Determination of Glutathione and Glutathione Disulphide in Lichens: a Comparison of Frequently Used Methods

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The determination of glutathione (GSH) and glutathione disulphide (GSSG) is subject to substantial problems when analysing plants containing high amounts of phenolic compounds, since the phenolics may react with the GSH present during the extraction. Three frequently used methods for the analysis of GSH and GSSG in the lichen Pseudevernia furfuracea have been compared. Glutathione seemed to be underestimated by the enzymatic recycling assay. Derivatization by high-performance liquid chromatography after derivatization of glutathione with 2,4-dinitro-1-fluorobenzene (DNFB) or labelling with monobromobimane (mBBr), respectively, gave similar results. Derivatization with DNFB gave the lowest values for GSSG expressed as a percentage of total glutathione. Cyst(e)ine and γ-glutamyl-cyst(e)ine were only detected with the mBBr assay.

In order to improve the yield of GSH, polyvinylpolypyrrolidone (PVP), which should serve to bind phenolics, was added during the extraction. The use of PVP improved the yield of glutathione dramatically in lichens harvested in June but not in October. However, addition of PVP led to oxidation of a portion of the GSH. This was demonstrated for each method and seemed to be independent of the thiol blocking agents used for the determination of GSSG, namely 2-vinylpyridine, iodoacetic acid or N-ethylmaleimide. For extensive studies on glutathione metabolism in Pseudevernia furfuracea we recommend that the use of PVP be avoided, by investigating thalli collected in autumn, that probably contain less phenolics than those harvested in summer.

Keywords: 2,4-dinitro-1-fluorobenzene (Sanger's reagent); glutathione; glutathione disulphide; lichens; monobromobimane; phenolics; 2-vinylpyridine.

INTRODUCTION

Glutathione (γ-glutamyl-cysteinyl-glycine) is the main low molecular weight thiol in most living cells. This tripeptide plays a critical role in detoxification and cellular defence against oxidative damage. Moreover, it influences the synthesis of DNA and proteins (Meister and Anderson, 1983). In plants, glutathione is the transport form of reduced sulphur (Rennenberg, 1982) and a role for this tripeptide in detoxifying heavy metals and xenobiotics has been postulated (Rennenberg and Lamoureux, 1990; Smith et al., 1990).

Although in most tissues glutathione is maintained in the reduced form (GSH), the relative amount of oxidized glutathione (GSSG) may rise during stress conditions (Guy et al., 1984). Furthermore, an increase in GSSG content has been reported during dormancy of seeds (Fahey et al., 1980; Kranner and Grill, 1993) and spores of fungi (Fahey et al., 1975), and in resurrection plants under drought (Dhindsa, 1987, 1991). Since redox reactions of glutathione may play an important role during desiccation and rehydration of poikilohydric plants (Dhindsa, 1987, 1991; Kranner and Grill, 1994), our investigations on desiccation tolerance of lichens (Kranner and Grill, 1994) call for a sensitive method for analysing both forms of glutathione. However, sample preparation is a critical point in glutathione analysis (Roberts and Francetic, 1992). It must be considered carefully that side reactions or oxidation of GSH may occur during the extraction. Moreover, plant tissues often contain high amounts of phenolic compounds that may react with GSH. At the present time, there are more than 600 known secondary products in lichens, the largest group being phenolics (Elix et al., 1984; Fahselt, 1994). It is therefore of interest to study the use of reagents that serve to bind phenolics during the GSH-assay.

In this paper, we compared three assays frequently used for the determination of glutathione. First, we used the labelling procedure with monobromobimane (mBBr) (Newton et al., 1981; Fahey and Newton, 1987; Schupp and Rennenberg, 1988; Svardal et al., 1990) for analysing total glutathione (GSH+GSSG). This was performed in combination with the method described by Fenton and Fahey (1986) for the determination of GSSG. In this case, N-ethylmaleimide (NEM) was used for the blocking of thiols. Disulphides were then reduced with dithiothreitol (DTT) and subsequently labelled with mBBr. To the best of our knowledge this method has not been applied for determining GSSG in plant tissues.

The second method was based on the enzymatic recycling assay (Owens and Belcher, 1965; Tietze, 1969; Griffith, 1980; Anderson, 1985) in which GSH is oxidized by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) with stoichiometric formation of 5-thio-2-nitrobenzoic acid (TNB), and GSSG is reduced by glutathione reductase (GR, EC 1.6.4.2) and NADPH. The rate of TNB formation monitored at 412 nm is proportional to the sum of GSH and GSSG present. GSSG is determined after blocking of thiols with 2-vinylpyridine.

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Finally, we used an assay, first described by Reed et al. (1980), which was evaluated in animal tissues (Fariss and Reed, 1987) and later adapted for use in woody plant tissues (Siller-Cepeda et al., 1991). This procedure uses S-carboxymethylation of thiols followed by the derivatization of amino groups with 2,4-dinitro-1-fluorobenzene (DNFB; Sanger's reagent). We compared the three assays for the determination of GSH and GSSG in dry lichens and discuss the use of polyvinylpyrrolidone (PVP) which serves to bind phenolics during the extraction.

EXPERIMENTAL

All reagents were of the highest quality available from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO, USA), with the exception of γ-glutamyl-cysteine, which was obtained from Nakalai-Chemicals (Kyoto, Japan), and mBBr, which was purchased from Calbiochem (La Jolla, CA, USA). All buffers were prepared in double-distilled water and stored at 4°C, whilst enzyme and co-factor solutions were prepared immediately before use. For spectrophotometric procedures a Shimadzu UV-visible recording spectrophotometer UV-2100 was used. High-performance liquid chromatographic (HPLC) analyses were conducted using an HPLC system consisting of two Knauer model 64 pumps and a graphic (HPLC) analyses were conducted using an HPLC UV-2100 was used. High-performance liquid chromatography (HPLC) was tested in procedures 1, 2 and 4.

HPLC analysis of low molecular weight thiols and disulphides as mBBr derivatives (method 1). Analysis of total glutathione was completed as previously described by Kranner and Grill (1993) with the exception that thalli were extracted using four different procedures. Procedure 1 required the extraction of 50 mg of the lyophilized material with 4 mL 0.1 M HCl containing 1 mm EDTA. Procedure 2 involved extraction with 4 mL 0.1 M HCl containing 1 mm EDTA and 0.5% Triton X-100 to test whether the use of a detergent would improve the yield of glutathione. Procedure 3 involved extraction with 4 mL 0.1 M HCl containing 1 mm EDTA and 1.25% PVP: the latter should serve to bind phenolics that may react with GSH during the extraction. Procedure 4 required extraction with 4 mL 0.1 M HCl containing 1 mm EDTA, 0.5% Triton X-100, and 1.25% PVP. Extracting P. furfuracea samples in 0.1 M HCl did not affect the pH value of the extract. All procedures were tested with GSH standard solutions: none interfered with the assay.

For determination of total glutathione, a 400 μL aliquot of the extracts was mixed with 600 μL 200 mm 2-(N-cyclohexylamino)ethane-sulfonic acid (CHES) buffer (pH 9.3) and 100 μL DTT. The subsequent procedures for labelling of thiols and for HPLC analysis were completed as previously described (Kranner and Grill, 1993). For determination of GSSG, a 400 μL aliquot was treated with 30 μL 50 mm NEM to block free thiols and 600 μL 200 mm CHES (pH 9.3) immediately after extraction. The reaction was carried out at room temperature for 15 min. Afterwards, excess NEM was removed by extracting five times with equal volumes of toluene (rather than benzene as used by Fenton and Fahey (1986)). Thereafter, 30 μL 3 mm DTT were added to a 300 μL aliquot of the NEM-treated extract to reduce disulphides. These aliquots were labelled with mBBr and analysed as previously described (Kranner and Grill, 1993).

Enzymatic recycling assay (method 2). The cyclic DTNB–GR assay was used to determine total glutathione (GSH + GSSG) in terms of GSH equivalents and, after the derivatization of free thiols with 2-vinylpyridine, oxidized glutathione as previously described (Kranner and Grill, 1993). Fifty mg of the lyophilized material was extracted with 4 mL 0.1 M HCl containing 1 mm EDTA. Again, the use of Triton X-100 and PVP was tested in procedures 1 to 4 as described for method 1.

HPLC analysis of low molecular weight thiols and disulphides as DNP-derivatives (method 3). The method of Fariss and Reed (1987) was used as described for woody plant tissues by Siller-Cepeda et al. (1991). This procedure uses iodoacetic acid to form S-carboxymethyl derivatives of free thiols, followed by the conversion of the free amino groups to DNP-derivatives. Thereafter, sulphur-containing amino acids and peptides can be separated by reversed-phase ion exchange HPLC and measured by using UV detection at 365 nm. Special attention must be paid to the presence of acetic acid in the mobile phase in order to maintain the bonded-phase groups of the 3-aminopropyl column (Spherisorb S5 NH2) in the protonated form (Fariss and Reed, 1987). Fifty mg of lyophilized lichens were extracted in 4 mL 10% perchloric acid (PCA) containing 1 mm bathophenanthroinedisulphonic acid (BPDS), as recommended by Fariss and Reed (1987). Again, the use of Triton X-100 and PVP was tested in procedures 1 to 4 as described for method 1.

RESULTS AND DISCUSSION

Determination of low molecular weight thiols and disulphides as mBBr derivatives

Since lichens show seasonal variations in glutathione content (Kranner and Grill, 1992), we compared the four extraction methods at the time of maximum content in June and of minimum content in October. P. furfuracea collected in June had about twice as much glutathione as in October and this is consistent with earlier results (Kranner and Grill, 1992). However, the glutathione contents described in the present study are
Table 1. Effect of sample preparation on glutathione analysis of Pseudevernia furfuracea samples harvested in October (A) and June (B). Glutathione and glutathione disulphide were determined by the mBBr method

<table>
<thead>
<tr>
<th>Procedure Employeda</th>
<th>Total Glutathione[^a,c] (nmol/g dry weight fSD)</th>
<th>GSSG[^b,c] (calculated[^a]) (nmol/g dry weight fSD)</th>
<th>GSSG (expressed as % of total[^f])</th>
</tr>
</thead>
<tbody>
<tr>
<td>October harvest (A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1029±153</td>
<td>363±71</td>
<td>666±128</td>
</tr>
<tr>
<td>2</td>
<td>1008±94</td>
<td>406±89</td>
<td>602±74</td>
</tr>
<tr>
<td>3</td>
<td>938±173</td>
<td>486±104[^a]</td>
<td>452±99[^a]</td>
</tr>
<tr>
<td>4</td>
<td>963±203</td>
<td>483±59[^a]</td>
<td>480±153[^a]</td>
</tr>
<tr>
<td>June harvest (B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1670±351</td>
<td>718±157</td>
<td>952±209[^a]</td>
</tr>
<tr>
<td>2</td>
<td>1829±151</td>
<td>750±195</td>
<td>1079±173</td>
</tr>
<tr>
<td>3</td>
<td>2182±567[^a]</td>
<td>1309±301[^a]</td>
<td>873±218</td>
</tr>
<tr>
<td>4</td>
<td>2315±468[^a]</td>
<td>1343±241[^a]</td>
<td>972±340[^a]</td>
</tr>
</tbody>
</table>

^a Procedure 1: homogenization in HCl containing EDTA; procedure 2: homogenization in HCl containing EDTA and Triton X-100; procedure 3: homogenization in HCl containing EDTA and PVP; procedure 4: homogenization in HCl containing EDTA, PVP and Triton X-100. (For details see Experimental section.)

^b Content expressed in nmol/g dry weight ±SD (n=6).

^c Values indicated with the same, or no, letters do not differ significantly one from another: where significant differences are indicated, a lower case letter (x, y) shows differences at P<0.05, an upper case letter (X) shows a differences at P<0.001.

Determination of glutathione and glutathione disulphide by the cyclic DTNB-GR assay

Extraction of glutathione from P. furfuracea seemed to be more problematic in thalli collected in June than for those collected in October. We therefore conducted further assays for thalli collected in June with the same procedures as described for the mBBr method to test whether these problems are due to extraction or to the analytical method. Results of the comparative study for sample preparation are shown in Table 2. All values were significantly lower (P<0.05) compared with the mBBr assay. This is in contrast to the experiments of

Table 2. Effect of sample preparation on glutathione analysis of Pseudevernia furfuracea samples harvested in June. Glutathione and glutathione disulphide were determined by the enzymatic recycling assay

<table>
<thead>
<tr>
<th>Procedure Employeda</th>
<th>Total Glutathione[^a,c] (nmol/g dry weight fSD)</th>
<th>GSSG[^b,c] (calculated[^a]) (nmol/g dry weight fSD)</th>
<th>GSSG (expressed as % of total[^f])</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1219±146</td>
<td>490±74</td>
<td>728±109</td>
</tr>
<tr>
<td>2</td>
<td>1390±243</td>
<td>515±93</td>
<td>835±134</td>
</tr>
<tr>
<td>3</td>
<td>1858±472[^a]</td>
<td>598±119[^a]</td>
<td>1259±227[^a]</td>
</tr>
<tr>
<td>4</td>
<td>1920±400[^a]</td>
<td>1081±227[^a]</td>
<td>839±184</td>
</tr>
</tbody>
</table>

^a Procedure 1: homogenization in HCl containing EDTA; procedure 2: homogenization in HCl containing EDTA and Triton X-100; procedure 3: homogenization in HCl containing EDTA and PVP; procedure 4: homogenization in HCl containing EDTA, PVP and Triton X-100. (For details see Experimental section.)

^b Content expressed in nmol/g dry weight ±SD (n=6).

^c Values indicated with the same, or no, letters do not differ significantly one from another: where significant differences are indicated, a lower case letter (x, y) shows differences at P<0.05, an upper case letter (X) shows differences at P<0.001.
**Table 3. Effect of sample preparation on glutathione analysis of *Pseudoterranova furfuracea* samples harvested in June. Glutathione and glutathione disulphide were determined after derivatization with Sanger's reagent.**

<table>
<thead>
<tr>
<th>Procedure Employed</th>
<th>Total Glutathione</th>
<th>GSSG</th>
<th>GSH (calculated)</th>
<th>GSSG (expressed as % of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1489±268</td>
<td>551±72</td>
<td>938±114</td>
<td>37±5</td>
</tr>
<tr>
<td>2</td>
<td>1717±227</td>
<td>721±107</td>
<td>996±115</td>
<td>42±7</td>
</tr>
<tr>
<td>3</td>
<td>1923±423*</td>
<td>962±183*</td>
<td>961±183</td>
<td>50±9</td>
</tr>
<tr>
<td>4</td>
<td>2203±381*X</td>
<td>1057±202*X</td>
<td>1146±229*</td>
<td>48±8</td>
</tr>
</tbody>
</table>

* Procedure 1: homogenization in PCA containing BPDS; procedure 2: homogenization in PCA containing BPDS and Triton X-100; procedure 3: homogenization in PCA containing BPDS and PVP; procedure 4: homogenization in PCA containing BPDS, PVP and Triton X-100. (For details see Experimental section.)

**Content expressed in nmol/g dry weight ±SD (n = 6).**

**Values indicated with the same, or no, letters do not differ significantly one from another: where significant differences are indicated, a lower case letter (x, y, z) shows differences at P<0.05, an upper case letter (X) shows differences at P<0.001.**

Loh *et al.* (1990) who showed significant discrepancies between glutathione concentrations determined by the enzymatic assay compared to those measured by the mBBr assay. In this case, the presence of acid-soluble sulphhydryl proteins in the extracts of murine tumours led to an overestimation of glutathione content in the enzymatic assay. Lichen extracts, homogenized with procedure 1, were bright yellow, those homogenized with procedures 2 to 4 were dark yellow in colour, and this may cause interference with the spectrophotometric assay. Some co-extracted, unidentified lichen dyes showed a distinct absorption maximum at 410 nm, which may decrease the instrument sensitivity for measurements at 412 nm. Nevertheless, addition of Triton X-100 and/or PVP showed a similar tendency in improving the assay of both GSH and GSSG compared with the labelling procedure with mBBr. Addition of PVP improved the yield of total glutathione by 52%, and addition of both BPDS and Triton X-100 by 58%. The recoveries for GSH were 77±8, 79±5, 75±5, and 69±7% from procedures 1 to 4 (n = 6 for all procedures). However, the percentage of GSSG significantly increased only when thalli were extracted using procedure 4.

**Determination of low molecular weight thiols as DNP derivatives**

Results of the comparative study for sample preparation are shown in Table 3. The yield of total glutathione was improved from procedures 1 to 4 and values were similar to those from the mBBr assay for lichens harvested in June. Again, addition of Triton X-100 seemed to improve the yield of total glutathione slightly but not significantly. GSH was recovered in 95±5% (procedure 1) and 91±7% (procedure 2) yields (n = 6 for both procedures). For procedures 3 and 4, 85±8% and 82±7% of the GSH was recovered, respectively. Addition of PVP caused GSSG, expressed as a percentage of total glutathione, to increase, but values were significantly lower (P<0.05) than those obtained from the mBBr assay for lichens homogenized with procedures 1, 3 and 4. From the point of view the extraction of glutathione with PCA and BPDS, in combination with blockage of thiols with iodoacetic acid and subsequent derivatization of the amino groups with Sanger's reagent, may be the best alternative for the determination of GSH and GSSG in lichens containing high amounts of phenolics. However, in contrast to the mBBr assay, we could not determine cyst(e)ine and γ-glutamyl-cyst(e)ine with this method: this could be explained by the more sensitive detector that is used in the mBBr assay. By determining low molecular weight thiols as DNP derivatives, no peaks were found after homogenization of lichens with procedures 1 and 2. After homogenization using procedures 3 and 4, cyst(e)ine and γ-glutamyl-cyst(e)ine appeared in trace amounts only: accurate determination was not possible because the peaks were only twice as high as the baseline. A second disadvantage of this method is the use of high acetic acid concentrations in the mobile phase which leads to destruction of the pump seals.

**CONCLUSIONS**

The glutathione content of lichens is dependent on several environmental factors. It was demonstrated that the total glutathione content of *P. furfuracea* shows seasonal variations, is dependent on elevation (Kraner and Grill, 1992) and probably the age of thalli. Moreover, thalli seem to contain large amounts of natural products that may react with GSH during the extraction. These compounds may also show seasonal variations. For this reason, these must be measured for each sampling time, in order to determine whether the harvested material should be treated with PVP during the extraction. In the case of *P. furfuracea* harvested in October, the use of PVP did not improve the yield of total glutathione, but probably interfered with the assay by partly oxidizing the GSH present. Because of this the use of PVP is disadvantageous. On the other hand, the use of PVP made detection of cyst(e)ine and γ-glutamyl-cyst(e)ine in the mBBr assay possible. *P. furfuracea* harvested in June probably contained more phenolics than that collected in October. Addition of PVP considerably improved the yield of total glutathione in these samples which is certainly an advantage. However, parts of GSH are oxidized in the presence of PVP during the extraction. When analysing the contents of low molecular weight thiols of lichens collected in summer, we recommend the compromise of using PVP. For extensive investigations on the metabolism of low molecular weight thiols in *P. furfuracea* we suggest that thalli be collected in autumn, when PVP is not needed. In any case, we prefer the mBBr assay for determining the redox couple of glutathione in lichens, thus allowing the detection of the whole spectrum of low molecular weight thiols.

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