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BRIEF COMMUNICATION

Identification and analysis of proteins sharing dehydroascorbate reductase activity

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Abstract

Dehydroascorbate (DHA)-reducing proteins were observed using a native-PAGE/activity staining method, and identified in soybean and rice by Western analysis using antibodies against homologous proteins known to catalyse DHA reduction. Administration of 1 mM DHA apparently did not trigger DHA reductase activity in lupin and onion, whereas activity was increased in rice and barley.

Additional key words: *Allium cepa*, ascorbate regeneration, *Glycine max*, *Lupinus albus*, *Oryza sativa*.

Ascorbate (AA) is widely utilised in plants as an electron donor in different metabolic reactions (De Tullio and Arrigoni 1999). AA utilisation yields dehydroascorbate (DHA) *via* the disproportionation of the intermediate oxidised product ascorbate free radical (AFR). DHA can be reduced back to AA both enzymatically and non-enzymatically. Glutathione (GSH)-dependent DHA reductase activity has been detected in many plant species, and the enzyme (EC 1.8.5.1) purified from different sources. The list of animal and plant proteins sharing DHA reductase activity, beside catalysing different reactions, presently includes thioredoxin, thioredoxin reductase, GSH peroxidase, Kunitz-type trypsin inhibitor and even rat serum albumin (De Tullio *et al.* 1998, Vethanayagam *et al.* 1999, Washburn and Wells 1999). Virtually all these proteins have in common the presence of thiols, frequently arranged as dicysteinyl motifs (C-X-X-C). This point suggests the possibility that, at least in some cases, DHA reduction could be just a non-specific side-reaction of thiol-containing proteins, with little physiological value. These findings opened the debate on the presence and function of "real" DHA reductases. We have previously described a native PAGE/activity staining technique for the identification of GSH-dependent DHA reductases (De Tullio *et al.* 1998). In order to face the problem of the functional analysis of DHA-reducing proteins (DRPs), we tested the specificity and reliability of this method. In addition, data concerning the effects of high DHA concentration on DRP activity in different plants are presented.

Soybean (*Glycine max* L.), barley (*Hordeum vulgare* L.), and rice (*Oryza sativa* L.) seeds were sown in glass Petri dishes with two layers of wet filter paper in dark room at 25 °C. After 48 - 72 h of germination, embryos were excised using a scalpel and either immediately used for protein extraction, or subjected to DHA treatment. Seeds of white lupin (*Lupinus albus* L.) were sown in moist vermiculite. Onion (*Allium cepa* L.) bulbs were suspended over plastic trays so that only their basal plates were immersed in distilled water. When roots had reached the length of 2 - 4 cm, lupin seedlings and onion bulbs were placed onto beakers containing distilled water (control) or 1 mM DHA. Solutions were aerated by continuous bubbling and their pH set at 5.8 with 1 M HCl

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Abbreviations: AA - ascorbate; AFR - ascorbate free radical; DHA - dehydroascorbate; DRP - dehydroascorbate-reducing protein(s); GSH - glutathione; PAGE - polyacrylamide gel electrophoresis; PVDF - polyvinylidene difluoride.

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or 1 M KOH. After 24 h of incubation, root tips (0.3 cm) were cut and used for protein extraction. Proteins were extracted and DHA reductase activity assayed both electrophoretically and spectrophotometrically as previously described (De Tullio *et al.* 1998). Proteins were determined according to Bradford (1976), using bovine serum albumin as a standard. Immunoblots were performed according to Harlow and Lane (1988) using PVDF membranes, peroxidase-conjugated secondary antibodies and diaminobenzidine as the peroxidase substrate.

Western blots confirmed the identity of DRP bands evidenced by gel electrophoresis (Fig. 1*A*). Both DRP bands observed in soybean embryos and cotyledons were recognised by antibodies against homologous trypsin inhibitor. On the other hand, antibodies against rice DHA reductase cross-reacted only with the upper DRP band observed by gel analysis of rice extracts, apparently indicating the presence in rice of additional DRPs, immunologically distinct from the DHA reductase described by Ushimaru and co-workers (Kato *et al.* 1997). Several attempts were made to assess whether the anti-soybean trypsin inhibitor and anti-rice DHA reductase antibodies could cross-react with DRPs from other dicots (white lupin, pea, spinach) and monocots (onion, maize) evidenced by the native PAGE/activity staining method. Unfortunately, none of these DRPs was recognised by the antibodies used (not shown).

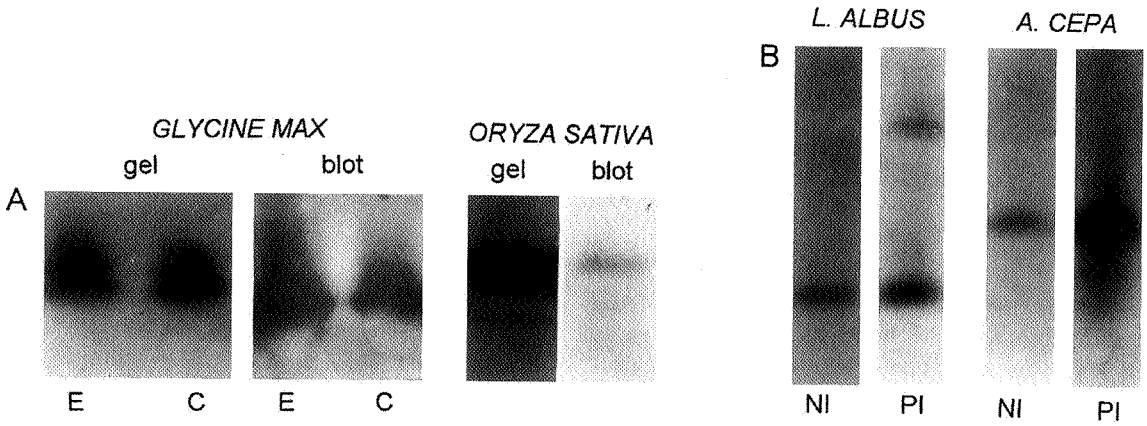

Fig. 1. Identification of DRPs and effect of gel pre-incubation in GSH. *A* - proteins extracted from soybean (*Glycine max*) embryos (E) or cotyledons (C), and proteins extracted from rice (*Oryza sativa*) embryos were electrophoresed in duplicate on the same gel. Half gel was stained for DHA reductase activity, the remaining half was transferred to PVDF membranes for immunoblotting with antibodies against soybean Kunitz-type trypsin inhibitor or rice DHA reductase. *B* - gel pre-incubation in GSH elicits DHA reductase activity. Protein samples extracted from root tips of *Lupinus albus* and *Allium cepa* were used for electrophoresis. After the run, gels were either incubated as usual with 2 mM DHA and 4 mM GSH (indicated with NI for normal incubation) or pre-incubated for 15 min in 5 mM GSH before standard incubation (indicated with PI for pre-incubated).

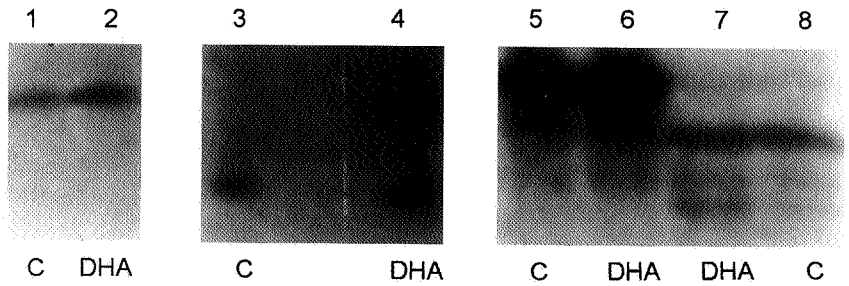

Fig. 2. Effect of DHA administration on DHA reductase activity in different plants. Root tips (0.3 cm) of white lupin (*lanes 1* and *2*) and onion (*lanes 3*, *4*) were removed from seedlings after 24 h of incubation in water (C, for control) or 1 mM DHA. Barley (*lanes 5*, *6*) and rice (*lanes 7*, *8*) embryos were excised from endosperm and incubated either in water or in 1 mM DHA in glass dishes under mild agitation. Equal protein loading was ensured for each material. White lupin: 80 μg total proteins per lane; onion: 150 μg total proteins per lane; barley and rice 100 μg total proteins per lane.

Enhanced DRP activity and/or new DRP bands were evidenced when, after the electrophoretic run, gels were pre-incubated in 5 mM GSH (Fig. 1*B*). This could be interpreted as an indication that some DRPs have low affinity for their substrate GSH. Alternatively, GSH treatment could simply activate potential DRPs by

shifting the protein thiol/disulfide balance towards the reduced form, responsible for DHA reduction. It should be considered that GSH pre-incubation was necessary to elicit DHA reducing capability in bovine erythrocyte GSH peroxidase (Washburn and Wells 1999). It cannot be excluded that in some cell compartments GSH could actually be present at higher concentration, thus enabling these "facultative DRPs" to play a role in enzymatic DHA reduction.

The concept that DHA reductase and AFR reductase have an important function in the replenishment of the AA pool due to their capability to recycle its oxidised forms DHA and AFR is still widely accepted, although it has been observed that AA regeneration cannot keep pace with AA utilisation under conditions limiting AA biosynthesis (De Tullio *et al.* 1998). We have suggested that the function of both AA-regenerating enzymes could be related to the avoidance of DHA accumulation, rather than quantitative AA regeneration (De Tullio and Arrigoni 1999). In this view, a "real" DHA reductase is supposed to be activated when DHA content is increased. This hypothesis was tested by challenging different plants with 1 mM DHA and analysing DHA reductase activity (Fig. 2). Our results show that DHA administration did not change DRP activity in lupin and onion, whereas the *Gramineae* barley and rice showed increased DRP band intensity in response to DHA, although to different extent.

In conclusion, functional DRP analysis performed by means of our native-PAGE method seems to confirm previous observations concerning the non-specificity of at least some proteins sharing DHA reductase activity. On the other hand, the induction of DRP activity in response to DHA in advanced monocots is a promising field deserving further investigation. It should be considered that Ushimaru and co-workers have purified an apparently specific DHA reductase from rice and found immunologically related proteins in monocots, but not in dicots (Kato *et al.* 1997) and have reported the identification of a cDNA coding for this protein (Urano *et al.* 2000). In addition, Shimaoka *et al.* (2000) recently reported the presence of two spinach DRPs with high affinity for both GSH and DHA. The future developments of these studies will shed new light on the role of catalysed DHA reduction.

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