A MACROMOLECULAR INHIBITOR OF GLUTAMINE SYNTHETASE ACTIVITY IN TOMATO ROOT EXTRACTS

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Key Word Index—Lycopersicon esculentum; Solanaceae; enzyme regulation; glutamine synthetase inhibitor.

Abstract—In vitro glutamine synthetase (GS; EC 6.3.1.2) activity is very low in the roots of tomato plants (Lycopersicon esculentum). Extracts prepared from roots substantially reduced the GS activity in a preparation from tomato leaves. The GS inhibitory activity could be separated from GS activity by gel filtration chromatography with a parallel recovery of enzyme activity. The results obtained indicate that the GS inhibitory activity detected in tomato roots is due to a protein factor with an apparent molecular mass of 110 kD. The protein was inhibitory to both cytosolic (GS1) and chloroplastic (GS2) glutamine synthetases from green leaves of different plant species. The GS inhibitor was found to competitively inhibit GS activity with respect to ATP. The physiological relevance of this protein in the regulation of glutamine synthesis, remains to be determined.

INTRODUCTION

In higher plants, the glutamine synthetase (EC 6.3.1.2) (GS)/glutamate synthase (EC 1.4.1.13-14; EC 1.4.7.1) (GOGAT) cycle is the main pathway for ammonia assimilation [1]. Glutamine synthetase has been extensively studied because of its central role in the nitrogen metabolism in plants. Although GS activity can be detected in all plant tissues, amounts from one tissue to another are variable [2]. It is well established that glutamine synthesis in plant cells is catalysed by cytosolic (GS1) and plastidial (GS2) isoenzymes [2]. The activities of GS isoenzymes can be feedback-regulated by end products of glutamine metabolism, such as amino acids and nucleotides [2, 3]. Glutamine synthetase genes have been cloned and characterized [4]. GS1 and GS2 are encoded by different nuclear genes which are differentially expressed during plant development [5]. The photocontrol of glutamine synthetase isoforms has also been documented [6–8]. In green leaves of tomato the total GS activity corresponds to only one molecular form that has been purified [9] and localized to chloroplast stroma by immunoelectron microscopy [10]. In tomato roots, GS activity is very low when compared to the levels found in the leaves [11]. In this study, we have identified and partially characterized a macromolecular factor in tomato root extracts which acts as an inhibitor of glutamine synthetase activity.

RESULTS AND DISCUSSION

In a previous study it has been reported that the glutamine synthetase activity was very low in the roots of tomato plants growing with nitrate and ammonium as fertilizers [11]. To investigate if the reduced GS activity could be explained by the presence of an inhibitor in the tissue, we added increasing amounts of dialysed root extracts to a fixed amount of an extract prepared from the leaves. The resulting GS activity was quantified in the mixture. Both activities of GS, transferase and synthetase, are sensitive to inhibition by root extracts although transferase activity was inhibited more extensively (Fig. 1). The addition of 5 μg of root extract protein to the reaction volume decreased by about 65% the total transferase activity, whereas more than 50 μg of root extract protein was needed to reach similar levels of inhibition of the synthetase activity. Fig. 1 also shows that the inhibitory effect on both activities was abolished by boiling the root extracts for 5 min. Therefore, the GS inhibitory activity is due to a non-dialysable, heat-labile factor present in the roots of tomato.

In contrast, the addition of increasing amounts of root extract to extracts prepared from the leaves did not
appreciably affect the activities of several enzymes present in the same leaf extract: glycolate oxidase, catalase and glutamate dehydrogenase (Table 1). These data suggest that the inactivating factor is not a general inhibitor of all enzymes present in the leaf extracts as might have been expected in the case of a nonspecific proteolytic activity. However, a specific GS protease cannot be eliminated as a possible explanation for these results. Previous studies have shown that proteolytic degradation of GS in certain bacteria occurs in two successive steps [12]. In the first step, an oxidation modifies the GS protein and in the second step the oxidized enzyme, having no enzymic activity, is sensitive to the degradation by intracellular proteases. Moreover, a specific protease that cleaves oxidized GS has recently been purified and characterized [13].

In order to investigate if the loss of GS activity can be explained by specific proteolytic degradation or if it is mediated by the action of a factor that can be separated from the protein (restoring the GS activity), we performed the fractionation of the dialysed root extract by gel filtration chromatography in Sephacryl S-300 (Fig. 2). Endogenous GS eluted from the column as only one peak of enzymic activity (Fig. 2A). In contrast, the GS inhibitory activity eluted as two peaks of different molecular weights (Fig. 2B). The first peak co-eluted with the GS peak and its elution profile showed a doublet marked by an inflexion point at the fraction exhibiting the maximal GS activity. A second peak was clearly separated from the GS profile. The molecular weight (M₀) of both peaks was estimated by calibration of the column with protein markers of a known size (Fig. 3). The first peak showed an apparent molecular mass of 440 kD and the M₀ of the second peak was estimated to be approximately 110,000. Rechromatography of the second peak in the absence of GS activity showed the same molecular mass of 110 kD. These data suggest that the higher M₀ peak of inhibitory activity could either represent a polymeric form of the second smaller one, or that the lower M₀ form was initially bound to GS as a complex. The existence of macromolecular inhibitors that specifically regulate enzyme activity is well documented, particularly in the case of proteases [14, 15]. Moreover, a non-proteolytic enzyme which is regulated by complexing with an inhibitor (antizyme) is ornithine decarboxylase (ODC) [16]; the active enzyme can be recovered by dissociation of the ODC antizyme complex in the presence of high salt concentrations [17, 18]. In order to test for a possible interaction between GS and the inhibiting factor, the

Table 1. Activity of several enzymes extracted from tomato leaves in mixtures with root extracts

<table>
<thead>
<tr>
<th>Root extract in assay (μg protein)</th>
<th>Glutamine synthetase Enzyme activities (nkat mg⁻¹ protein)</th>
<th>Catalase X 10</th>
<th>Glycolate oxidase</th>
<th>Glutamate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>18.53 (100)</td>
<td>0.21 (100)</td>
<td>0.46 (100)</td>
<td>0.32 (100)</td>
</tr>
<tr>
<td>17</td>
<td>11.67 (63)</td>
<td>0.19 (90)</td>
<td>0.45 (97)</td>
<td>0.29 (90)</td>
</tr>
<tr>
<td>42</td>
<td>7.41 (40)</td>
<td>0.21 (100)</td>
<td>0.45 (97)</td>
<td>0.32 (100)</td>
</tr>
</tbody>
</table>
*Synthetase activity.
The values in parentheses represent the percentage of the observed maximum activity.
fractions corresponding to the first peak of GS inhibitory activity (that also contain GS activity) were pooled and KCl added to active fractions to a final concentration of 0.2 M. The extract was then applied to a Sephacryl S-300 column equilibrated with standard buffer plus 0.2 M KCl. Only one peak of GS inhibitory activity eluted from the column at an apparent molecular mass of 110 kD (results not shown). These findings suggest that, as occurs with the ODC antizyme, GS inhibitor detected in tomato roots may interact with GS forming a complex susceptible to dissociation by high salt concentrations. The existence of the GS inhibitor complex could also explain the appearance of a doublet in the first peak of the GS inhibitory activity elution profile (Fig. 2). An additional possibility is that the 110 kD fraction may form aggregates of higher $M_r$, which dissociate at 0.2 M KCl. Nevertheless, the separation of GS inhibitor from GS by gel filtration led to a simultaneous recovery of GS activity (Table 2). The enzyme activity obtained after the second Sephacryl S-300 chromatography is similar to the levels normally found in leaves.

The above results indicate that the GS inhibitor detected in tomato roots is non-dialysable and heat labile (Fig. 1). In addition, Fig. 4 shows that the GS inhibitory activity is susceptible to proteolytic degradation. When partially purified root extracts are incubated at 37°C in the presence of trypsin, a gradual loss of inhibitory activity was observed when compared to a control incubated in the same conditions but in the absence of trypsin. Therefore, we conclude that the GS inhibitory activity is due to a factor that is protein in nature.

To study the inactivating capacity of the GS inhibitor on the enzymic activity of different glutamine synthetases, we selected the isoenzymes from the leaves of several plant species, including tomato. The chloroplastic isoform (GS2) from spinach [19] and the isoenzymes GS1 and GS2 from maize [20] and barley [21] were partially purified by ion exchange chromatography on DEAE-Sephadex A-25. Mixing extracts of GS isoforms and partially purified GS inhibitor decreased the synthetase activities of both cytosolic (GS1) and chloroplastic (GS2) isoenzymes (Table 3). The observed inhibitory effect was stronger when the transferase activity was measured (data not shown). An inhibition of about 50% was recorded in the case of barley GS1 using the transferase assay.

In order to further determine the specificity of the inhibitory activity towards glutamine synthetase, we firstly tested the possibility that there may be an interaction of some component present in extracts from tomato roots with hydroxamates affecting both GS assays, transferase and synthetase. However, the addition of GS inhibitory activity to authentic $\gamma$-glutamyl hydroxamate did not modify the absorbance at 540 nm observed in the absence of the GS inhibitor. We secondly tested the possibility that the GS inhibitory activity may be due to a protein that competes with the GS enzyme for one of the substrates in the incubation mixture. To check this possibility, GS synthetase activity was assayed at variable amounts of GS inhibitor in the presence of several concentrations of ATP. A Dixon plot showed that the inactivating factor appears to act as a competitive inhibitor with respect to ATP (Fig. 5). According to these results, the GS inhibitor activity was due to a factor that is protein in nature.

Table 2. Recovery of GS activity in tomato root extracts by Sephacryl S-300 gel filtration chromatography

<table>
<thead>
<tr>
<th>Step</th>
<th>Total GS* (nkat)</th>
<th>Recovery (%)</th>
<th>Specific activity (nkat mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>21.3</td>
<td>100</td>
<td>7.1</td>
</tr>
<tr>
<td>Sephacryl S-300</td>
<td>85.6</td>
<td>403</td>
<td>214.0</td>
</tr>
<tr>
<td>Sephacryl S-300 + 0.2 M KCl</td>
<td>251.5</td>
<td>1181</td>
<td>838.3</td>
</tr>
</tbody>
</table>

*Transferase activity.
Table 3. Effect of partially purified root extract on the activities of glutamine synthetase isoenzymes from the leaves of different plant species

<table>
<thead>
<tr>
<th>Plants</th>
<th>Root extract in assay (µg protein)</th>
<th>GS activity* (nkat mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GS1</td>
</tr>
<tr>
<td>L. esculentum</td>
<td>0</td>
<td>38.0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>31.5</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>22.8</td>
</tr>
<tr>
<td>S. oleracea</td>
<td>0</td>
<td>14.2</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>13.2</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>10.7</td>
</tr>
<tr>
<td>H. vulgare</td>
<td>0</td>
<td>26.1</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>25.6</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>23.8</td>
</tr>
<tr>
<td>Z. mays</td>
<td>0</td>
<td>16.6</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>7.1</td>
</tr>
</tbody>
</table>

*Synthetase activity.
Values in parentheses represent the percentage of the observed maximum activity.

In summary, this work reports the occurrence of a GS inhibitory activity in tomato roots. The inhibitor appears to be a protein with an apparent molecular mass of 110 kD. The mechanism of the inhibitory effect observed may be a competition of both proteins, GS and inhibitor, for the substrates, although our results also suggest that a glutamine synthetase—GS inhibitor complex may be involved. More investigation is needed to clarify if this factor has any physiological role in the regulation of glutamine synthesis in vitro, or if it is released during tissue extraction and exerts its inhibitory action only in vitro. Whatever may be the biological function of this protein, we recommend extreme caution when the GS activity is evaluated in roots by the synthetase and transferase assays because it may be underestimated. Further work will be needed to obtain homogenous preparations of this protein as a critical step in the study of its molecular characteristics and localization within the cell, and its possible occurrence in other plant species.

EXPERIMENTAL

Plant material. Tomato (Lycopersicon esculentum L. var. Hellfrucht Frühstamm) was grown on the laboratory bench under natural light from June to November. Plants were watered regularly with a nutrient solution containing: 5 mM KNO₃, 0.8 mM MgSO₄, 1.3 mM (NH₄)₂HPO₄ and 2.5 mM NH₄NO₃. Roots and leaves from 40–50-day-old plants were used for extract preparations. Barley (Hordeum vulgare) and maize (Zea mays) plants were cultivated in the same conditions above indicated for tomato plants. Spinach (Spinacia oleracea) was purchased in a local market.

Extract preparation. Leaves were cut into small pieces and homogenized in a Sorvall Omni-mixer at maximum speed for 8 periods of 30 sec each in 50 mM Tris–HCl (pH 8.0), 2 mM EDTA, 10 mM 2-mercaptoethanol and 10% glycerol. The ratio of extraction buffer/tissue was 2:1 (v:w). Roots were ground in a mortar with a pestle using washed sea sand in the same buffer, as described above. We used a ratio of extraction buffer/sand/root tissue of 2:0.5:1 (v:w:w). The extracts were filtered through 2 layers of muslin and the filtrate was centrifuged at 22 000 g for 20 min. Ammonium sulphate, as a fine powder, was added to the supernatants to reach a final concc of 85% (w/v). The pellets, containing the majority of total proteins, were resuspended in standard buffer: 50 mM Tris–HCl (pH 8.0), 2 mM EDTA, 10 mM 2-mercaptoethanol and 10 mM MgSO₄. The resulting extracts were extensively dialysed against standard buffer. Dialysed extracts were used in all studies. All the steps were carried out at 4°C.

Enzyme assays. Glutamine synthetase (EC 6.3.1.2) activity was measured by the synthetase and transferase assays. For GS synthetase determinations in addition to the extract sample, the final reaction mixture contained in a volume of 750 µl: 88 mM Tris–HCl (pH 8.0), 250 mM sodium glutamate, 60 mM hydroxylamine (neutralized pH 7.0), 22 mM MgSO₄ and 6.25 mM ATP. After 15 min of incubation at 37°C the reaction was stopped by adding 250 µl of a mixture containing, 10% FeCl₃, 6H₂O in 0.2 N HCl. 24% trichloroacetic acid and 50% HCl in a ratio 1:1:1. The absorbance at 540 nm was measured and the γ-glutamyl hydroxamate produced was quantified using commercial γ-glutamyl hydroxamate as standard. The transferase assay described by Shapiro and Stadiman [23] was adapted using a final reaction volume of 750 µl.

The GS inhibitory activity was determined as the ability to depress the GS activity present in the samples. Mixed extracts containing both GS activity and GS inhibitory activity were
incubated at room temp. for 15 min. They were then transferred to an ice bath until GS activity remaining after treatment was measured by the synthetase or transferase assays described above.

Glyceraldehyde 3-phosphate dehydrogenase (EC 1.1.1.12) activity was measured following the method of Wieland [27].

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.1.1.12) activity was measured following the method of Wieland [27].

Glutamate dehydrogenase (EC 1.4.1.2) activity was measured following the method of Yamaya et al. [26].

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REFERENCES