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Ophthalmic acid is a marker of oxidative stress in plants as in animals

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ABSTRACT

Background: Ophthalmic acid (OPH), γ-glutamyl-L-2-aminobutyryl-glycine, a tripeptide analogue of glutathione (GSH), has recently captured considerable attention as a biomarker of oxidative stress in animals. The OPH and GSH biosynthesis, as well as some biochemical behaviors, are very similar. Here, we sought to investigate the presence of OPH in plants and its possible relationship with GSH, known to possess multiple functions in the plant development, growth and response to environmental changes.
Methods: HPLC-ESI-MS/MS analysis was used to examine the occurrence of OPH in leaves from various plant species, and flours from several plant seeds. Different types of oxidative stress, i.e., water, dark, paraquat, and cadmium stress, were induced in rye, barley, oat, and winter wheat leaves to evaluate the effects on the levels of OPH and its metabolic precursors.
Results: OPH and its dipeptide precursor, γ-glutamyl-2-aminobutyric acid, were found to occur in phylogenetically distant plants. Interestingly, the levels of OPH were tightly associated with the oxidative stress tested. Levels of OPH precursors, γ-glutamyl-2-aminobutyric acid and 2-aminobutyric acid, the latter efficiently formed in plants via biosynthetic pathways absent in the animal kingdom, were also found to increase during oxidative stress.
Conclusions: OPH occurs in plants and its levels are tightly associated with oxidative stress.
General significance: OPH behaves as an oxidative stress marker and its biogenesis might occur through a biochemical pathway common to many living organisms.

1. Introduction

Glutathione (GSH, γ-glutamyl-cysteinyl-glycine) is an essential metabolite, present in most organisms, where it plays a key role in protecting the cell against oxidative stress thanks to its redox potential. The protective function of GSH in cells occurs essentially through oxidation/reduction or conjugation routes. In the oxidation/reduction route, glutathione peroxidase reduces hydroperoxides by forming the oxidized glutathione form (GSSG), which is then reduced by NADPH in a reaction catalyzed by glutathione reductase. In the conjugation route, glutathione transferase catalyzes the conjugation to reactive molecules. In animals, GSH is involved in modulation of cell proliferation, immune response and cell signaling [1]. In plants, GSH also possesses multiple functions contributing to plant development, growth and response to environmental changes [2]. Overall, GSH acts as an important defense against specific reactive oxygen species (ROS) and preservation from oxidative damage induced under conditions of abiotic stresses such as drought, salinity, cold, and heavy metals. The GSH involvement in regulation of sulfur assimilation and the synthesis of phytochelatins, polymeric compounds able to sequester toxic heavy metal ions by chelation, is peculiar to plants [3].

Ophthalmic acid (OPH) or γ-glutamyl-L-2-aminobutyryl-glycine is a tripeptide analogue of GSH, in which cysteine is substituted by 2-aminobutyric acid (2-ABA). OPH and the tripeptide γ-glutamyl-alanyl-glycine, also called norophthalmic acid (NOP), firstly isolated from calf eye lens, have been identified in organs of several animals [3,4], including humans [5]. Except for the noticeable difference represented by the absence of the cysteine residue that deprives OPH of reducing

Abbreviations: 2-ABA, 2-aminobutyric acid; 2-KBA, 2-ketobutyric acid; GCS, γ-glutamylcysteine synthetase; GS, glutathione synthetase; GSH, reduced glutathione; NOP, norophthalmic acid; OPH, ophthalmic acid; ROS, reactive oxygen species; γ-Glu-Cys, γ-glutamyl-cysteine; γ-glutamyl-2-ABA, γ-glutamyl-2-aminobutyric acid; γ-GT, γ-glutamyltranspeptidase

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properties, the biosynthesis and some of the biochemical behaviors are very similar to those of GSH [6]. Similar to GSH, OPH can be donor of the glutamate moiety to other amino acids in the reaction catalyzed by γ-glutamyltranspeptidase (γ-GT) [7]. Important links correlate GSH and OPH. Both peptides are formed under the reactions catalyzed by γ-glutamylcysteine synthetase (GCS), which promotes linkage of glutamic acid to cysteine (or to 2-aminobutyric acid), forming the corresponding dipeptides, and glutathione synthetase (GS) that, subsequently, links the dipeptides to glycine to form GSH or OPH [6]. The substrates of the two reactions are however different: GSH is synthesized from glutamate and cysteine, OPH from glutamate and 2-ABA. Interestingly, increased OPH levels have been proposed as liver and heart markers of GSH depletion elicited by oxidative stress [8–10]. Decrease in the intracellular GSH levels and increase of the OPH levels were also recently evidenced in endothelial cells exposed to high-glucose [11]. Among its physiological activities, OPH inhibits insulin degradation in rat adipose tissues [12], GCS [13] and glyoxalase I [14]. A study on oxidative-stress detoxification in cyanobacterium Synechocystis showed that OPH is accumulated in the bacterial cells stressed by glucose [15]. OPH competitively inhibits the GSH uptake across the rat canalicular liver plasma membranes, thus indicating the possibility that it is a substrate of the GSH carrier. In this way OPH, by using the same carrier as GSH, decreases the cellular GSH efflux protecting cell integrity [16].

Prompted by the results from our previous in vitro studies showing inverse intracellular changes of OPH and GSH levels during oxidative damage [11], here we sought to investigate the possible presence and role of OPH in plants where, to date, only the multiple functions of GSH, some of which are similar to those carried out in animal cells, have been described.

2. Materials and methods

2.1. Reagents

Reduced and oxidized glutathione, glutamine, 2-aminobutyric acid, γ-GT from equine kidney, Raney-Ni 2800 (slurry in H_2O), paraquat dichloride (PQT), Amicon Ultra 0.5 mL centrifugal filters with 3 kDa molecular weight cut off, and 0.1% solution of formic acid in water used for the LC-ESI-MS analyses were from Sigma-Aldrich (Milan, Italy). Ophthalmic acid (OPH) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other solvents and reagents used were of analytical grade. Purified water was obtained with a Milli-Q system from Millipore (Milan, Italy).

2.2. Vegetable sources

Flours of chickpeas (*Cicer arietinum*), buckwheat (*Fagopyrum esculentum*), quinoa (*Chenopodium quinoa*), barley (*Hordeum vulgare*), durum wheat (*Triticum durum*), winter wheat (*Triticum aestivum*), *Triticum dicoccum*, rye (*Secale cereale*), oat (*Avena sativa*) were purchased in local markets. Leaves of different tree species were supplied by the city botanical gardens. Rye, barley, oat and durum wheat, whose leaves were examined before and after oxidative stress induction, were grown in the laboratory as described below.

2.3. Plant growth conditions

Gramineae plant seeds of various species were washed in sterile water, drained and immersed for 3 min in 20% (v/v) sodium hypochlorite solution. Successively, seeds were thoroughly washed with sterile water and grown in vermiculite for 15–20 days at 24 °C under daylight fluorescent lamp. Fully grown leaves were harvested and 5 cm of the subapical segments were floated with the abaxial side (convex side) downwards in 35 × 10 mm Petri dishes containing test solutions, or were floated in water, as controls, in the same environmental conditions. After the due time, depending on the type of stress given, leaf segments were subjected to the extraction procedure.

2.4. Preparation of leaf extracts

Shredded leaves (250 mg) were treated in a Precellys 24 homogenizer (Bertin Instruments, France) in 2 mL tubes with 750 μL of 0.1% formic acid in Milli-Q-grade water. The homogenates, after centrifugation at 18000*g* for 30 min at 4 °C, were filtered sequentially through 5 μm and 0.45 μm Millipore filters and then stored frozen at −20 °C until used for the mass spectrometric measurements.

2.5. Preparation of flour extracts

Commercial refined flours were purchased in local markets. Three different lots of each type of flour were employed for sample preparations. Extracts were prepared by weighting 500 mg of flour in 20 mL centrifuge tube containing a magnet bar and extracted with a solution containing 0.1% formic acid in water 1:5 or 1:10 w/w. The suspension was kept under agitation for 3 h and then centrifuged at 18,000*g* for 30 min at 4 °C. The clarified supernatant was filtered sequentially through a 5 μm and 0.45 μm Millipore filters and then stored frozen at −20 °C until used for the mass spectrometric measurements. Flour extracts were prepared in triplicate.

2.6. Enzymatic synthesis of γ-glutamylpeptides

As some γ-glutamylpeptides were not available, that is, γ-glutamyl-2-aminobutyryc acid and γ-glutamylglutamine, we prepared the reference standard substances through enzymatic synthesis by employing γ-GT from equine kidney. In suitable conditions, this enzyme is able to transfer the γ-glutamyl moiety from glutathione to an acceptor amino acid. Briefly, the incubation mixture contained 20 mM Tris-HCl pH 8.4, 2 U of γ-GT, 20 mM glutathione and 5 mM of the acceptor amino acid (2-aminobutyric acid, or glutamine) in a final volume of in 1.0 mL. The reaction was conducted at 37 °C for 1 h by monitoring over time the product formation by mass spectrometric analysis. Finally, the reaction mixture was filtered by employing Amicon Ultra 0.5 mL centrifugal filters of 3 kDa molecular weight cut off in order to remove the enzyme from the products, and stored at −20 °C.

2.7. Norophtalmic acid synthesis

NOP was prepared by reacting glutathione with nickel Raney, a catalyst which is able to produce rapidly and under very mild condition the desulfurization of the cysteine residue, that is converted into an alanine residue. Briefly, the reaction was conducted for 30 min at 40 °C by stirring 5 mL of an aqueous solution of GSH (20 mg) at pH 7.4 in the presence of nickel Raney (500 mg). Mass spectrometric analysis of the mixture after 30 min of reaction showed the complete disappearance of GSH and NOP as the unique reaction product.

2.8. Induction of oxidative stress in rye, barley, oat, and winter wheat leaves

2.8.1. Water stress

Detached leaves were kept at 20 °C for various times at 55% relative humidity to induce water stress. For comparison with the water stressed leaves, controls were made by keeping leaves for the same times in a chamber containing water saturated air. Experiments were carried out under daylight fluorescent lamp. Leaves were weighted before and after wilting in order to correct the compound concentrations for weight loss.

2.8.2. Dark stress

Dark stress was induced by keeping for various times in darkness leaves, or leaf segments, while floating in Petri dishes containing water. Controls for comparison were made by keeping, under daylight