Activity of the tetramer and octamer of glutamine synthetase isoforms during primary leaf ontogeny of sugar beet (Beta vulgaris L.)

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Abstract. In extracts from the primary leaf blade of sugar beet (Beta vulgaris L.) we separated a chloroplastic isoform (GS 2) of glutamine synthetase (GS, EC 6.3.1.2) and one or two (depending on leaf age) cytosolic isoforms (GS la and GS lb). The latter were prominent in the early (GS la) and late stages of leaf ontogeny (GS 1a and GS 1b), whereas during leaf maturation GS 2 was the predominantly active GS isoform. The GS 1 isoforms were active exclusively in the octameric state although tetrameric GS 1 protein was detected immunologically. Their activity stayed at a relatively constant level during leaf ontogeny; an increase was observed only in the senescent leaf. The activity of GS 2, however, changed drastically during primary leaf ontogeny and was modulated by changes in the oligomeric state of the active enzyme. In the early and late stages of leaf ontogeny when GS 2 activity was low (lower than that of the GS 1 isoforms), GS 2 was active only in the octameric state. In the maturing leaf, when GS 2 activity had reached its maximum level (much higher than that of the GS 1 isoforms), 80% of total GS 2 activity was due the activity of the tetrameric form of the enzyme and 20% was due to octameric GS 2. Tetrameric GS 2 was a hetero-tetramer and thus not the unspecific dissociation product of homo-octameric GS 2. In addition, GS 2 activity was modulated by an activation/inactivation of the tetrameric GS 2 protein. Due to an activation of the GS 2 tetramer, the activity of tetrameric GS 2 increased during leaf maturation from zero level 23-fold compared with that of GS 1a and 18-fold compared with that of GS 1b. Possible activators of tetrameric GS 2 are thiol-reactive substances. During leaf senescence, GS 2 activity decreased to zero; this decrease was due to an inactivation of the tetrameric GS 2 protein probably caused by oxidation.

Key words: Beta – Glutamine synthetase isoforms – Glutamine synthetase oligomers (octamer, tetramer) – Glutamine synthetase subunit composition – Leaf ontogeny

Introduction

Different catalytically active glutamine synthetase (GS; EC 6.3.1.2) oligomers have been reported for the mammalian (Wilk et al. 1969) and fungal enzyme (Dávila et al. 1980) and also for higher plants (Mäck and Tischner 1990). In all cases, GS was active in the octameric or tetrameric state; the oligomeric state depended on the availability of cofactors (mammalian GS; Wilk et al. 1969) or of nitrogen (fungal GS; Dávila et al. 1980). In the present paper, we analyze the oligomeric state of active GS in relation to its intracellular localization in leaves of sugar beet.

The GS isoforms of higher plants are important in different aspects of NH₄⁺ metabolism: GS 1 plays a role in translocation of seed storage reserves during germination, in remobilization of N-reserves during ear development of maize and during leaf senescence (cited by Ta 1991); GS 2 functions in the re-assimilation of photorespiratory NH₄⁺, in the assimilation of NH₄⁺ derived from NO₃⁻ reduction, and in amino acid catabolism (cited by Redinbaugh and Campbell 1993). In the work presented here, the activity patterns of the GS isoforms and their oligomeric states have been studied during the entire life span of the primary leaf of sugar beet, and the temporal separation of the activity maxima has been discussed in the context of leaf development and its sink-source conversion with respect to carbon (Giaquinta 1978) and nitrogen (Joy 1967).

The conformational modification of Lemma GS by thiol-reactive substances has been studied by Rhodes et al. (1978). Mann et al. (1979) reported that the cytosolic and chloroplastic GS isoforms of barley respond differently to thiol-reactive substances. In sugar beet, β-mercaptoethanol has been found to activate tetrameric
Glutamine synthetase activity and total protein content of the crude extract. After grinding the primary leaf and centrifugation of the extract, which was done as previously reported (Mäck and Tischner 1990) except that β-mercaptoethanol was omitted, the supernatant was used for assay of GS activity [synthetase reaction; the formation of γ-glutamyl hydroxamate (GHA) was measured as previously described, Mäck and Tischner 1990] and determination of total soluble protein according to Bradford (1976).

Separation of the GS isoforms. After centrifugation of the crude extract at 100000 × g for 15 min, the supernatant (8 mg protein) was applied to a Superose 12 column (Mono Q, FPLC system; Pharmacia, Freiburg, Germany) equilibrated with 0.1 M imidazole buffer (pH 7.3), and 1 mM MgSO4. Protein was eluted with a two-step NaCl gradient: from 0 to 250 mM NaCl the fraction size was 0.5 ml and from 250 to 400 mM NaCl it was 0.8 ml. The flow rate was 0.4 ml min⁻¹. Fifty microlitres of each fraction was assayed for GS activity (synthetase reaction) and protein content as mentioned above. Peak fractions containing the isoforms were pooled, desalted on a Sephadex G 25 column and immediately submitted to non-denaturing gel electrophoresis.

Separation of octa- and tetrameric GS. Crude extract (500 µg protein) and the fast protein liquid chromatography (FPLC)-separated GS isoforms (20 to 60 µg protein) were loaded onto a non-denaturing polyacrylamide gel (5–8.5% gradient gel) in a Tris-glycine buffer system modified after Laemmli (1970). The gels were run at 100 V for 3 h. Thereafter the marker lane (high-molecular-weight markers, Pharmacia) was stained with Ponceau-Red (0.5% Ponceau S; Sigma in 1% acetic acid). The primary antibody cross-reacted with ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) protein. This was an unspecific reaction which occurred only if high amounts of leaf protein were applied to the gels.

Separation of the GS1 and GS2 subunits. The subunit compositions of GS1 and GS2 were determined by two-dimensional gel electrophoresis. The proteins of the desalted FPLC peak fractions were precipitated with acetone:ethanol (1:1, v/v). Solubilization of the precipitate and isoelectric focusing of 30 µg protein in capillary gel rods (1.5 mm diameter) at pH 4–9 was done in the presence of 9 M urea according to Dunbar et al. (1990). After focusing (3000 V-h), the gel rods were equilibrated in 0.1 M Tris buffer (pH 6.8), 2% SDS (w/v), 6 M urea and loaded onto a 7.5–20% polyacrylamide gradient gel with 0.4% SDS (Laemmli 1970) for separation in the second dimension. The proteins of the SDS slab gel were electrophobotted onto a nitrocellulose membrane. The immunodetected GS subunits (procedure as described above) were visualized with alkaline phosphatase linked to a goat anti-rabbit IgG (BioRad, München, Germany). The markers (low-molecular-weight markers; Pharmacia) were stained with Ponceau-Red (0.5% Ponceau S; Sigma in 1% acetic acid).

Isolation of chloroplasts. Chloroplast isolation from approx. 40 g leaf blades was done according to Heldt and Sauer (1971), but with some modifications. The chloroplasts were purified on a Percoll gradient according to Mourou and Douce (1981) and osmotically shocked in 0.1 M imidazole-HCl (pH 7.3), with 1 mM MgSO4. After centrifugation at 100000 × g for 15 min, the supernatant was applied to the anion-exchange column.

Calculations. The data presented are the average of three independent experimental sets.

Results

Ontogenetic stages of the primary leaf. The physiological state of the expanding leaf was determined by the lamina length (according to Giacinta 1978); when leaf expansion had ceased, the degree of senescence was determined by the chlorophyll content. Maximum chlorophyll content (measured at 80% final lamina length; FLL) was defined as 0% senescence and minimum chlorophyll content was defined as 100% senescence (Table I). At that stage the leaf was yellow and wilted.

Glutamine synthetase activity and protein content of the crude extract. During maturation of the primary leaf, the content of specific protein increased concomitantly with GS activity, reaching a maximum at 30–40% senescence (Fig. 1).