabolising enzymes*

In the soluble fraction of nodules, the activity of alkaline invertase was significantly higher than that of other sucrose cleavage enzymes (Table 2). The specific activity of acid and alkaline invertase declined in mature nodules (50 DAS), whereas activity of sucrose synthase was high in nodules of this stage (Table 2). Activities of alkaline invertase and sucrose synthase were very low (less than 15 pkat $g^{-1}$ fr. wt) at all stages of root development. The specific activities of acid invertase increased from 20 to 30 DAS and thereafter declined sharply.

### *Purification of alkaline invertase*

Alkaline invertase from nodules at 30 DAS was purified using differential ammonium sulphate fractionation, DEAE cellulose and Sephadex G-150 column chromatography (Table 3). During anion-exchange chromatography, alkaline invertase eluted at approximately 0.28 M NaCl. After Sephadex G-150 chromatography, the enzyme could be purified by 59 fold over the starting crude enzyme (Table 3). The purified preparation was electrophoretically homogenous on a 7.7% polyacrylamide gel.

### *Properties of alkaline invertase*

The invertase showed sharp pH optima in the region 7.0 to 7.5. The maximum activity of mungbean invertase was observed at 40°C but was not very stable to heat. Although heating the purified preparation at 40°C for 1 h did not cause much loss of enzyme activity, when the enzyme was kept at 45° for 1 h, it lost 66% of its original activity. 1 h heating at 50°C caused about 80% loss of activity, whereas at 55°C it lost its complete activity. Thermal denaturation of invertase was biphasic. Pre-incubation of the enzyme at 50°C for 2 min caused approximately 50% loss of activity. Thereafter, heating at this temperature caused a gradual loss of activity from approximately 50% after 2 min heating to 80% after 1 h heating.

The data on the effect of increasing sucrose concentrations at different temperatures, i.e. 25, 30, 35 and 40°C, on invertase activity showed typical Michaelis–Menten kinetics. The effect of increasing the temperature of the assay system on $K_m$ and $V_{max}$ of alkaline invertase was determined from hyperbolic enzyme activity vs substrate concentration graphs and Lineweaver–Burk plots at different temperatures. The $K_m$ of invertase varied from 3.0 to 4.3 mM depending upon the temperature of the assay system. The energy of activation for sucrose was approximately 3.9 kcal $mol^{-1}$ and for raffinose and stachyose was calculated to be approximately 6.4 and 7.1 kcal $mol^{-1}$ of substrate, respectively. Raffinose and stachyose at 20 mM

Table 2. Activities of enzymes involved in sucrose cleavage in roots and nodules of mungbean

| Days after sowing | Nodules (pkat $g^{-1}$ fr. wt) | | | Roots (pkat $g^{-1}$ fr. wt) |
|---|---|---|---|---|
| | sucrose synthase | acid invertase | alkaline invertase | acid invertase |
| 20 | 1500 ± 220 (60 ± 5) | 820 ± 130 (107 ± 5) | 14600 ± 145 (2020 ± 25) | 280 ± 15 (187 ± 10) |
| 30 | 12800 ± 2200 (550 ± 30) | 13800 ± 250 (1280 ± 235) | 25400 ± 200 (2210 ± 290) | 446 ± 20 (230 ± 10) |
| 40 | 3500 ± 135 (270 ± 20) | 410 ± 127 (60 ± 2) | 12990 ± 1580 (1910 ± 160) | 60 ± 6 (30 ± 3) |
| 50 | 11280 ± 450 (990 ± 50) | 1520 ± 120 (108 ± 10) | 16490 ± 1895 (1240 ± 60) | 25 ± 2 (23 ± 2) |

Values in parentheses represent pkat $mg^{-1}$ protein. Values are mean ± S.D. of data obtained from triplicate extracts.

Table 3. Partial purification of alkaline invertase from mungbean nodules

| Steps of purification | Volume (ml) | Enzyme activity (nkat) | Protein (mg) | Specific activity (nkat $mg^{-1}$ protein) | Fold purification |
|---|---|---|---|---|---|
| Crude | 35 | 296 | 900 | 0.33 | 1.0 |
| 30–60% $(NH_4)_2SO_4$ saturation | 9.0 | 209 | 100 | 2.09 | 6.3 |
| DEAE cellulose | 10 | 89 | 10 | 8.90 | 26.9 |
| Sephadex G-150 | 10 | 39 | 2 | 19.5 | 59.1 |

8 ml of the enzyme obtained from a DEAE cellulose column was loaded on to a Sephadex G-150 column having a void volume of 55 ml.

concentration were hydrolyzed at 10.7 and 8.9%, respectively, of the rate for sucrose hydrolysis.

In a preliminary experiment, it was observed that different salts at 5 mM concentration had no effect on the photometric determination of hexoses. $Na^+$, $Ba^{2+}$, $NH_4^+$, $Ca^{2+}$ and $SO_4^{2-}$ ions had almost no effect on invertase activity (Table 4). Alkaline invertase was inhibited non-competitively by $HgCl_2$ with a $K_i$ of approximately 40 $\mu$M. Pyridoxine–HCl showed activation (Table 4). The $M_r$ of alkaline invertase determined by Sephadex G-150 chromatography was approximately 355 000.

## DISCUSSION

The relatively lower concentrations of fructose as compared with glucose in mungbean nodules seem to be a reflection of either the faster utilization of fructose over glucose or conversion of some fructose into glucose. The former appears possible because of the reported high fructokinase activity in the host cytoplasm [12]. Fructose-6-phosphate is then utilized through the glycolytic pathway and the tricarboxylic acid cycle for producing organic acids which serve as a carbon source for the bacteroids [13]. In nodules of *Sesbania grandiflora* and *Cicer arietinum*, sucrose is the principal sugar and the fructose content was lower than that of glucose at all stages of nodule development [14, 15].

Table 4. Effect of various exogenous compounds on the activity of alkaline invertase

| Compound | Activity (%) |
|---|---|
| Sodium chloride | 110 |
| Barium chloride | 106 |
| Ammonium chloride | 114 |
| Manganese chloride | 124 |
| Sodium nitrate | 148 |
| Sodium sulphate | 118 |
| Calcium chloride | 128 |
| Pyruvate | 96 |
| Pyridoxine HCl | 154 |
| Iodoacetamide | 114 |
| Mercuric chloride | 0 |
| Control | 100 |

Enzyme was incubated with 5 mM of each compound for 15 min before adding the substrate. Values are mean of three independent assays.

Two peak activities of nitrogenase, one at 25 DAS and the other at 47 DAS, in developing mungbean nodules have been reported [16]. The main sucrose cleaving enzyme in the cytosol appears to be an alkaline invertase, whereas sucrose synthase becomes active during the phase of active nitrogen fixation. High activity of all the sucrose cleaving enzymes at 30 DAS could be responsible for the sudden fall in nodular sucrose content at this stage of development (Table 1). The simultaneous action of these enzymes in the nodules ensures a hexose supply for meeting the energy and reducing power demands of nodules during the stage of active nitrogen fixation. The low level of sucrose-cleaving enzymes in the roots (Table 1) indicated almost intact transport of sucrose from leaves to the nodules. However, the activity of sucrose synthase has been indicated in the phloem tissue [17], the acid invertase, because of its apoplastic and vacuolar localization, in cells outside the phloem, probably does not coming into direct conflict with sucrose transport [18]. Our results indicate that most of the sucrose is transported as such through the roots to nodules. The observed decline in acid invertase activity in mungbean roots with development (Table 2) has also been reported in soybean roots [4]. The decline in sucrose content between 40–50 DAS of root development could be due to onset of flowering in mungbean, which will divert the photosynthate to the developing reproductive organs.

The 59-fold purification of alkaline invertase obtained in the present study compares favourably with the 39-fold purification of this enzyme achieved in soybean nodules [4]. Compared with other $\beta$-linked fructose oligosaccharides, the invertase showed maximum activity with sucrose. The low energy of activation of alkaline invertase with sucrose as a substrate, compared with raffinose and stachyose, might be a factor for more specificity of this enzyme with sucrose than raffinose and stachyose. The enzyme was a true $\beta$-fructosidase, because it could not hydrolyse maltose and *p*-nitrophenyl-$\alpha$-D-glucopyranoside. The pH optimum of 7.0–7.5 of this enzyme is well in the range of 6.8 to 8.0 reported from various sources [4, 19–21].

The $K_m$ of alkaline invertase varied from 3 to 4 mM depending upon the temperature. From other plants, $K_m$ values for this enzyme in the range of 9

to 65 mM have been reported [4, 20–26]. Alkaline invertase of mungbean nodules had a lower $K_m$ than that of 10 mM of alkaline invertase from soybean nodules [4]. Although the content of sucrose in different compartments of the nodular cell is not known, from the data presented in Table 1, the sucrose content in nodules varied from 30 to 60 mM depending upon its stage of development. Because of active metabolism of sucrose in the nodules, it is expected that actively metabolising sucrose will be in the cytoplasm and not in the vacuoles. In fact, sucrose synthase from different plant tissues has been reported to have a $K_m$ varying from 30 to 130 mM [5, 27, 28]. Therefore, in mungbean, an alkaline invertase with low $K_m$ and low energy of activation with a near saturated substrate environment appears to be better placed physiologically for sucrose cleavage.

Since NaCl did not show any effect on invertase activity, the activating effect of $NaNO_3$ could be due to $NO_3^-$ ions (Table 4); $NO_3^-$ ions have been shown to be one of the physiological activators of invertase from *Hevea brasiliensis* [29] and sweet potato [23]. Alkaline invertase from leaves of *Citrus sinensis* was inhibited completely with 10 mM Tris, whereas it had hardly any effect on acid invertase [25]. However, mungbean alkaline invertase was only partly inhibited at high concentrations (50–70 mM) of Tris. Complete inhibition of alkaline invertase by $HgCl_2$ (Table 4) with a $K_i$ of 40 $\mu$M suggests that the sulphydryl group might be essential for its activity. Iodoacetamide could alkylate both imidazole and sulphydryl groups of side-chain amino acids of proteins. Since iodoacetamide did not affect alkaline invertase activity, it appears that iodoacetamide alkylates preferably histidine which did not seem to affect the alkaline invertase activity.

The $M_r$ of the purified alkaline invertase was *ca.* 355 000. Alkaline invertase present in *C. sinensis* [25], sugar beet [20], soybean hypocotyl [26], chicory root [21] and mungbean hypocotyl [30] showed native masses of 200 000, 280 000, 240 000, 260 000 and 450 000, respectively.

## EXPERIMENTAL

### *Materials*

Mungbeans (*Vigna radiata* L.) (cv. ML267) were raised in the field following recommended agronomic practices. Uniformly growing plants were uprooted from the wet field at 10 days interval of growth from 20 to 50 DAS. Roots with intact nodules were thoroughly washed first with running tap water and then with deionized $H_2O$ and uniformly developing nodules were collected. Roots were also taken for preservation and enzyme assay purposes.

### *Extraction and estimation of free sugars*

Free sugars were quantitatively extracted twice with hot 80% EtOH and then twice with 70% EtOH. EtOH extracts of each sample were combined and concentrated by evaporation at 50°C under vacuum. Reducing sugars were determined colorimeterically using reaction with arsenomolybdate [31]. Sucrose content was determined after hydrolysing the sucrose present in the extract with acid invertase (Sigma I 4504) and then estimating the glucose using the glucose oxidase and peroxidase reaction [32]. From glucose obtained after invertase hydrolysis, the quantity of free glucose present in the sugar extract was subtracted. Free fructose was determined by subtracting bound fructose estimated after destroying free fructose with 30% NaOH from the total fructose determined by the resorcinol–HCl procedure [33].

### *Extraction of enzymes*

For extracting sucrose synthase (EC 2.4.1.13), fr. nodules were homogenized in cold (3–4°C) 100 mM HEPES buffer (pH 8.2) containing 10 mM EDTA, 15 mM KCl, 5 mM $MgCl_2$, 2 mM sodium diethyl dithiocarbamate and 5 mM $\beta$-mercaptoethanol. PVP (100 mg $g^{-1}$ tissue) was also added while extracting the enzyme. The homogenate was filtered through double layered cheese-cloth and centrifuged at 10 000*g* for 15 min. The supernatant was freed from soluble sugars by chromatographing it on a Sephadex G-25 column using 10 mM HEPES buffer (pH 7) containing 5 mM $MgCl_2$. For extraction of acid invertase (EC 3.2.1.26) and alkaline invertase (EC 3.2.1.27), 0.02 M $NaP_i$ buffer (pH 7) was used and the supernatant freed from the soluble sugars by passing it through a Sephadex G-25 column.

### *Enzyme assays*

The assay system for sucrose synthase in the direction of sucrose breakdown consisted of 0.2 M HEPES buffer (pH 6.5), 4 mM UDP and 0.1 M sucrose; the reaction was initiated by adding enzyme. The total volume of the assay system was 0.5 ml. In control assays, UDP was absent. The assay mixture was incubated for 30 min at 37°C the reaction stopped by adding 1 ml of alkaline copper tartrate reagent and ther fructose released estimated [31]. Acid invertase activity was measured by incubating 0.1 M NaOAc buffer (pH 5.0), 0.1 M sucrose and enzyme extract in a total volume of 1 ml at 37°C for 30 min. Reducing sugars formed after sucrose hydrolysis were estimated [31]. For estimation of alkaline invertase activity, the assay system was the same as that described above, except that the NaOAc buffer was replaced by $NaP_i$ buffer (pH 8). The rate of product formation in sucrose synthase and invertases was linear for at least 40 min.

*Purification of alkaline invertase*

Nodules (12.5 g) were harvested from mungbean roots 30 DAS and crushed with a pestle and mortar using chilled extraction buffer consisting of 25 ml of 50 mM $NaP_i$ buffer (pH 8) containing 1 mM EDTA and 5 mM $\beta$-mercaptoethanol. PVP (2 g) was also added during extraction of the enzyme. The extract was filtered through double layered cheese cloth and centrifuged at 10 000*g* for 15 min. Proteins were differentially precipitated at 0–30, 30–60 and 60–100% saturation of $(NH_4)_2SO_4$. The fr. obtained with 30–60% saturation contained maximum invertase activity and was freed from sulphate ions by passing through a Sephadex G-25 column. Thereafter, it was loaded onto a DEAE cellulose column. The enzyme was eluted using a linear gradient of NaCl (0–1 M) in eluting buffer (0.02 M $NaP_i$, pH 7.2). Each fr. (5 ml) obtained from DEAE-cellulose column was assayed for alkaline invertase activity. The fr. with the highest specific activity of invertase was further purified by Sephadex G-150 CC. Purity of the enzyme was checked by electrophoresising it on 7.7% polyacrylamide gel [34]. For $M_r$ determination, the elution volumes ($V_e$) of the purified enzyme and standard proteins were determined on a Sephadex G-150 column. Carbonic anhydrase (29 000), BSA (66 000), alcohol dehydrogenase (150 000), $\beta$-amylase (200 000) and apoferritin (440 000) were used as markers.

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