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S-nitrosoglutathione reductase (GSNOR) activity is down-regulated during pepper (*Capsicum annuum* L.) fruit ripening

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ABSTRACT

Pepper (*Capsicum annuum* L.) is an annual plant species of great agronomic importance whose fruits undergo major metabolic changes through development and ripening. These changes include emission of volatile organic compounds associated with respiration, destruction of chlorophylls and synthesis of new pigments (red/yellow carotenoids plus xanthophylls and anthocyans) responsible for color shift, protein degradation/synthesis and changes in total soluble reducing equivalents. Previous data have shown that, during the ripening of pepper fruit, an enhancement of protein tyrosine nitration takes place. On the other hand, it is well known that *S*-nitrosoglutathione reductase (GSNOR) activity can modulate the transnitrosylation equilibrium between GSNO and *S*-nitrosylated proteins and, consequently, regulate cellular NO homeostasis. In this study, GSNOR activity, protein content and gene expression were analyzed in green and red pepper fruits. The content of *S*-nitrosylated proteins on diaminofluorescein (DAF) gels was also studied. The data show that, while GSNOR activity and protein expression diminished during fruit ripening, *S*-nitrosylated protein content increased. Some of the protein candidates for *S*-nitrosylation identified, such as cytochorme c oxidase and peroxiredoxin II E, have previously been described as targets of this posttranslational modification in other plant species. These findings corroborate the important role played by GSNOR activity in the NO metabolism during the process of pepper fruit ripening.

1. Introduction

Peppers (*Capsicum annuum* L.), tomatoes and potatoes, belonging to the Solanaceae family, are of great agronomic and economic interest worldwide. Bell/sweet pepper fruits can be classified into three main types according to shape: California, Lamuyo and Dulce italiano. The California type is characterized by cube-shaped fruits of similar size along the transverse and longitudinal axes [1–3]. With regard to their culinary and nutritional values, pepper fruits have high levels of antioxidants, such as ascorbic acid (vitamin C), carotenoids, including β-carotene (provitamin A), and phenolic compounds [4–8]. Fruit ripening is a complex and irreversible process involving many changes at the phenotypic, structural and biochemical level [9,10]. In the case of pepper, fruits undergo multiple biochemical and cellular modifications which involve variations in antioxidant content and increased carotenoid synthesis [11–13], loss of cellular integrity, changes in β-galactosidase and peroxidase activity [14,15], carbon dioxide concentration [16] and increased NADPH content in mature fruits [1].

Nitric oxide (NO) is a radical molecule either directly or indirectly involved in many aspects of plant physiology under natural and environmental adverse conditions [17]. Thus, NO participates in reduction-oxidation processes which can affect signal transduction pathways, in which a family of NO-derived molecules, called reactive nitrogen species (RNS), plays a significant role. In many cases, this regulation process occurs through post-translational modifications (PTMs) of proteins, mainly nitration and/or *S*-nitrosylation (better known as *S*-nitrosation) [18–20]. The involvement of NO as a potential regulator of fruit ripening has been reported in different species such as strawberry [21], banana

Abbreviations: DAF, diaminofluorescein; GSNOR, S-nitrosoglutathione reductase; NO, nitric oxide; SNOs, S-nitrosothiols.

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[22] and olive [23]. Although pepper fruits have a very active oxidative metabolism [2,7], it has recently been demonstrated that NO metabolism could play an important role during the ripening process in this crop species. Specifically, protein nitration content has been shown to increase throughout this physiological process, with the antioxidant enzyme catalase being a prominent target which is negatively modulated by this posttranslational modification [3]. Given that *S*-nitrosothiols (SNOs) are important mediators of NO signaling, the main goal of this study was to analyze the activity, protein and gene expression of the *S*-nitrosoglutathione (GSNO) reductase, an enzyme directly involved in SNO regulation. Additionally, we studied the content of total *S*-nitrosylated (S-nitrosated) proteins with the aid of diaminofluorescein (DAF) gels. The data show that, during sweet pepper ripening, GSNOR activity is down-regulated, with a consequent increase in *S*-nitrosylated proteins, suggesting that NO metabolism plays a crucial role in this process.

2. Material and methods

2.1. Plant material

California-type sweet pepper (*Capsicum annuum* L., cv. Melchor) fruits, obtained from Syngenta Seeds Ltd. (El Ejido, Almería, Spain), were used at two differentiated ripening stages: green immature and ripe red.

2.2. Preparation of crude extracts

The fruits were ground in liquid N_2 using a mortar and pestle, and the resulting powder was resuspended in 0.1 M Tris-HCl buffer, pH 8.0, containing 1 mM EDTA, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol to a final ratio 1:1 of plant material/buffer (w/v). Homogenates were centrifuged at 15,000 *g* for 27 min. The supernatants were used for assays.

2.3. GSNOR activity assay and protein determination

GSNOR activity was determined spectrophotometrically at 25 °C by monitoring the oxidation of NADH at 340 nm according to the technique described by Barroso et al. [24] with some minor modifications. Mainly, the Sephadex G-25 gel filtration columns were not used before the spectrophotometric enzymatic assay because, unlike other plant samples, pepper fruit extracts did not show any interference in the slope of the GSNOR enzymatic reaction between pepper samples before and after elution through the columns.

GSNOR activity was also assayed by non-denaturing polyacrylamide gel electrophoresis using 6% acrylamide gels in Tris-HCl, pH 8.9, pH 8.0. Staining for GSNOR activity was carried out by soaking the gels in a solution of 0.1 M sodium phosphate, pH 7.4, containing 2 mM NADH, for 15 min in an ice bath. Excess buffer was drained, and the gel was covered with filter paper strips soaked in freshly prepared 5 mM GSNO (Calbiochem). After 10 min, the filter paper was removed, and the gels were exposed to UV light and analyzed for the appearance of GSNOR activity bands [24,25].

Protein concentration was determined with the aid of the Bio-Rad Protein Assay, which is based on the Bradford dye-binding method [26], using bovine serum albumin as standard.

2.4. SDS-PAGE and immunoblot analyses

SDS-PAGE was carried out in 4–20% precast polyacrylamide gel using a Mini-Protean electrophoresis cell (Bio-Rad, Hercules, CA, USA). For immunoblot analyses, the proteins were transferred onto 0.45-μm PVDF membranes using a Trans-Blot® Turbo™ Transfer Starter System (Bio-Rad, Hercules, CA, USA). After transfer, the membranes were used for cross-reactivity assays with a rabbit polyclonal antibody against GSNO reductase [27]. For immunodetection, an affinity-purified goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad Laboratories) and an enhanced chemiluminescence kit (Clarity™ Western ECL Substrate, BioRad) were used. Chemiluminescence was detected using a Molecular Imager® Gel Doc™ XR documentation system. Band intensity was quantified with the aid of ImageJ 1.45 software.

2.5. Semiquantitative reverse transcription-PCR

Total RNA was extracted from whole fruit using Trizol, as described by the manufacturer (Gibco BRL, Life Technologies). Two micrograms of total RNA was used to produce cDNA by reverse transcription (RT)-PCR [28]. Semiquantitative RT-PCR amplification of *ACTIN* cDNA from pepper was used as control. *GSNOR* and *ACTIN* cDNAs (accession numbers EU652335 and AY572427, respectively) were amplified by the PCR as follows: 1 μl of each cDNA (30 ng) was added to 0.250 mM dNTPs, 2.5 mM $MgCl_2$, 1xPCR buffer, 0.5 U of HotMaster TaqTM DNA polymerase (Eppendorf, Madrid, Spain) and 0.5 mM of each specific primer (*GSNOR*, 5′-CTTGACAAAGTATGTGTCC-3′ and 5′-GTGAGTGTAGAACTTCTCC-3′; *ACTIN*, 5′-ACTCTTAATCAATCCCTCC-3′ and 5′-GCACTGTATGACTGACACC-3′) in a final volume of 20 μl. Reactions were carried out in the Hybaid thermocycler. A first step lasting 10 min at 95 °C was followed by 28–33 cycles (depending on the gene) of 30 s at 95 °C, 30 s at 55 °C and 45 s at 65 °C. PCR products were then detected by electrophoresis in 1% (w/v) agarose gels and by staining with Gel-red. The bands were quantified using a Gel Doc system (Bio-Rad Laboratories) coupled with a high-sensitivity charge-coupled device camera. Band intensity was expressed as relative absorbance units. The ratio between the *GSNOR* gene and *ACTIN* amplification was calculated to normalize for initial variations in the sample concentration [29].

2.6. Protein S-nitrosothiols (SNOs) on diaminofluorescein (DAF) gels

Protein *S*-nitrosothiols were separated and detected on DAF gels [30]. Briefly, SDS-PAGE was run using gradient gels, as indicated above, with standard running buffer containing 1 mM EDTA. The gels were coated with 28 μM 3-amino,4-aminomethyl-2′7′-difluorescein (DAF-FM) and incubated in the dark for 10 min at room temperature. After electrophoresis, the gels were exposed to UV light for 5 min and then imaged using a fluor imager (BioRad) (excitation wavelength of 488 nm and emission wavelength of 530 nm). As internal controls, before being loaded on DAF gels, the pepper samples (25 μg proteins per treatment) were pre-treated at 25 °C for 3 h with different chemicals capable of: 1) decomposing SNOs, such as 20 mM ascorbate (AsA) and 0.1 mM CuCl; 2) blocking free thiols, such as 5 mM *N*-ethylmaleimide (NEM); and 3) reducing agents, such as 20 mM dithiothreitol (DTT), 20 mM reduced glutathione (GSH) and 100 mM β-mercaptoethanol (ME).

2.7. Electro-elution and proteomic analyses by MALDI-TOF/TOF

After electrophoresis, the identified bands were cut out from gels and placed in the glass tubes of a Bio-Rad 422 Electro-Eluter Module. Electro-elution was carried out at 10 mA per tube, at constant current, for 5 h in 25 mM Tris/192 mM glycine buffer (pH 8.6) containing 0.1% (w/v) SDS, with membrane caps (12,000 Da cut-off). Finally, the electro-eluted proteins were analyzed by Matrix-Assisted Laser Desorption Ionization - Time Of Flight (MALDI - TOF/TOF) [31].