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Changes in sugar content and activities of sucrose metabolizing enzymes in roots and nodules of lentil

J. CHOPRA, N. KAUR and A.K. GUPTA*

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

Abstract

Activities of acid and alkaline invertases and sucrose synthase were determined in roots and nodules of lentil at various stages of development. Alkaline invertase and sucrose synthase were both involved in sucrose metabolism in the nodule cytosol, but there was only a small amount of acid invertase present. Activity of sucrose metabolizing enzymes in roots was significantly less than that observed in the nodules. Amongst sugars, sucrose was found to be the main component in the host cytosol. Lentil neutral invertase (LNI) was partially purified from nodules at 50 days after sowing (DAS). Two forms of invertase were identified, *i.e.*, a major form of 71 kDa which was taken for enzyme characterization and a minor form of 270 kDa which was not used for further studies. The purified enzyme exhibited typical hyperbolic saturation kinetics for sucrose hydrolysis. It had a Km of 11.0 to 14.0 mM for sucrose depending upon the temperature, a pH optimum of 6.8 and an optimum temperature of 40 °C. Compared with raffinose and stachyose, sucrose was better substrate for LNI. The enzyme showed no significant hydrolysis of maltose and *p*-nitrophenyl-α-D-glucopyranoside, showing its true β-fructosidase nature. LNI is completely inhibited by $HgCl_2$, $MnCl_2$ and iodoacetamide but not by $CaCl_2$, $MgCl_2$ or $BaCl_2$.

Additional key words: invertase, *Lens culinaris*, sucrose metabolism, sucrose synthase.

Introduction

Legume nodules are primarily dependent on the import and metabolism of sucrose to provide the energy and carbon skeletons for biological nitrogen fixation, the assimilation of ammonia, and the export of nitrogenous products. Sucrose entering the legume nodule is metabolized by cytosolic alkaline invertase and/or sucrose synthase (Morell and Copeland 1984, 1985, Copeland *et al.* 1995). In contrast, the bacteroids had only limited capacity for sugar metabolism (Kouchi *et al.* 1988, Copeland *et al.* 1989). The initial hydrolysis of sucrose is a key step in nitrogen fixation. Sucrose synthase in legume nodules is reported to be essential for nitrogen fixation (Gordon *et al.* 1999). Less is known about alkaline invertase, the other nodule enzyme capable of sucrose hydrolysis. Most of the information about the major enzymes involved in sucrose cleavage comes from work on soybean (Kouchi *et al.* 1988, Copeland *et al.* 1989, Anthon and Emerich 1990), chickpea (Sawhney *et al.* 1988, Singh *et al.* 1994) and pea (Sukalo and Krugova 1993). On the basis of our work on mungbean nodules, we suggested alkaline invertase to be the major enzyme of sucrose metabolism (Chopra *et al.* 1998). To our knowledge, no information is available about sugar metabolism in lentil nodules. The present study was, therefore, initiated to identify the potential contribution of these sucrolytic enzymes during development of nodules and, for comparison, in roots of lentil. This study also reports the purification and characterization of invertase from lentil nodules, which has earlier been purified from three legumes, *i.e.*, soybean (Morell and Copeland 1984), chickpea (Asthir and Singh 1997) and mungbean (Chopra *et al.* 1998).

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Abbreviations: DAS - days after sowing; LNI - lentil neutral invertase; SS - sucrose synthase.
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*Corresponding author; fax: (+91) 161 400945, e-mail: akgupta99@rediffmail.com

Materials and methods

The lentil (*Lens culinaris* L. cv. L4076) was raised in the field. Uniformly growing plants in the wet field were uprooted at random at 10 d interval from 50 to 90 DAS. The roots with intact nodules were thoroughly washed first with running tap water and then with deionized water and the uniformly developing nodules were collected. Roots were also taken for sugar content determination and enzyme assay purposes.

Free sugars were extracted twice with 80 % ethanol and then twice with 70 % ethanol at 80 °C for 30 min. Ethanol extracts of each sample were combined and concentrated by evaporation at 50 °C under vacuum. Sugars were identified by descending paper partition chromatography using authentic standard sugars in *n*-butanol:acetic acid:water solvent system (4:1:5 v/v/v) as described previously (Singh *et al.* 1994). Reducing sugars were determined colorimetrically using reaction with arsenomolybdate (Nelson 1944). Sucrose content was determined after hydrolysing the sucrose with acid invertase (*Sigma*, St. Louis, USA) and then determining the glucose using the glucose oxidase and peroxidase reaction (Gascon and Lampen 1968). Fructose was determined by using the resorcinol-HCl procedure (Williard and Slattery 1945).

For the extraction of sucrose synthase (EC 2.4.1.13), fresh nodules (0.5 - 1 g) were homogenized in 3 - 4 cm^3 of 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 β-mercaptoethanol. Polyvinylpyrrolidone [100 mg g^{-1} (tissue)] was added. 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 passing it through a *Sephadex G-25* column using 10 mM HEPES buffer (pH 7) containing 5 mM $MgCl_2$. For extracting acid invertase (EC 3.2.1.26) and alkaline invertase (EC 3.2.1.27), the procedure was same as for sucrose synthase except that 0.02 M sodium phosphate buffer (pH 7) was used instead of HEPES buffer. There were three replicates for each enzyme extract. Two crops were harvested for confirmation of the results for enzyme activities during the time-course study.

Assay system of sucrose synthase (0.5 cm^3) contained 0.2 M HEPES buffer (pH 6.5), 4 mM UDP and 0.1 M sucrose and the enzyme extract (0.1 cm^3). In the control assays, UDP was absent. The assay mixture was incubated for 30 min at 37 °C, the reaction stopped by adding 1 cm^3 of alkaline copper tartrate reagent and the fructose released was estimated (Nelson 1944). Acid invertase activity was measured by incubating 0.1 M sodium acetate buffer (pH 5.0), 0.1 M sucrose and enzyme extract (0.4 cm^3) in a total volume of 1 cm^3 at 37 °C for 30 min. Reducing sugars formed after sucrose hydrolysis were estimated (Nelson 1944). For estimation of alkaline invertase activity, the assay system was the same as that described above, except that the sodium acetate buffer was replaced by sodium phosphate buffer (pH 8.0). The rate of product formation in sucrose synthase and invertases was linear for at least 40 min. The activities of the above enzymes were estimated in cytosolic and bacteroid fractions of nodules (Singh *et al.* 1994).

For purification of alkaline invertase, the nodules (40 g) were harvested from lentil roots at 50 DAS and homogenized with a chilled pestle and mortar in 150 cm^3 of 50 mM sodium phosphate buffer (pH 8.0) containing 1 mM EDTA and 5 mM β-mercaptoethanol. Polyvinyl-pyrrolidone [100 mg g^{-1}(tissue)] was also added during extraction of the enzyme. The homogenate was squeezed through double layered cheese cloth and the filtrate centrifuged at 10 000 *g* for 15 min at 3 - 4 °C. Proteins from the supernatant were differentially precipitated with ammonium sulphate at its 0 - 30, 30 - 60 and 60 - 100 % saturation. The fraction obtained at 30 - 60 % salt 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 (0.35 M) in eluting buffer (0.02 M sodium phosphate, pH 7.2). Fractions (5 cm^3 each) collected from DEAE-cellulose column were assayed for alkaline invertase activity. Active enzyme fraction with the highest specific activity of invertase was further purified by *Sephadex G-150* column chromatography. For molecular mass determination, the elution volume (Ve) of the purified enzyme and standard proteins were determined on a *Sephadex G-150* column. Carbonic anhydrase (29 kDa), BSA (66 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa) and apoferritin (440 kDa) were used as reference proteins.

Results

The content of free sugars was relatively high at 60 and 80 DAS stages in the nodules during development (Table 1). By paper partition chromatography, sucrose was the only detectable non reducing sugar. Amongst sucrose, glucose and fructose, sucrose constituted the major free sugar in nodules. Sucrose was also the dominant sugar in roots with higher values at 60 and 80 DAS (Table 1).

Alkaline invertase showed higher activities than sucrose synthase in nodule cytosolic fraction during 50 and 60 DAS, however, at 70 and 80 DAS the sucrose

synthase showed equal or/and higher contributions in cleaving the incoming sucrose (Table 2). There was only a small amount of acid invertase in the nodules (Table 2). Activities of acid, alkaline invertase and sucrose synthase were not detected in the bacteroids.

Alkaline invertase at 50 DAS was highest in the roots (Table 2) and thereafter it declined but did not vary significantly between 70 to 90 DAS. However, the activity of sucrose synthase increased between 60 and 70 DAS, remained constant till 80 DAS and thereafter declined. The activities of all the three sucrolytic enzymes were much higher in nodules than in roots.

Table 1. Changes in sugar contents [mg g^{-1}(f.m.)] in the nodules and roots. Means ± SD of data obtained from triplicate extracts.

DAS	Nodules			Roots		
	fructose	glucose	sucrose	fructose	glucose	sucrose
50	0.18 ± 0.002	0.33 ± 0.01	2.40 ± 0.06	0.07 ± 0.00	0.18 ± 0.01	2.16 ± 0.27
60	0.25 ± 0.016	0.22 ± 0.01	3.24 ± 0.01	0.23 ± 0.03	0.21 ± 0.00	3.19 ± 0.07
70	0.28 ± 0.005	0.06 ± 0.01	2.68 ± 0.07	0.19 ± 0.01	0.16 ± 0.01	2.02 ± 0.11
80	0.42 ± 0.040	0.48 ± 0.02	2.80 ± 0.40	0.24 ± 0.01	0.36 ± 0.02	2.77 ± 0.06
90	0.08 ± 0.001	0.17 ± 0.00	2.03 ± 0.12	0.13 ± 0.01	0.33 ± 0.03	2.09 ± 0.08

Table 2. Activities [nmol g^{-1}(f.m.) s^{-1}] of enzymes involved in sucrose cleavage in roots and cytosolic fraction of nodules of lentil. Means ± SD of data obtained from triplicate extracts.

DAS	Nodules			Roots		
	acid invertase	alkaline invertase	sucrose synthase	acid invertase	alkaline invertase	sucrose synthase
50	365.2 ± 67.5	1351.4 ± 28.5	582.7 ± 52.2	210.5 ± 23.6	240.1 ± 13.1	231.5 ± 11.1
60	45.9 ± 13.3	488.7 ± 35.4	377.8 ± 21.0	17.6 ± 4.0	35.5 ± 4.5	104.9 ± 2.9
70	43.4 ± 2.9	987.7 ± 123.2	1284.3 ± 107.6	21.8 ± 1.7	84.1 ± 10.6	457.9 ± 62.7
80	43.8 ± 0.8	801.7 ± 56.9	1849.8 ± 117.6	49.8 ± 3.7	111.5 ± 4.2	406.5 ± 18.5
90	24.8 ± 3.7	1111.9 ± 73.9	561.4 ± 78.3	18.3 ± 2.5	75.5 ± 17.1	131.3 ± 11.6

Table 3. Partial purification of neutral invertase from lentil nodules (HMM - high molecular mass, LMM - low molecular mass).

Steps of purification	Enzyme activity [nmol]	Protein [mg]	Specific activity [nmol mg^{-1}(protein)]	Purification [fold]
Crude	32925	2051	16.05	1.0
30 - 60 % $(NH_4)_2SO_4$ saturation	15364	230	66.80	4.2
DEAE cellulose	3682	20	184	11.5
Sephadex G-150 minor peak (HMM)	280	0.8	350	21.8
Sephadex G-150 major peak (LMM)	1370	1.0	1370	85.4

The major alkaline invertase was purified to 85 folds over the starting crude extract (Table 3). This enzyme preparation showed typical Michaelis-Menten kinetics when the concentration of sucrose was increased at different temperatures, *i.e.*, 25, 30, 35, and 40 °C. The Km of invertase for sucrose varied from 11.0 to 14.0 mM depending upon the temperature of the assay system. The energy of activation for sucrose was about 24.3 kJ mol^{-1} and the enthalpy change was about 11.3 kJ mol^{-1}. Raffinose (final concentration 20 mM) and stachyose (20 mM) were hydrolyzed at 11.2 and 9.7 % respectively, of the rate for sucrose. The enzyme was unable to hydrolyze maltose and *p*-nitrophenyl-α-D-glucopyranoside.

The optimum pH was 6.8 and activities of 90 % or greater of the maximum were observed between pH 6.5 and 7.0. The maximum activity of lentil invertase was observed at 40 °C but was not very stable to heat. The enzyme exhibited stability on heating the purified preparation at 40 °C for 1 h, but when the enzyme was kept at 45 °C for 1 h, it lost 54 % of its original activity. Heating (1 h at 50 °C) caused about 96 % loss of activity, whereas 1-h heating at 55 °C caused complete loss of activity.

The addition of salts of Na^+, Ba^{2+}, NH_4^+, Ca^{2+}, Mg^{2+},

Cl^-, SO_4^{2-} ions (5 mM) had no effect on invertase activity. Pyridoxine-HCl inhibited enzyme activity by 71 %. Invertase is almost completely inhibited by manganese chloride, iodoacetamide and mercuric chloride at 5 mM. Lentil neutral invertase was inhibited non-competitively by $HgCl_2$ with a inhibitor constant Ki of about 140 μM.

Discussion

Sucrose is main sugar in the nodules of *Lens culinaris* as has been reported in nodules of *Sesbania grandiflora*, *Cicer arietinum* and *Vigna radiata* (Singh *et al.* 1980, 1994, Chopra *et al.* 1998). In roots also, sucrose is the dominant sugar (Table 1). Essentially, all of the acid, alkaline invertase and sucrose synthase was in the soluble fraction of the nodules indicating that the cleavage of sucrose takes place in host cytosol. The simultaneous action of alkaline invertase and sucrose synthase in the nodules ensures a hexose supply for meeting the energy and reducing power demands of nodules during the stage of active nitrogen fixation and these enzymes probably may regulate carbon metabolism and nitrogen fixation. In soybean and pea nodules, it is reported that in addition to the provision of carbon for nodule N fixation, control of sucrose synthase gene expression may be an important means of regulating carbon and nitrogen metabolism as well as development of functional nodules (Gordon *et al.* 1997, 1999). The peak activity of nitrogenase in developing lentil nodules was observed at around 90 DAS (Awan 1994).

The 85-fold purification of neutral invertase obtained in the present study compares favourably with the 80-fold purification of neutral invertase from the roots of *Cichorium intybus* (Van den Ende and Van Laere 1995). Sucrose hydrolysis by lentil neutral invertase obeys hyberbolic saturation kinetics, as would be expected from single substrate enzyme catalyzed reaction obeying simple Michaelis-Menten kinetics. The Km of LNI varied from 11.0 to 14.0 mM depending upon the temperature. The Km for sucrose of alkaline invertase from soybean and mungbean nodules have been reported to be 10 mM (Morell and Copeland 1984) and 3 - 4 mM (Chopra *et al.* 1998). The Km for sucrose of other neutral invertases are 10-20 mM (Chen and Black 1992, Van den Ende and Van Laere 1995, Lee and Sturm 1996, Vorster and Botha 1998). The LNI had a low affinity for raffinose and stachyose as compared with sucrose, resembling alkaline invertases from soybean and mungbean. The pH optimum of LNI is 6.8, which is consistent with other neutral invertases (Ranwala *et al.* 1991, Van den Ende and Van Laere 1995, Vorster and Botha 1998). The optimum temperature of 40 °C for LNI was same as that for mungbean alkaline invertase previously reported by us (Chopra *et al.* 1998).

LNI is almost completely inhibited by $HgCl_2$, $MnCl_2$ and iodoacetamide at 5 mM. Complete inhibition by Hg^{2+} is consistent with other reports on neutral invertases (Chen and Black 1992, Van den Ende and Van Laere 1995, Vorster and Botha 1998) suggesting that a reduced sulphydryl group might be essential for the activity. Sugarcane neutral invertase was not inhibited by $MnCl_2$ and the neutral invertase from muskmelon was only 20 % inhibited by Mn^{2+}. Differences in metal ion inhibition profiles between neutral invertases indicate differences at the structural level. Major enzyme activity was eluted from a gel filtration column at 71 kDa and a minor peak at 270 kDa position, suggesting a monomer of mass 71 kDa aggregating to form a tetramer of about 270 kDa. Evidence from other sources support this hypothesis; the monomeric form of other neutral invertases are 57 - 66 kDa, being homotetramers of native mass 238 - 260 kDa (Chen and Black 1992, Van den Ende and Van Laere 1995, Ross *et al.* 1996, Vorster and Botha 1998). The characteristics of lentil neutral invertase were different from the alkaline invertase purified from soybean (Morell and Copeland 1984) and mungbean (Chopra *et al.* 1998) which showed more alkaline pH optimum, lesser Km for sucrose and different molecular mass. However LNI and neutral invertase from other sources (Van den Ende and Van Laere 1995, Vorster and Botha 1998, Gallagher and Pollock 1998) showed similar molecular mass and Km values. There appears, therefore, to be more than one class of enzyme within the umbrella of alkaline/neutral invertases.

References

Anthon, G.E., Emerich, D.W.: Developmental regulation of enzymes of sucrose and hexose metabolism in effective and ineffective soybean nodules. - Plant Physiol. **92**: 346-351, 1990.

Asthir, B., Singh, R.: Purificaton and characterization of neutral invertase from chickpea nodules. - Indian J. Biochem. Biophys. **34**: 529-534, 1997.

Awan, M.F.M.: Ontogenic variation of nodulation, nitrogen fixation and nitrogen assimilation in lentil (*Lens culinaris* Medic). VI. Nitrogenase, nitrate reductase and glutamine synthetase activities. - Acta Physiol. Plant. **16**: 255-262, 1994.

Chen, J.Q., Black, C.C.: Biochemical and immunological properties of alkaline invertase isolated from sprouting

soybean hypocotyls. - Arch. Biochem. Biophys. **295**: 61-69, 1992.

Chopra, J., Kaur, N., Gupta, A.K.: Carbohydrate status and sucrose metabolism in mungbean roots and nodules. - Phytochemistry **49**: 1891-1895, 1998.

Copeland, L., Lee, H.S., Cowlishaw, N.: Carbon metabolism in chickpea nodules. - Soil Biol. Biochem. **27**: 381-385, 1995.

Copeland, L., Vella, J., Hong, Z.: Enzymes of carbohydrate metabolism in soybean nodules. - Phytochemistry **28**: 57-61, 1989.

Gallagher, J.A., Pollock, C.J.: Isolation and characterization of a cDNA clone from *Lolium temulentum* L. encoding for a sucrose hydrolytic enzyme which shows alkaline/neutral invertase activity. - J. exp. Bot. **49**: 789-795, 1998.

Gascon, S., Lampen, J.O.: Purification of internal invertase of yeast. - J. biol. Chem. **243**: 1567-1572, 1968.

Gordon, A.J., Minchin, F.R., James, C.L., Komina, O.: Sucrose synthase in legume nodules is essential for nitrogen fixation. - Plant Physiol. **120**: 867-877, 1999.

Gordon, A.J., Minchin, F.R., Skot, L., James, C.L.: Stress-induced declines in soybean N_2 fixation are related to nodule sucrose synthase activity. - Plant Physiol. **114**: 937-946, 1997.

Kouchi, H., Fukai, A., Katagiri, H., Minamisawa, K., Tajima, S.: Isolation and enzymological characterization of infected and uninfected cell protoplasts from root nodules of *Glycine max*. - Physiol. Plant. **73**: 327-334, 1988.

Lee, H., Sturm, A.: Purification and characterization of neutral and alkaline invertase from carrot. - Plant Physiol. **112**: 1513-1522, 1996.

Morell, M., Copeland, L.: Enzymes of sucrose breakdown in soybean nodules. - Plant Physiol. **74**: 1030-1034, 1984.

Morell, M., Copeland, L.: Sucrose synthase of soybean nodules. - Plant Physiol. **78**: 149-154, 1985.

Nelson, N.: A photometric adaptation of Somogyi method for determination of glucose. - J. biol. Chem. **153**: 375-380, 1944.

Ranwala, A.P., Iwanami, S.S., Masuda, H.: Acid and neutral invertases in the mesocarp of developing muskmelon *Cucumis melo* L. cv. Prince fruit. - Plant Physiol. **96**: 881-886, 1991.

Ross, H.A., Mc Rae, D., Davies, H.V.: Sucrolytic enzyme activities in cotyledons of the faba bean. Developmental changes and purification of alkaline invertase. - Plant Physiol. **111**: 329-338, 1996.

Sawhney, V., Sheoran, I.S., Amarjit, Singh, R.: Ontogenic changes in enzymes of sugar metabolism in relation to nitrogen fixation in chickpea (*Cicer arietinum* L.). - Plant Physiol. Biochem. (India) **15**: 49-57, 1988.

Singh, R., Karamdeep, L., Bhullar, S.S., Gupta, A.K.: Metabolism of free sugars in relation to the activities of enzymes involved in sucrose metabolism and nitrogen assimilation in the developing nodules of chickpea. - Plant Physiol. Biochem. **32**: 875-882, 1994.

Singh, R., Sidhu, P.S., Vadhera, S., Sital, J.S., Bhatia, I.S.: Extracellular invertase of *Rhizobium japonicum* and its role in free sugar metabolism in the developing root nodules of *Sesbania grandiflora*. - Physiol. Plant. **48**: 504-508, 1980.

Sukalo, V.D., Krugova, E.D.: [Enzymes of sucrose metabolism in pea nodule fractions incubated by an active strain of nodule bacteria.] - Fiziol. Biokhim. kul't. Rast. **25**: 581-587, 1993. [In Russ.]

Van den Ende, W., Van Laere, A.: Purification and properties of a neutral invertase from roots of *Cichorium intybus*. - Physiol. Plant. **93**: 241-248, 1995.

Vorster, D.J., Botha, F.C.: Partial purification and characterization of sugarcane neutral invertase. - Phytochemistry **49**: 651-655, 1998.

Williard, L.M., Slattery, M.: The colorimetric determination of fructosan in plant material. - J. biol. Chem. **157**: 161-167, 1945.