J. Rubb. Res., **4**(1), 56–68

Glutamine Synthetase of Hevea Brasiliensis *Latex: Characterisation and Physiological Regulation*

ANNE CLÉMENT*#, JEAN-CLAUDE PRÉVÔT*, VALÉRIE PUJADE-RENAUD*, BERTRAND HIREL** AND JEAN-LOUIS JACOB*

Regular exploitation of rubber tree causes intense metabolic activity in latex cells located in the phloem, associated with the regeneration of the latex cellular components removed by tapping. In this context, glutamine synthetase (GS) plays a prominent role by ensuring ammonium fixation necessary for the regeneration of latex nitrogenous components.

In latex, GS activity was detected in the cytosol only. The enzyme was purified and its main physico-chemical characteristics were determined. Its molecular mass was 450 kDa for the native form and 45 kDa ± 5 kDa for the sub-unit. The pH optimum was found to be 8.0 and variations in the physiological pH range (6.4 to 7.2) strongly affected GS activity. K_m for ammonium, glutamate and ATP were estimated at 8 µM ± 2 µM, 4 mM ± 0.5 mM and 0.6 mM ± 0.05 mM, respectively. Comparison with the cytosol physiological parameters showed the importance of ATP concentration for GS activity regulation. Functioning of the purified enzyme in a paraphysiological (ultrafiltrated, protein-free) medium, as compared with an optimised control medium, demonstrated that physiological ATP concentrations were significantly limiting for GS potential activity.

Treatment with ethephon increases latex production both by increasing latex flow duration and by stimulating the regeneration of metabolism. In this paper we demonstrated that ethephon treatment induced a concomitant increase in pH, ATP concentration and GS activity. These results suggest that biochemical mechanisms involving pH and ATP participate in the regulation of GS activity in response to ethylene, in addition to the previously observed stimulation of gene expression.

Key words: glutamine synthetase; *Hevea brasiliensis*; latex stimulation; characterisation; physiological regulation; ethephon; purification; biochemical analysis; GT 1

In *Hevea brasiliensis*, latex is the rubber-containing cytoplasm of specialised cells called laticifers, located in the secondary phloem. Laticifers are concentric rings of anastomosed cells regularly differentiated from the cambium[1]. Upon tapping (half spiral

* CIRAD/AMIS-CP, TA 40/01, Avenue d'Agropolis, 34398 Montpellier Cedex 5, France
** INRA – Centre de Versailles, Laboratoire du métabolisme, route de Saint Cyr, 78026 Versailles, France
# Corresponding author (e-mail: anne.clement-vidal@cirad.fr)

incision of the bark up to 1 mm from the cambium), latex is expelled from the laticifer rings, driven by turgor pressure. Coagulation involving the rubber particles finally plugs the wound and stop latex flow. The cellular material lost during tapping is subsequently regenerated inside the laticifers, and within two or three days, most of the latex is renewed, allowing repeated tapping. In regularly tapped trees, the efficiency of the latex regeneration metabolism is therefore of fundamental importance for rubber yield. This metabolism involves not only rubber (*cis*-polyisoprene) biosynthesis, but also the reconstitution of sub-cellular components and structures. One central enzyme necessary for the regeneration process is glutamine synthetase (GS; EC 6.3.1.2) which takes part in ammonium incorporation into organic molecules[2].

$$\text{Glutamate} + \text{ATP} + \text{ammonium} \xrightarrow{Mg^{2+}} \text{Glutamine} + \text{ADP} + \text{Pi}$$

Glutamine is one of the major nitrogenous intermediate involved in the formation of amino acids and nucleotides necessary for the synthesis of proteins, nucleic acids and other nitrogen-containing molecules.

Ammonium is the only form of nitrogen that plants can incorporate into organic molecules. It may originate from the reduction of nitrates in the roots, or can be released during various metabolic reactions (photo-respiration, protein turnover *etc.*). It is toxic for the cell if accumulated above a threshold level. For all these reasons, glutamine synthetase plays a key role both in detoxification and general metabolism. Its localization is tightly linked to the metabolic origin of the ammonium used as a substrate. Various GS isoforms are therefore present in all parts of the plant.

The latex yield can be increased by treating the trees with the ethylene generator chloro-ethylphosphonic acid (ethephon)[3]. In such intensive exploitation systems, larger quantities of latex are exported from the laticifers, increasing the demand for latex regeneration between tappings. Efficient functioning of the nitrogen metabolism, and hence of GS activity, are therefore required to ensure full regeneration of the exported latex.

Stimulation with ethephon both increases the volume of exported latex and stimulates the latex regeneration metabolism between tappings. In a previous paper[4], we demonstrated that treatment with ethephon increases GS activity in the latex cell. The regulation of GS activity by ethylene seems to involve several mechanisms. One is through a marked increase of gene expression, although the observed gain in GS protein concentration after ethephon treatment was not proportional to the GS mRNA increase, suggesting the occurrence of regulatory processes other than transcriptional activation. Biochemical regulation of the GS enzyme activity is also likely to occur.

To further characterise the regulation of GS activity in the latex, it was important to first purify the enzyme. In this paper, we present the purification and biochemical analysis of the latex glutamine synthetase *in vitro* as well as in paraphysiological conditions.

METHODS

Plant Material

The *Hevea brasiliensis* latex used in all experiments was from the IDEFOR rubber tree plantation, at Bimbresso (Ivory Coast). For the ethylene experiments 2 groups of 25 trees, genotype GT 1 were selected. One group

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included control trees and the other treated trees with Ethrel 2.5% on the tapping cut (Rhone Poulenc, France). The latex used for GS purification was pooled from trees of the same genotype (GT 1).

The latex was collected on ice and centrifuged at 16 000 × *g* for 15 min at 4°C. The pellet, composed of lutoids and Frey-Wyssling particles, was washed in a buffer (25 mM TRIS-MES: morpholinoethanesulfonic acid), pH 7.2 containing 1 mM $MgCl_2$ and 0.33 M mannitol and freeze dried (bottom fraction). The supernatant was centrifuged (45 000 × *g*, 90 min). The upper phase (rubber particles) was discarded and the cytosolic fraction was freeze-dried. The freeze-dried fractions were kept at –30°C until used for enzyme assay.

The paraphysiological medium was obtained by dissolving freeze-dried cytosol in distilled water (50 mg mL^{-1}) and ultrafiltration of this solution through a 10 kDa membrane (Filtron, Northborough, MA., USA).

Enzyme Assays

GS activity was determined by colorimetric measurement of Pi formed after incubation at 30°C in 1 mL buffer (0.1 M Tris HCl pH 7.5) containing 20 mM glutamate, 3 mM to 5 mM ATP, 5 mM to 10 mM $MgSO_4$ and 5 mM $(NH_4)_2SO_4$, using the Taussky and Shorr method[5]. The enzyme reaction was stopped by the addition of 6 M perchloric acid and the measurements were carried out after removing the protein pellet by centrifugation (5 000 × *g*, 10 min). This method was used to estimate K_m values for glutamate and ATP-Mg and then for the pH response curve, substrate specificity studies, and effector determination.

GS activity was also measured directly by spectrophotometric measurement of ADP formed coupled with NADH oxidation *via* pyruvate kinase[6]. This protocol was used to determine Km values to NH_4^+.

GS activity in the paraphysiological medium was determined by ADP measurement as described above after incubation at 30°C. The reaction was stopped by the addition of 6 M perchloric acid and neutralised with 6 M KOH.

Protein Assay

Protein contents were determined by the Bradford method[7] using bovin serum albumin as standard.

Substrates and Co-factor Determinations

The ATP concentration in the cytosol was measured by chemiluminescence using a biocounter (model M2500, Lumac, Landgraaf, the Netherlands) with a measurement kit provided by Lumac (Lumit PM 8, Perstorp analytical company, Bezons, France). Ammonium concentration in the cytosol was determined using the method of Berthelot modified by Fallavier[8]. Determination of glutamate concentration in the cytosol was carried out while following the transformation of NAD+ in NADH by glutamate dehydrogenase at 340 nm[9]. The magnesium concentration in the cytosol was measured using eriochrome black T as described by Pujarniscle[10].

GS Purification

All purification steps were conducted at 4°C. Crude extract: 1 g of freeze-dried cytosol

was resuspended (50 mg mL^{-1}) in buffer [20 mM Hepes-KOH pH 7.6 containing 5 mM $MgSO_4$ and 0.02% NaN_3 (Buffer A)]. The suspension was stirred for 20 min and then centrifuged at 20 000 × *g* for 15 min.

Proteins were precipitated between 35% and 45% ammonium sulphate saturation, collected by centrifugation at 20 000 g for 15 min and resuspended in Buffer A. The protein extract was applied to a 100 × 1.6 cm column (Sephacryl S300 Pharmacia) equilibrated and eluted with Buffer A at 0.8 mL min^{-1}. The fractions containing GS activity were pooled. The extract was applied to a 1.5 cm × 10 cm column (Fractogel DMAE, Merck) pre-equilibrated with Buffer A. After washing the column with Buffer A containing 0.08 M KCl, GS elution was carried out by KCl linear gradient (0.08 M – 0.2 M). The flow rate was 0.7 mL min^{-1}. The fractions displaying GS activity were pooled and the KCl was removed by successive washings in an ultrafiltration cell with Buffer A. The desalted protein extract was applied to a 0.8 cm × 8 cm column (Ultrogel hydroxyapatite, IBF) equilibrated with Buffer B (10 mM potassium phosphate, pH 7.2) at 0.5 mL min^{-1}. GS was eluted with a linear gradient of potassium phosphate buffer (10 mM – 500 mM). The fractions displaying GS activity were used for native PAGE, SDS-PAGE and western blots.

Electrophoresis and Western Blot Analysis

Non-denaturing polyacrylamide electrophoresis was performed on 4% – 15% gradient ready gels (Mini protean II, Bio Rad) for 2.5 h at 120 V. GS activity was detected by transferase activity as described by Winter *et al.*[11] Proteins were stained with silver nitrate as per Wray *et al.*[12] Pharmacia molecular mass markers were used.

SDS-PAGE was carried out on polyacrylamide gradient ready gel using the Laemmli[13] method. Protein markers were provided by Bio-Rad. Proteins were electrotransferred to nitro-cellulose membranes using a Mini transblot unit from Bio-Rad. Immunodetection was performed as described previously by Hirel *et al.*[14] using a polyclonal antibody directed against soybean GS. Heterologous antibodies can often be successfully used for GS study owing to the high degree of conservation of this enzyme, as evidenced by phylogenetic comparison of their primary structure[15].

RESULTS AND DISCUSSION

Detection and Localisation of GS in Latex

The search for GS activity was conducted in the two main fractions obtained after latex centrifugation: the cytosolic serum and the pellet (see methods). Although mostly composed of lutoids (vacuo-lysosomes), the pellet fraction also contains plastid-like organelles called Frey-Wyssling particles[1]. Its was therefore justified to check for the possible existence of a plastidial GS isoform in this latex fraction. However, GS activity could only be detected in the cytosol.

Purification of GS from the Cytosol

The different steps of enzyme purification are shown in *Table 1*. After step 4 (DMAE), the degree of enzyme purification was around 87 fold. For the final step on hydroxyapatite gel, the quantity of protein remaining in the extract was below the threshold of protein measurement, although GS activity was still detectable.

Native polyacrylamide gel electrophoresis of the protein extract after the last purification

TABLE 1. PURIFICATION OF GS FROM THE LATEX CYTOSOL OF *HEVEA BRASILIENSIS*

STEPS	Total protein (mg)	Total activity (μ kat)	Specific activity (μkat mg^{-1})	Purification (Fold)	Recovery (%)
Crude extract	141.6	2.93	0.021	–	100
$(NH_4)_2SO_4$ 35% – 45%	32.6	1.22	0.037	1.8	41.6
S300 HR	4.9	0.8	0.163	7.9	27.3
DMAE	0.264	0.47	1.81	87.4	16
Hydroxyapatite	UD[a]	0.35	–	–	11.9

[a] Undetectable

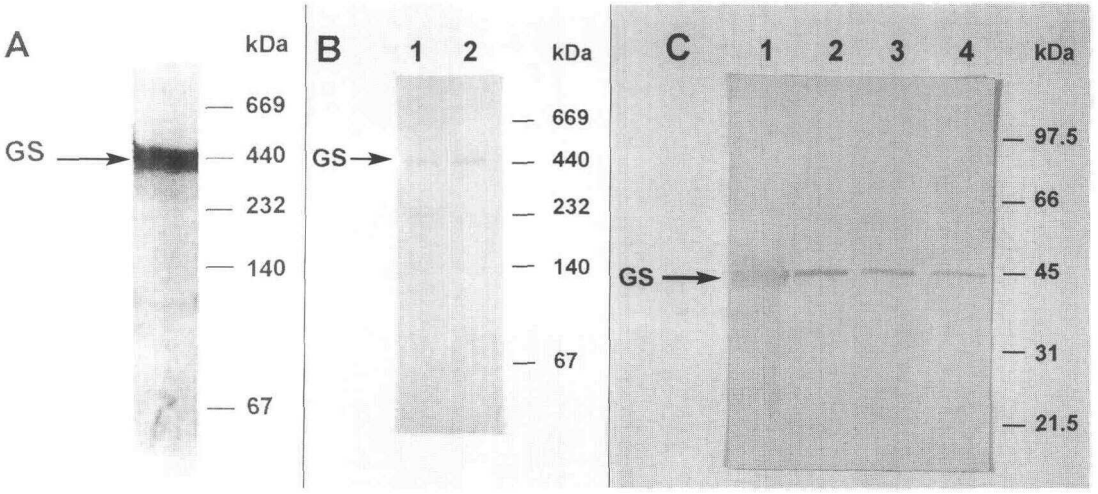


Figure 1. Determination of GS molecular mass by native and SDS PAGE.

A: native PAGE of the purified GS and silver staining; B: specific activity of the purified GS (1) and DMAE purification step (2) in native PAGE; C: western blot analysis in SDS-PAGE. Crude extract (1), and different purification steps, S300 (2), DMAE (3) and hydroxyapatite (4).

step revealed a single band after silver staining (*Figure 1 A*), which matched the band detected by specific activity staining (*Figure 1 B*), indicating satisfying purity. The presence of the GS enzyme in the extract at each purification step, including the final one, was also confirmed by Western blot analysis performed on SDS-PAGE with an antibody directed against a cytosolic GS from soybean (*Figure 1 C*).

GS Physico- and Biochemical Characteristics

Migration of glutamine synthetase (pI about 4.5) following non-denaturing polyacrylamide gel electrophoresis indicated its molecular weight to be approximately 450 kDa (*Figures 1 A and 1 B*). This estimate was confirmed by gel filtration of the protein on a Sephacryl S300 column calibrated against molecular weight standards of 67 kDa to 669 kDa (results not presented). Such a value is similar to that of the rice leaf GS[16]. Under denaturing conditions (*Figure 1 C*), only one band at 45 ± 3 kDA was observed in the purified extracts. In a previous immunoblot experiment performed on crude cytosol, a second band corresponding supposedly to a sub-unit of slightly higher molecular mass had been detected[4]. In the present work, a single but thicker band was observed in the crude extract, which does not allow either to confirm or infirm the existence of a second isoform in the latex. Under our purification protocol, only one detectable isoform could be recovered.

Above 50°C, latex GS was rapidly and totally denatured. This threshold is apparently a characteristic of cytosolic GS, as it is much lower for plastidial GS[17,18].

The optimum pH was around 8.0 and an increase from 6.4 to 7.2, corresponding to the physiological pH range in the latex cytosol[19] resulted in increased GS activity (*Figure 2*). Analysis of the latex GS specificity for its substrates and co-factors confirmed a high specificity for ATP, L-glutamate, $(NH_4)_2SO_4$ and magnesium salts, compared to other analogs tested at equivalent concentrations. Polyphosphates or pyrophosphates (used at 5 mM) could replace ATP but with a much lower efficiency (about 10%). D-glutamate and monohydroxamate reacted poorly as compared to L-glutamate (at 20 mM). Hydroxylamine, a transferase activity substrate, attained only 60% of the efficiency of ammonium sulphate (at 5 mM). With respect to divalent cations, none of the other cations tested (Mn^{2+}, Zn^{2+}, Ca^{2+}, Cd^{2+}, Cu^{2+}, used at 10 mM) could replace Mg^{2+} at the pH used, with the exception of Co^{2+} which showed 20% of the Mg^{2+} control activity.

Biosynthetic activity proved irreversible under our experimental conditions, confirming that the constant of equilibrium (K=1.2 10^{-3}, at pH 7, 37°C) was highly propitious to glutamine formation[20].

The affinity of the purified latex GS for the different substrates in the reaction was measured (*Table 2*). The K_m for ammonium was extremely low (8 ± 2 µM) considering the physiological concentration of that cation in the latex cytosol (between 0.5 and 5 mM). No inhibition was observed at the concentrations tested (up to 20 mM). The K_m for glutamate, measured in the presence of various ATP

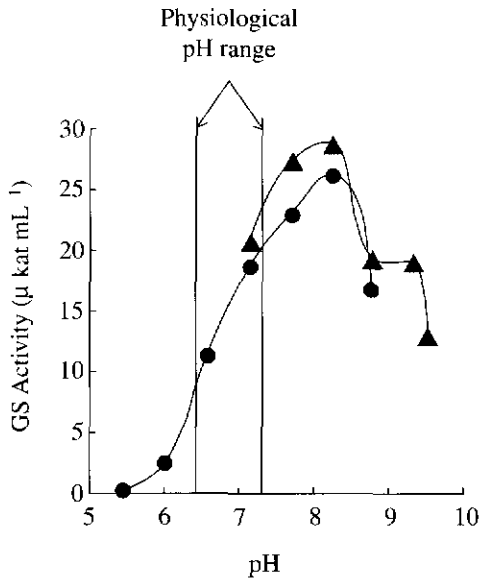


Figure 2. pH effect on the purified GS activity in a buffer: 0.1 M Tris-maleate-NaOH (●), 0.1 M Tris-HCl (▲).

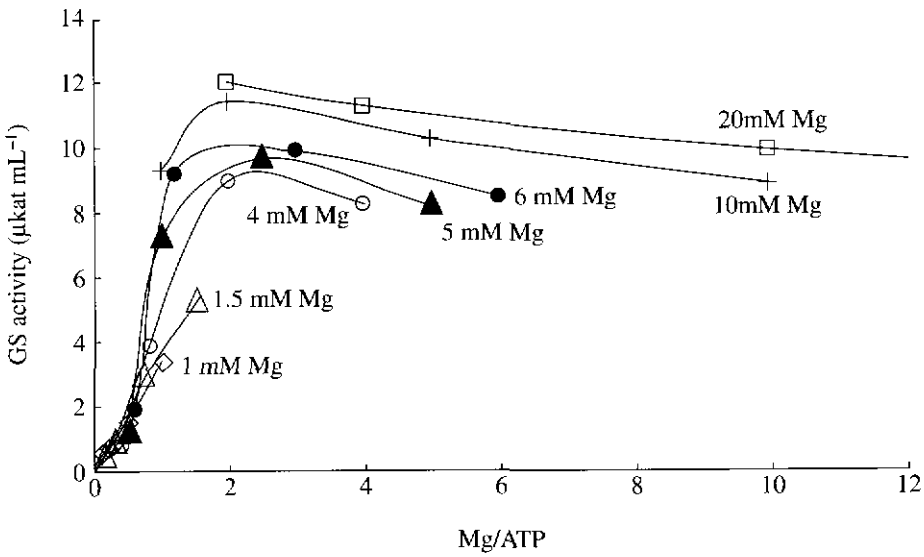
TABLE 2. COMPARATIVE ANALYSIS OF THE GS K_m FOR ITS SUBSTRATES AND OPTIMUM pH WITH THE PHYSIOLOGICAL PARAMETERS OF THE CYTOSOL

Item	Purified GS characteristics	Physiological parameters of the cytosol		GS activity inhibition threshold
		– Stimulation	+ Stimulation	
Ammonium	$K_m = 8 \pm 2$ µM	0.5 – 5 mM	No variation	> 20 mM
Glutamate	$K_m = 4 \pm 0.5$ mM	10 – 15 mM	No variation	>100 mM
ATP	$K_m = 0.6 \pm 0.05$ mM	0.12 – 0.20 mM	0.20 – 0.40 mM	
Magnesium		5 – 10 mM	No variation	[MG]/[ATP] ≤ 2
pH	Optimum pH : 8.0	6.4 to 7.2[19]	6.6 to 7.4	≥ 8

concentrations, was estimated at 4 ± 0.5 mM. It was similar to that of GS of various origins[21]. Glutamate should not be limiting for GS activity in latex cytosol considering its physiological concentration (*Table 2*). It is necessary to consider the Mg:ATP ratio when analysing GS affinity for ATP. For Mg/ATP values up to 2 (*Figure 3A*), the ATP saturation kinetics in the presence of different Mg^{2+} concentrations (1, 1.5, 4, 5, 6, 10 and 20 mM) gave similar GS activity levels. Under these conditions, the apparent K_m for ATP was 0.6 ± 0.05 mM. Inhibition occurred when the Mg:ATP ratio fell below 2, the free ATP not chelated by Mg^{2+} probably acting as inhibitor. The Mg^{2+} saturation kinetics in the presence of various ATP concentrations (1, 2, 5 and 10 mM) (*Figure 3 B*) were sigmoid in form. This was particularly evident at higher ATP concentrations. These kinetics confirmed the inhibiting effect of free ATP. When the ATP content was lower than or equal to that of Mg^{2+} the saturation kinetics were quasi Michaelian because only the ATP-Mg form was present. The same results were obtained for GS in pine[22] and Douglas fir[23], which has led to suggest that allosteric mechanisms may occur at the active site of the enzyme[22]. Our data neither confirm nor disprove this. However, the absence of Mg^{2+} in the GS solubilization buffer rapidly led to irreversible denaturation of the enzyme, suggesting that Mg^{2+} play a significant role in maintaining the structure of the active site of GS. *In situ*, given the ATP and Mg^{2+} content that were measured in the cytosol (0.17 ± 0.2 mM and 10.4 ± 1 mM, respectively), and the physiological pH range, only the complexed ATP-Mg form is present and no inhibitory effect is expected. By contrast, the physiological concentration in ATP appears limiting for GS activity considering the very low affinity of the enzyme for that co-factor.

The effect of various chemicals on latex GS functioning was studied. Among the different divalent cations studied (at 1 mM), Mn^{2+}, Zn^{2+} and Cd^{2+} proved to be powerful inhibitors, whereas Co^{2+} acted as an activator. The fact that Co^{2+} could replace Mg^{2+} and still ensure a similar level of activity to that observed when added in conjunction with Mg^{2+}, suggests that its binding site is different from that of Mg^{2+}. However, the very low concentration of the tested cations in the latex suggests that they have no physiological effect on GS activity *in vivo*. ADP and GDP, at 1 mM concentration, decreased GS activity down to respectively 33% and 23% that of the control but physiological

A



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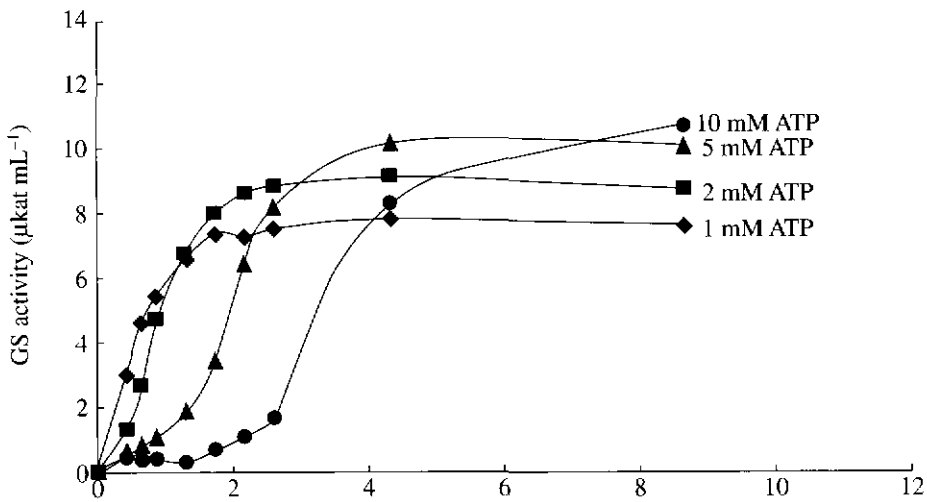

Figure 3. GS activity related to the concentration ratio ATP/Mg (A) GS activity related to the Mg concentration with different ATP contents (B).

concentrations are below 0.3 mM. Two non physiological chemicals reacting with thiol groups, p-chloromercuribenzoic acid (PCMB) and N-ethylmaleimid (NEM), decreased GS activity down to 82% and 34%, respectively when used at 1 mM. This suggested the presence of a thiol group at the active site of the enzyme. Finally, the non-physiological specific GS inhibitor methionine sulphoximine (MSO) indeed inhibited the latex enzyme by 50% at 100 μM.

Enzyme Properties in a Paraphysiological Medium

Filtering the cytosolic serum through a membrane that retained all molecules larger than 10 kDa produced a paraphysiological medium that was virtually free of proteins, hence of enzymes likely to interfere in the reactions studied. This enabled the analysis of the purified GS properties in a medium containing all the effectors found in plant with a molecular mass lower than 10 kDa. The effect of these effectors was analysed in comparison with the GS potential activity obtained in an optimised reaction buffer, at a pH identical to that of the paraphysiological medium (7.2). *Figure 4* shows that GS activity in the paraphysiological medium alone (Column B) was only 10% of that measured under optimum conditions in the buffer medium (Column A). Given the low natural ATP content of the paraphysiological medium (0.17 mM) and the low GS affinity for ATP (0.6 ± 0.05 mM), ATP

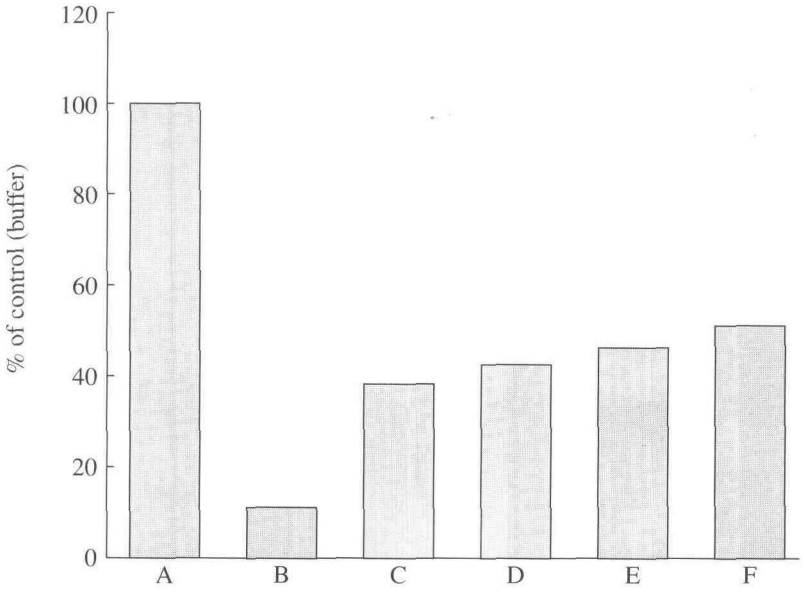

Figure 4. Comparative analysis between GS activity in an optimised buffer and in paraphysiological medium.

A: Buffer medium: 0.1 M Tris-HCl pH 7.2 + 3 mM ATP + 5 mM $MgSO_4$ + 5 mM $(NH_4)_2SO_4$ + 20 mM glutamate; B: Paraphysiological medium alone; C: B + 3 mM ATP; D: C + 5 mM $MgSO_4$; E: D + 5 mM $(NH_4)SO_4$ and F: E + 20 mM glutamate. The paraphysiological medium contained: 0.17 ± 0.02 mM ATP, 8.9 ± 0.5 mM glutamate, 10 ± 0.8 mM ammonium and 10.4 ± 1 mM Mg^{2+}.

was added up to the saturating concentration (3 mM). This resulted in a 3.6 fold increase of GS activity, equivalent to 36% that of the control (Column C). Successive and cumulated addition of Mg^{2+} (Column D), NH_4^+ (Column E) and glutamate (Column F), at saturating concentrations identical to those of the control, resulted in more modest additional increases. The final levels of activity obtained after addition of all the effectors (Column F) was 50% that of the control. These results demonstrate that ATP concentration in normal physiological conditions is indeed limiting for GS activity and that any variation in ATP concentration may have a significant impact on GS activity. The influence of the other effectors, as expected from the K_m study, is not as significant.

The reason why 50% only of the control activity was obtained is still unclear. The physicochemical influence of the buffer used as control comparatively to the paraphysiological medium should be checked. GS activity may also be specifically inhibited by unknown substances of small molecular mass present in the paraphysiological medium.

Effect of Ethylene

Our previous work demonstrated that the ethylene-induced increase in latex production is coupled with a concomitant increase in GS potential activity, as well as a small increase (18% to 40% of the control) in ammonium concentration in the latex[4]. Our present work indicates, from the K_m analysis and tests in paraphysiological medium, that variations in ammonium concentrations are not likely to have a significant effect on GS activity through biochemical regulatory mechanisms. This is probably also true for Mg^{2+} and glutamate. On the other hand, ATP concentration seems to play a major role and may be one of the intermediate factors involved in GS regulation by ethylene. This hypothesis is supported by Amalou *et al.*[24] who described an ethylene-induced increase of the entire adenylic pool, including ATP. To confirm our hypothesis, ethylene influence on the concentrations of the main substrates and co-factors of GS reaction (ATP, glutamate, ammonium, Mg^{2+}) was

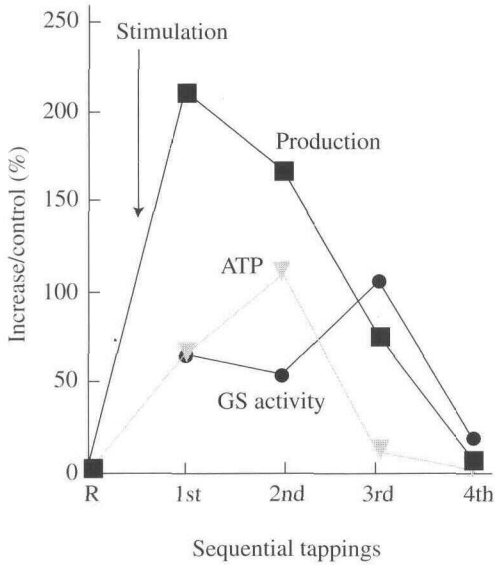

Figure 5. Variation of the latex production (■), cytosolic ATP concentration (▼) and cytosolic GS activity (●) in response to ethylene treatment. The expressed results are taking into account control trees. Calculation for each parameter (production, ATP and GS activity) was:

$$\left[\frac{(S_N - S_R)}{S_R} - \frac{(C_N - C_R)}{C_R} \right] * 100$$

S_N: Value of the parameter for the treated trees, 1st, 2nd or 3rd tapping after treatment.
S_R: Value of the parameter for the treated trees before stimulation (reference tapping).
C_N: Value of the parameter for the control trees, 1st, 2nd or 3rd tapping after treatment.
C_R: Value of the parameter for the control tree (reference tapping).

analysed on the same samples, in parallel with the effect on GS activity and latex production (*Figure 5*). We found a simultaneous, transient increase of both ATP content and GS activity at the first tapping after ethylene treatment. The concentrations of the other GS substrates were not significantly modified. The GS activity increase was also effective on the first tapping after treatment but peaked later (3rd tapping) and lasted longer. These kinetics support the key role played by ATP in the regulation of GS activity in response to ethylene.

Another parameter likely to be involved in GS regulation by ethylene is pH. Indeed, ethylene treatment is known to induce an alkalisation by up to 0.4 pH units of the cytosol[18] as a result of a general metabolic activation, and more specifically due to the activation of H^+–ATPases located on the plasmalemma[25] and tonoplast[26]. Owing to the characteristic of latex GS determined in the present study (*Figure 2*), such alkalisation in the physiological range is likely to induce a significant increase of GS activity.

CONCLUSION

This study describes the purification and biochemical characterisation of a latex cytosolic glutamine synthetase. Comparative analysis of the main characteristics of the enzyme *in vitro* with the physiological parameters of the latex, together with a test in paraphysiological medium, shed some light on possible biochemical mechanisms controlling GS activity *in situ*.

Magnesium, ammonium and glutamate contents appeared not limiting for GS functioning *in situ*, considering that their values titrated in the cytosol which were well above the enzyme K_m values for these compounds. ATP concentration, on the other hand, may vary within a range centring on GS affinity for the substrate[19], which confers to this substrate an important role in the biochemical regulation of the latex GS activity, as confirmed by our test in paraphysiological medium. Variations in pH within the physiological range of latex cytosol, are also likely to significantly modify the enzyme activity. However, there are probably other as yet unidentified low molecular mass molecules in the cytosol that regulate GS activity, either directly or *via* the biochemical characteristics of the cytosol (ionic strenght, osmotic potential, *etc.*). This was demonstrated by the fact that the purified GS, functioning in paraphysiological medium saturated with its substrates, could only achieve 50% of its potential activity.

The present study led to clearer understanding of the mechanisms ruling the ethylene-induced increase in GS activity *in situ*. Beyond the transcriptional regulation process observed previously, the present work suggests that variations in ATP[24] and pH[19] may be an intermediary phenomena involved in GS regulation by ethylene.

The existence of concomitant and synergic regulatory processes gives to GS activity substantial flexibility, enabling it to adapt to the varying conditions imposed by rubber exploitation, specially in the case of intensive exploitation systems where latex production is stimulated by ethylene.

ACKNOWLEDGEMENTS

We wish to express our gratitude to Prof d'Auzac (Université Montpellier II, France) and Dr Dingkuhn (CIRAD-AMIS-Agronomie) for their invaluable remarks on the manuscript.

Date of receipt: December 2000
Date of acceptance: February 2001

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