*Arch. Biol. Sci*., Belgrade, 63 (4), 965-969, 2011 DOI:10.2298/ABS1104965D

# COUPLING NATIVE PAGE/ACTIVITY-STAINING WITH SDS-PAGE/IMMUNODETECTION FOR THE ANALYSIS OF GLUTAMINE SYNTHETASE ISOFORMS IN SPINACH

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*Abstract* - Glutamine synthetase (GS) is a key nitrogen-assimilating enzyme in plants and a target for the broad-spectrum herbicide glufosinate. Understanding its kinetic and structural properties is of major agricultural importance. Spinach (*Spinacia oleracea*) is classified as a plant expressing only chloroplastic GS activity. We have analyzed soluble proteins in the spinach by coupling native polyacrylamide gel electrophoresis (PAGE)-activity detection, based on phosphate precipitation, with SDS-PAGE/immunoblotting. One cytosolic (GS1) isoform from the roots and two chloroplastic (GS2) isoforms expressed in leaves were resolved by native PAGE. The identity of the obtained bands was established by the application of GS-specific inhibitors, L-methionine sulfoximine and glufosinate. Examination by sodium dodecyl sulfate (SDS)-PAGE/ Western analysis with anti-GS antibodies, confirmed the identity of the active bands and revealed that both chloroplastic isoforms are composed of 44 kDa subunits, while the cytosolic isoform consists of 40 kDa subunits. The presence of more GS2 isozymes than encoded in the spinach genome is discussed in terms of posttranslational modifications.

*Key words:* Glutamine synthetase, *Spinacia oleracea,* Western blot, phosphate precipitation

UDC 582.661.15:577.15

## INTRODUCTION

Glutamine synthetase (GS, EC 6.3.1.2) facilitates the assimilation of inorganic nitrogen in the form of ammonia with glutamate, forming glutamine. As a key N-assimilation enzyme and a target for herbicides such as glufosinate, GS has been well studied, both structurally and kinetically (Forde and Cullimore, 1989; Eisenberg et al., 2000). While the primary sequence of GS proteins is conserved (Pesole et al., 1991; Eisenberg et al., 2000), their quaternary structures differ, including bacterial homododecamers, known as GS class I (Eisenberg et al., 2000), as well as eukaryotic octamers (Stahl and Jaenicke, 1972; Eisenberg et al., 2000; Llorca et al., 2006) or decamers (Unno et al., 2006) which comprise GS class II (found also in some prokaryotes). Higher plants typically express one chloroplast (GS2) and one or more cytosol (GS1) isoforms (Hirel and Gadal, 1980; Lam et al., 1996). GS2 is predominant in leaves, with a primary role in the reassimilation of photorespiratory ammonia (Wallsgrove et al., 1987). GS1 isoforms can be expressed throughout the plant, but primarily in nonphotosynthetic tissues (Hirel and Gadal, 1980; Hirel et al., 1984; Lam et al., 1996), being involved either in primary N assimilation or N remobilization (Muhitch, 2003). *Spinacia oleracea* has been classified as a plant expressing only GS2 activity in leaves (Hirel et al., 1982, 1984; McNally et al., 1983; Ericson, 1985), even though both GS1 and GS2 sequences have been deposited to the GenBank (EU057984 and EF143582). Hereinwith we present high-resolution spinach GS zymograms: one GS1 expressed in the roots and two distinct GS2 activity bands.

## MATERIALS AND METHODS

Spinach (*Spinacia oleracea*) plants were purchased at a local market. The leaves and roots were separated and individually tested.

For protein isolation, 1 g of plant tissue was ground in liquid nitrogen, followed by homogenization in 2 ml buffer solution containing 50 mM Tris–HCl pH 8, 1 mM EDTA, 1.5% w/v polyvinylpolypyrrolidone (PVPP), 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 30% v/v glycerol. The crude extracts were centrifuged at 15,000xg for 5 min at 4°C to pellet the cell debris and the PVPP. The soluble protein content was determined by the Coomassie Blue dye binding method using bovine serum albumin as standard (Bradford, 1976).

Chloroplasts were isolated according to the method of Cerović and Plesničar (1984) from 20 g of spinach leaves. After two washes, the clean chloroplast pellet was ground in liquid nitrogen and the chloroplast proteins were extracted with 500 µl buffer solution, as described for total cellular proteins.

Proteins were separated on discontinuous nondenaturing 7% polyacrylamide slab gels using the Hoefer SE600 system, at 120 V for 20 h at 10°C. The gels with separated GS isoforms were first incubated in a buffer containing GS substrates. The sites of GS activity were visualized by the phosphate precipitation assay (Simonović et al., 2004). To verify that the obtained bands represent specific GS activity, not nonspecific phosphatase activity, parallel assays were performed with the addition of GS-specific inhibitors, L-methionine sulfoximine (5 mM, Sigma) and glufosinate (1 mM, Sigma), along with the substrates; structurally related L-buthionine-S,R-sulfoximine (5 mM, Sigma), which does not affect GS activity, served as a positive control.

The developed activity bands were photographed and then cut out from the gel, equilibrated in 50 mM Tris-HCl pH 8 for one hour at room temperature, homogenized by the syringe maceration method (Scheer et al., 2001) and extracted for one hour at 50°C with 200 µl buffer containing 50 mM Tris HCl pH 8, 1% SDS and 3% β-mercaptoethanol. The obtained supernatants containing GS proteins were separated by SDS-PAGE (Laemmli et al., 1970), transferred to a nitrocellulose membrane using a BioRad transfer unit and immune-detected by a modified protocol (Ristić et al., 2007) with GLN1 GLN2/GS1 glutamine synthetase global primary antibodies (Agrisera) and visualized by enhanced chemiluminescence.

The chloroplastic GS2 sequence was analyzed using ChloroP 1.1 Server (Emanuelsson et al., 1999).

## RESULTS AND DISCUSSION

The activity staining of GS isoforms used in this work is based on the physiological reaction of glutamine synthesis from glutamate and ammonia at the expense of ATP, resulting in phosphate release. The inorganic phosphate (Pi) is precipitated in the gel, forming white bands – evidence of enzymatic activity (Simonović et al., 2004). Phosphate precipitation-dyeing of native gels containing electrophoretically separated spinach leaf and chloroplast proteins revealed two GS activities that are referred to as GSα and GSγ based on their increasing mobility (Fig. 1). Since both bands appeared in the chloroplastic zymogram, they are GS2 moieties. The cytosolic GS1 activity, referred to as GSβ, was only observed in the root extracts (Fig. 1). To the best of our knowledge, this is the first report on GS1 activity in the spinach. Since the phosphate precipitation assay is not specific for GS but detects any activity that releases Pi, the identity of the obtained bands was confirmed in parallel assays using specific GS inhibitors, L-methionine sulfoximine and glufosinate. At 5 mM concentration both inhibitors completely inhibited GS activity while the structurally related L-buthionine-S,R-sulfoximine which does not inhibit GS due to its bulky side chain (Griffith, 1982), had no effect, as expected (data not shown). Comparison of ten different spinach samples, including several varieties, obtained from the green markets, showed no difference in the GS profile (not shown).

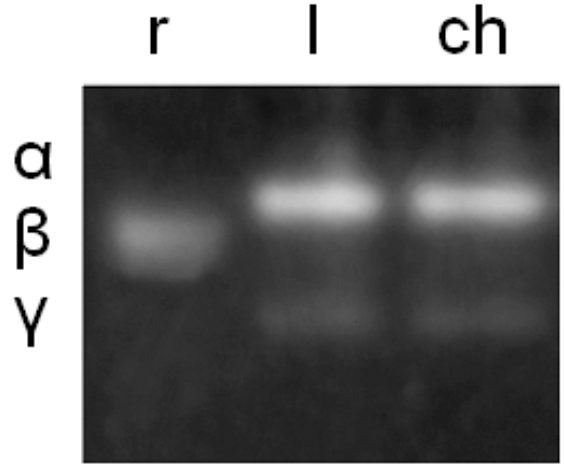
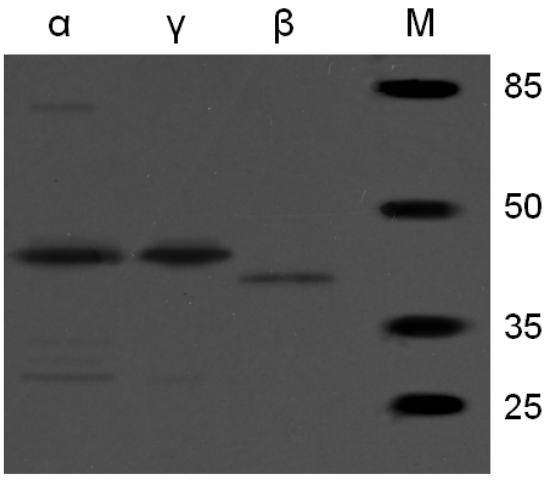



**Fig. 2.** Western analysis of spinach GS after SDS-PAGE. The GS activity bands α, γ, and β were extracted from native PAGE gel, denatured, separated by SDS-PAGE, transferred to a nitrocellulose membrane, and examined using anti-GS antibodies and subsequent enhanced chemiluminescence. M – MW markers.

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The proteins from the detected GS activity bands were extracted from the gel, denatured and analyzed by Western blotting using specific anti-GS antibodies. Immunoblotting revealed that both chloroplastic isoforms consisted of 44 kDa subunits, which is in agreement with previous reports (Ericson, 1985), while the cytosolic isoform was composed of 40 kDa subunits (Fig. 2). The electrophoretic sizing data for GS1 are in good correlation with the sequence-predicted MW of 39,225 Da according to the GenBank accession number EU057984. The sequence-predicted MW for the GS2 chloroplastic precursor (accession number EF143582) is 47,380 Da. Running the GS2 precursor sequence through a ChloroP 1.1 Server (Emanuelsson et al., 1999) which predicts the presence of chloroplast transit peptides (cTP) in protein sequences and the location of potential cTP cleavage sitesresulted in a processed GS2 subunit with a MW of 41,956 Da (Fig. 3).

The observation of two GS2 bands after native PAGE of spinach (Fig. 1) was unexpected in light of the fact that all plants encode a single chloroplast isoform (Llorca et al., 2006; Unno et al., 2006.). Even when it was found that *Arabidopsis* mitochondria also possess GS activity, the genome-wide search led to *GLN2* as the sole candidate for both organellar functions (Taira et al., 2004). The possibility of post-translational modifications of GS2 isoforms and/or their interaction with regulatory proteins that would result in shifts in electrophoretic mobility offers a plausible explanation for the appearance of more GS2 activity bands than are known to be encoded in spinach. Phosphorylation of GS isoforms has been documented in *Brassica napus* and *Medicago truncatula*. In both species the phosphorylated isoforms interact with regulatory 14-3-3 proteins (Finnemann and Schjoerring, 2000; Lima et al., 2006a, b). The GS2 in *Catharanthus roseus* is glycosylated (Miranda-Ham and Loyola-Vargas, 1992). Proteins other than 14-3-3 can also interact with GS. Thus, a 110-kDa protein from tomato roots was shown to inhibit both GS1 and GS2 from tomato and other species (Gallardo and Cánovas, 1992).

The phosphate precipitation assay is useful in the detection of GS activity in all the tested plants tested, including maize (Simonović et al., 2004; Simonović and Anderson, 2007), *Phaseolus vulgaris* (Estivill et al., 2010), *Lotus corniculatus* and *Arabidopsis* (Dragićević, unpublished data). In the case

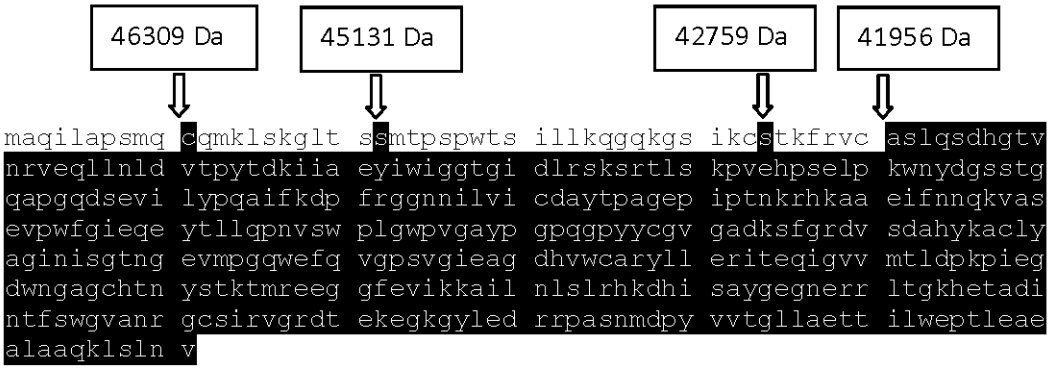


**Fig. 3.** Prediction of potential chloroplast transit peptides (cTP) cleavage sites (CS) in the unprocessed spinach GS2 transcript (EF143582). ChloroP 1.1 Server predicted four possible cleavage sites, indicated by arrows, before amino acids at positions: 11 (CS-score 3.560; the resultant GS2 peptide would have a MW of 46,309 Da), 22 (CS-score 3.089, MW 45,131 Da), 43 (CS-score 5.333, MW 42,759 Da) and at 51 (CS –score 6.563, MW 41,956 Da). Based on this prediction, the most likely candidate for cTP is the 50 amino acid N-terminal sequence (white), in which case the processed GS2 sequence (shaded black) would have just below 42 kDa.

of spinach, the assay could discriminate between two activity bands of the same chloroplast isoform, It was also sensitive enough to visualize GS1 activity that has not been detected in earlier analyses of this enzyme in spinach (Hirel et al., 1982; McNally et al., 1983; Ericson, 1985). Immunoblotting was used to confirm the identity of the enzymatic activities and to estimate the size of the contributing subunits. While enzymatic gel and Western blotting assays are widely used in enzymology, coupling these two procedures by extracting the activity bands and subsequently analyzing them by immunoblotting is a seldom used approach that could prove to be useful for examining other enzymes for which an appropriate gel assay has been developed.

*Acknowledgement* - This study was supported by the Ministry of Education and Science of the Republic of Serbia, contract No. ON173024.

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