GLUTAMINE SYNTHETASE ISOZYMES IN SUGARCANE (*SACCHARUM OFFICINARUM* CV. CO-740) CULTURED *IN VITRO*

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ABSTRACT

The glutamine synthetase isozymes were studied in nonshoot forming (non-chlorophyllous) and shoot forming (chlorophyllous) callus and fully differentiated green leaves of sugarcane grown *in vitro*. Two isozymes, one specific to chloroplast and the other to cytoplasm, were found in leaves and shoot forming callus. In nonshoot forming callus only one isozyme (corresponding to cytoplasmic form from leaves and shoot forming callus) was found. The data suggest that no different isozymes of glutamine synthetase appear during organogenesis. Further, the appearance of chloroplast specific isozyme is related to the differentiation of chloroplasts in the shoot-forming callus.

INTRODUCTION

PRESENCE of isozymes of glutamine synthetase (GS) has been demonstrated in leaves, seeds, cotyledons and hypocotyl[1-9]. The properties and physiological role of these isozymes of glutamine synthatase located in different parts of plants, are not understood. However, glutamine synthetase is known to play a key role in the nitrogen metabolism of various organisms[10]. In green tissues two forms of GS, one cytoplasmic and the other chloroplast specific, have been reported. Only cytoplasmic form was demonstrated in yellow seeds and etiolated (non green) structures[8,9]. Isozymes of glutamine synthetase, specific to both cytosol and chloroplast were different in seeds and in green leaves of pea, suggesting that there is a change in the isozyme species during organ development[7]. There is no information, on the isozymes of GS in relation to organogenesis from callus in culture.

The objectives of the present work are two fold. First, to analyze the isozymes of glutamine synthetase in non shoot forming, shoot forming callus and green leaves grown *in vitro*, and second, to examine if there are any new isozyme forms developing during organogenesis.

MATERIALS AND METHODS

Callus tissue was initiated from the leaf explants of sugarcane (*Saccharum officinarum* var. CO-740) and maintained in dark at 26 ± 2°C on a modified Murashige and Skoog[11] medium, containing the following organic additives: 2,4-dichlorophenoxy acetic acid (2,4-D), 3 mg/l; thiamine hydrochloride, 1.0 mg/l; myoinositol, 100 mg/l; coconut milk, 10% by volume; sucrose, 20 g/l and agar 9 g/l as reported previously[12]. Shoot differentiation was induced by omitting 2,4-D from the medium and keeping the tissue in light. Eight day old callus was used.

Shoot multiplication was achieved on the basal Murashige and Skoog medium supplemented with 0.1 mg/l kinetin, 0.2 mg/l benzyl aminopurine (BAP) and 10% coconut milk[13]. Leaves of plantlets (about 3 week old) were used.

Extraction: Sugarcane callus, freed from agar and sponge-dried under the folds of a filter paper, was homogenized with 20 mM Tris-HCl buffer, pH 7.4, containing 5 mM $MgCl_2$ and 2 mM β-mercaptoethanol. The extraction medium (2 ml) was used per gram fresh weight of the tissue. The homogenate was filtered through two layers of cheese cloth and the filtrate centrifuged for 30 min at 10,000 × g. The supernatant (S_{10}) was used for enzyme assays. The S_{10} preparation from leaves was similarly obtained.

Enzyme assay: Glutamine synthetase activity was estimated by the assay procedure of Boyer *et al*[14]. The assay mixture (pH 7.0) contained 100 mM Tris-HCl, 50 mM NH_4Cl, 10 mM ATP, 50 mM $MgCl_2$ and 100 mM glutamate in the final volume of 0.2 ml. Reaction was started by the addition of S_{10} and, after 15 min (or more) at 37 C, was stopped by adding 1.8 ml of 1.1% $FeSO_4$ solution in 0.3 NH_2SO_4. Colour was developed by adding 0.15 ml of 6.6% ammonium molybdate solution in 7.5 N H_2SO_4. Control reactions were carried out in the absence of glutamate. Absorbance was recorded at 660 nm on a Shimadzu spectrophotometer model UV-210 A.

Isolation of chloroplasts: Chloroplasts from callus and leaves were isolated[15]. The tissues were homogenized in Tris-HCl buffer, 50 mM (pH 7.5) containing 10 mM NaCl and 0.5 M sucrose. After filtration through four layers of cheese cloth the homogenate was centrifuged at 250 × g for 5 min. The supernatant was centrifuged for 10 min at 2500 × g in a Sorval refrigerated centrifuge and chloroplast pelletted. The pellet was suspended in Tris-HCl buffer 20 mM (pH 7.5) containing 2 mM β-mercaptoethanol and 5 mM $MgCl_2$, homogenized using sand and the homogenate was centrifuged at 20,000 × g. This supernatant (S_{20}) was used as glutamine synthetase preparation enriched in chloroplast specific enzyme.

Chlorophyll extraction and absorption spectra: Chlorophyll of the leaves, green callus (shoot forming) and non green callus (non shoot forming) was extracted with 80 % acetone, estimated by its absorption at 662 nm in a recording spectrophotometer (Shimadzu UV-210 A)

Protein was determined by the method of Lowry *et al*[16].

Polyacrylamide gel electrophoresis was done as described by Davis[17]. Separation of GS isozymes by electrophoresis was performed on 7 % polyacrylamide gels. Gels were electrophoresed for 3 to 4 hr (current 2–3 mA per tube), incubated in the assay buffer minus enzyme for 30 min at 37°C, and washed with cold water. GS specific activity staining was visualized when these gels were treated for 15 min with a mixture of 1.1 % $FeSO_4$ and 6.6 % ammonium molybdate in H_2SO_4, 18:1.5, v/v.

RESULTS AND DISCUSSION

Figure 1 shows the zymograms of glutamine synthetase isozymes from various tissues. Two activity bands for glutamine synthetase were obtained on the gels when extracts of green leaves and shoot forming callus were analyzed (figure 1a, b). Only one activity band was observed in non shoot forming callus extract (figure 1c). The slow moving activity band (band 1) was absent in this tissue. When extracts from chloroplast enriched preparation, isolated from green leaves, were analyzed only one major activity band at position of band 1, was observed (figure 1d) suggesting chloroplast origin of band 1 glutamine synthetase activity. Band 1 of GS, therefore, appears when callus acquires visible greenness and chloroplasts during very early stages of differentiation. Presence of a chloroplast specific GS that could be related to the greenness of the tissue has been reported in other tissues[2,4].

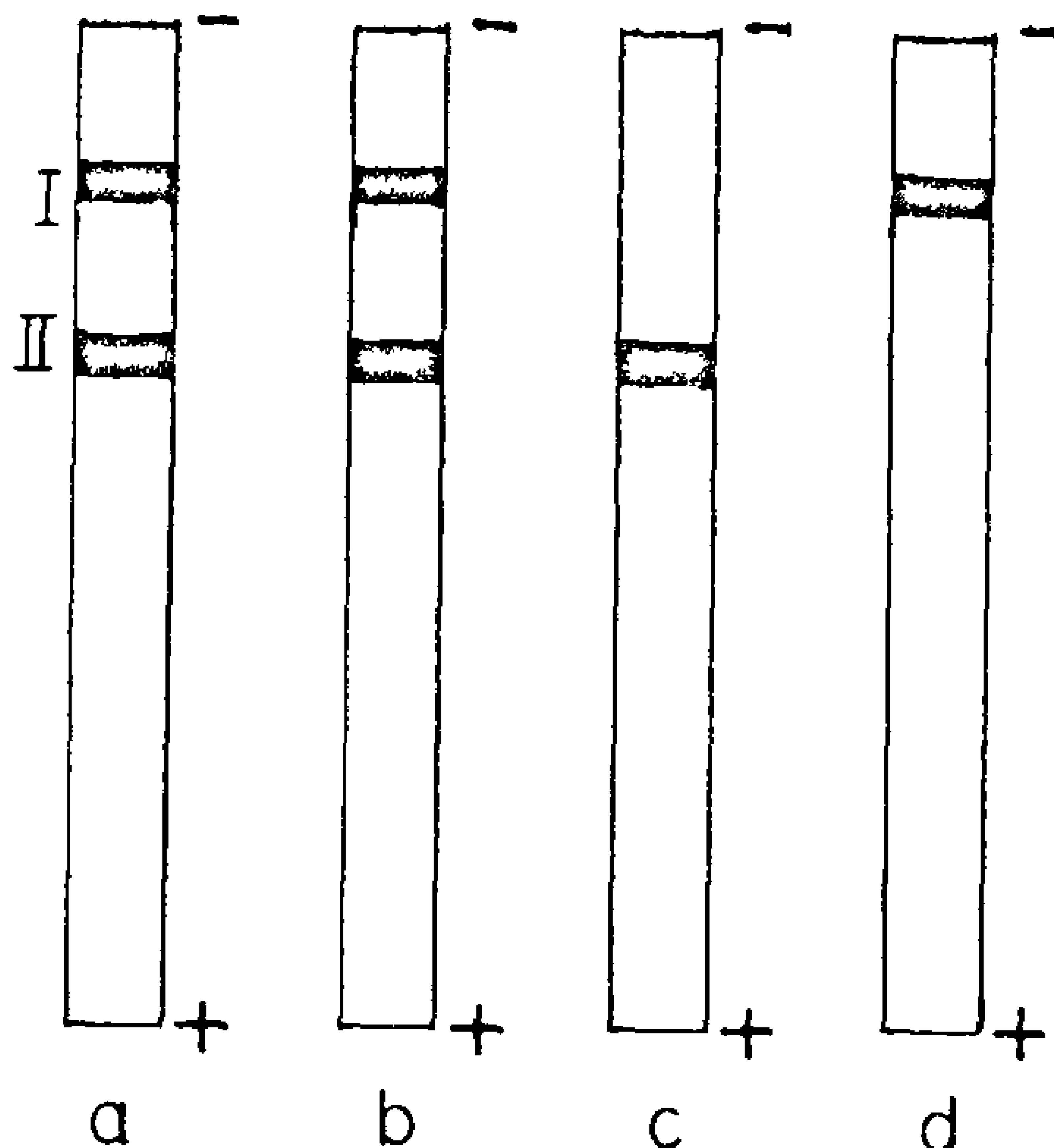

Figure 1. Electrophoretic separation of glutamine synthetase. Zymograms of glutamine synthetase activity staining patterns from various tissue extracts are represented as **a.** green leaves, **b.** shoot forming callus, **c.** non shoot forming callus, **d.** chloroplast enriched fraction from green leaves. 250 μg protein was electrophoresed in each case. Activity staining was performed as described in Materials and Methods.

Chlorophyll-a and chlorophyll-b were absent in non shoot forming callus, but were present in small amounts in the shoot forming callus tissue. The result that in non shoot forming callus both chlorophylls-a and -b were absent concomittant with the absence of band 1 enzyme GS activity further supported the localization of band 1 enzyme to chloroplasts.

It has been reported in pea[7] that isozyme species specific to both cytosol and chloroplast were not identical in the green leaves and seeds, suggesting a switch to new polypeptide chain synthesis during organogenesis. In sugarcane callus, however, identical isozymes appeared in shoot forming callus as well as in mature green leaves. Thus genomic information triggered on the onset of differentiation of chloroplast in callus remains unaltered when the microdifferentiation leads to organogenesis. In the case of pea[7] during seed maturation, it seems as though a separate locus or a set of loci coding for the same enzyme(s) gets activated and expressed in the preformed chloroplast. During germination and plant development, however,

another set of genes for chloroplast related macromolecules might be expressed. This hypothesis needs to be analyzed in more detail.

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NEWS

INSIDE EINSTEIN'S BRAIN

. . . "Einstein *was* different from you and me: At least one part of his brain was more developed than usual, according to the first published examination of it. . . . Marian Diamond [U. California] and co-workers got samples of the physicist's brain from Tom Harvey, a pathologist now in Weston, Mo., who performed the autopsy in 1955 and was given the brain for study. . . . The scientists looked at the ratio of two kinds of brain cells, neurons and glial cells. Neurons, which cannot divide, are the basic cells of the brain; glial cells, which can increase in number, provide support and nourishment to the neurons. Diamond's previous work has shown that animals put in environments that stimulate mental activity develop more glial cells per neuron. 'So we hypothesized that if Einstein's brain was more active in some areas, we would find more glial cells there,' [said] Diamond. Indeed, the scientists found that the 76-year-old physicist's brain contained more glial cells per neuron in [the upper front and lower rear portions of both hemispheres], compared with the brains of 11 normal males aged 47 to 80. The difference was statistically significant, however, only in the samples from the left rear portion. 'We don't know if Einstein was born with this or developed it later,' [said] Diamond. 'But it tells us that in one of the highest evolved areas of the brain, there is evidence that he had greater intellectual processing.' "

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