BIOLOGIA PLANTARUM 46 (2): 175-179, 2003

Brownish acidic protein induced in pumpkin callus by a high concentration of 2,4-dichlorophenoxyacetic acid

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Abstract

An unknown brownish protein was purified by ammonium sulfate precipitation and DEAE-cellulose column and hydroxyapatite column chromatographies from pumpkin callus treated with a high concentration of 2,4-D. The apparent molecular mass and isoelectric point of the purified protein were estimated to be 38 kD and 4.6, respectively. The absorption spectra of the protein showed a shoulder at around 280 nm and a sharp peak at 405 nm. In order to determine what the purified protein is, a cDNA library of the callus treated with a high concentration of 2,4-D was immunoscreened with antiserum raised against the purified protein. The obtained positive cDNA clone encoded a thioredoxin *h* having a predicted molecular mass of 13 123 D and a predicted isoelectric point of 5.24, suggesting that the purified protein might be a trimer that was formed by oxidative polymerization of the thioredoxin *h*.

Additional key words: antiserum, cDNA cloning, *Cucurbita maxima*, 2,4-D, immunoscreening, purification, thioredoxin *h*, Western blotting.

Introduction

Thioredoxins are small and ubiquitous proteins possessing a readily reducible disulfide bond in their active structure. They are thought to play an important role in the control of protein function in various physiological processes by reducing disulfide bonds in the proteins. In plants, *f*-type and *m*-type thioredoxins, localized in chloroplasts, and *h*-type thioredoxins, which are thought to be present in the cytosol, have been reported. The *f*-type and *m*-type thioredoxins have been demonstrated to be involved in the light regulation of enzymes in photosynthesis (Droux *et al.* 1987, Crawford *et al.* 1989, Scheibe 1991).

On the other hand, *h*-type thioredoxins have been reported to be involved in self-incompatibility systems in *Phalaris coerulescens* (Li *et al.* 1995) and *Brassica* (Bower *et al.* 1996, Cabrillac *et al.* 2001). In the rice plant, *h*-type thioredoxin has been shown to be one of the major proteins in sieve tubes (Ishiwatari *et al.* 1995, 1998). However, little is known about the physiological and biochemical properties of thioredoxin *h*.

The aim of this study was to purify the brownish acidic protein from pumpkin callus treated with a high concentration of 2,4-D and to identify it at molecular level.

Materials and methods

Plants: Callus was induced from sarcocarp tissues of pumpkin (*Cucurbita maxima* Duch. cv. Ebisu) fruit harvested at Kagawa University Farm on Murashige and Skoog's (MS) solid medium containing 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 μM kinetin, at 25 °C in the dark, as described previously (Fujita *et al.* 1994). The callus was cultivated for more than fifty generations under the same conditions except that 5.3 μM naphthalene-1-acetic acid (NAA) was used instead of 4.5 μM 2,4-D. Before being used for protein extraction,

Received 12 July 2001, *accepted* 4 March 2002.
Abbreviations: Ala - alanine; Cys - cysteine; ECL - enhanced chemiluminescence; 2,4-D - 2,4-dichlorophenoxyacetic acid; Gly - glycine; Mr - relative molecular mass; NAA - naphthalene-1-acetic acid; pfu - plaque-forming unit; Pro - proline; Trp - tryptophan.
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the callus was transferred to the same medium containing 180 μM 2,4-D instead of 5.3 μM NAA and incubated for 6 d.

Extraction of soluble protein: The callus and organs were homogenized in an equal volume of 25 mM Tris-HCl buffer (pH 8.0) that contained 1 mM EDTA and 1 % (m/v) ascorbate. The homogenate was squeezed through two layers of nylon cloth. The filtrate was centrifuged at 12 000 *g* for 15 min, and the supernatant was used as soluble protein solution for enzyme purification and Western blotting analysis.

Protein content was measured by the method of Bradford (1976).

Purification of brownish protein: Protein in the solution prepared from 250 g of callus was precipitated with ammonium sulfate between 30 and 70 % saturation. The protein was dialyzed against 5 mM Tris-HCl buffer (pH 8.0) that contained 0.01% (v/v) β-mercaptoethanol (buffer A) overnight. The dialyzate was put on a column (1.25 cm i.d. × 57 cm) of DEAE-cellulose (*DE-52*; *Whatman*, Kent, UK) that had been equilibrated with buffer A. The column was washed with 200 cm^3 of buffer A and eluted with a linear gradient of 0 to 180 mM KCl in 1 dm^3 of buffer A. Fractions of 5.8 cm^3 were collected. The fractions corresponding to 42.5 to 57.5 mM KCl were combined as a 50 mM KCl eluate. This process was carried out five times. The five eluates obtained were combined and put on a column (2 cm i.d. × 4 cm) of hydroxyapatite (*Wako*, Osaka, Japan) that had been equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 0.01 % (v/v) β-mercaptoethanol. The column was washed with the same buffer. The brownish protein fractions that passed through the column were combined. The protein was precipitated with ammonium sulfate of 70 % saturation and dialyzed against buffer A. The dialyzate was used as the purified protein.

Pumpkin callus cDNA library: Total RNA (1.29 mg) was obtained from 23 g of callus treated with 180 μM 2,4-D for 2 d according to the method of Vries *et al.* (1988). A 2,4-D-treated pumpkin callus cDNA library was constructed using 3 μg of purified poly(A)$^+$mRNA (*Oligotex-dT30 super*, *Takara*, Tokyo, Japan) with a ZAP-cDNA synthesis kit, a *Uni Zap XR* vector, and a *Gigapack II Gold* packaging extract (*Stratagene*, Ja Lolla, CA, USA), with a titer of 2.5×10^5 plaque-forming units (pfu) for the library. The library was used for screening as described below.

12 000 pfu of the pumpkin cDNA library were plated for primary screening. Plaques were formed on NZY top agarose at 42 °C for 4 h. To lift plaques, 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG)-treated nitrocellulose filter (*Hybond ECL*, *Amersham Pharmacia*, Little Chalfont, Buckinghamshire, UK) was put on the surface of the agarose where plaques had formed and incubated at 39 °C for 3.5 h. After incubation, the filter was removed from the agarose and subjected to immunodetection on the basis of *Amersham*'s ECL using antiserum against purified thioredoxin, as described previously (Fujita *et al.* 1994). Positives responsive to anti-thioredoxin antiserum were further purified through two more rounds of screening under the above-described conditions.

The purified cDNA clones were rescued from the phage following *Stratagene*'s *in vivo* excision protocol. The cDNAs rescued in pBluescript SK(-) were sequenced using an *ABI PRISM 310* genetic analyzer (*PE Applied Biosystems Japan*, Tokyo, Japan). Nucleotide sequences and deduced amino acid sequences were analyzed using a *GENETYX* software system (*Software Development Co.*, Tokyo, Japan).

Two-dimensional gel electrophoresis: A miniaturized version of O'Farrell's two-dimensional electrophoresis was performed as described previously (Fujita *et al.* 1994). The gel was stained with silver.

Western blotting analysis was carried out as described previously (Fujita *et al.* 1994).

Production of antibody: A rabbit (weighing about 3 kg) was given subcutaneous injections of a total of 1.18 mg of purified thioredoxin in Freund's complete adjuvant at several sites. After two and four weeks, the rabbit was given a booster injection of 1.18 mg of purified thioredoxin in incomplete adjuvant. Blood was taken from the ear vein.

Results and discussion

In studies on chemical stress-responsive proteins, we have purified various isozymes of 2,4-D-inducible glutathione S-transferase from pumpkin callus treated with a high concentration of 2,4-D. During hydroxyapatite column chromatography in purification of one of the isozymes, we found that there were fractions containing a brownish protein at homogeneity among waste fractions. We tried to determine the properties of this abundant protein in the callus. The brownish protein was readily purified through ammonium sulfate-precipitation (in fractions of 30 - 70 % saturation), DEAE-cellulose column chromatography (in fractions eluted with 42.5 - 57.5 mM KCl), and hydroxyapatite column chromatography (in nonadsorptive fractions) from the soluble protein fraction of the pumpkin callus treated with 180 μM 2,4-D. A total of 5.3 mg of the

brownish protein was finally purified from 1 250 g of the callus (corresponding to 4.05 g protein). Two-dimensional electrophoresis of the purified protein showed one protein spot corresponding to Mr 38 000 and pI 4.6 (Fig. 1). Absorption spectra of the protein showed a shoulder at around 280 nm and a sharp peak at 405 nm (Fig. 2*A*,*B*).

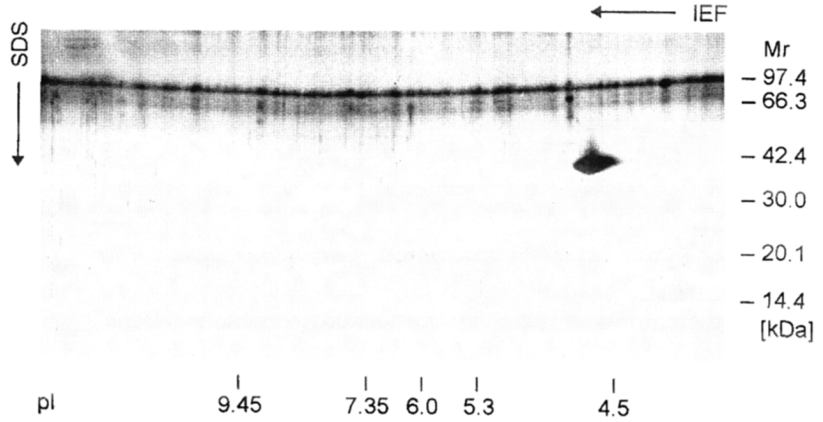

Fig. 1. Two-dimensional electrophoresis of brownish protein purified from pumpkin callus treated with a high concentration of 2,4-D. Two micrograms of the purified protein was applied. The gel was stained with silver.

In order to determine what the purified brownish protein is, we tried to produce an antiserum against the brownish protein and to clone the cDNA encoding the protein that was reactive to the antiserum. We obtained

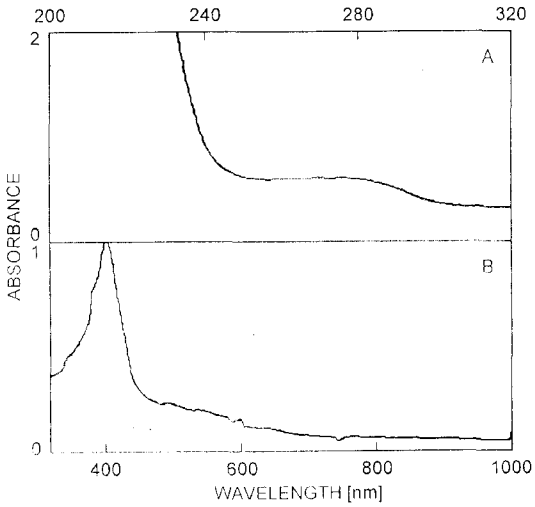

Fig. 2. Absorption spectra of the purified brownish protein (0.5 mg cm^{-3}) in the ultraviolet range (*A*) and in the visible range (*B*).

two positive clones through three rounds of immunoscreening from a cDNA library that was constructed using poly(A)$^+$mRNA from 2,4-D-treated pumpkin callus. Sequence comparison of the two clones showed that they are identical. The cDNA (Fig. 3) consists of 582-bp nucleotides encoding a polypeptide of 120 amino acids having a predicted molecular mass of 13 123 and a predicted isoelectric point of 5.24.

From nucleotide BLAST sequence similarity searching, the polypeptide was found to share high homologies with thioredoxins *h* and thioredoxin *h*-like proteins (rice thioredoxin *h* (52 % identity, 69 % similarity), *Arabidopsis thaliana* thioredoxin *h* (54 % identity, 72 % similarity), *Brassica rapa* thioredoxin (52 % identity, 69 % similarity), *Brassica oleracea* pollen coat protein (51 % identity, 69 % similarity), and *Brassica napus* thioredoxin-*h*-like-1 protein (51 % identity, 69 % similarity)).

Thioredoxins have been reported to have the most strictly conserved sequence, Trp-Cys-Gly-Pro-Cys (Marty and Meyer 1991, Brugidou *et al.* 1993), in which the reduced cysteine pair forms a very reactive center able to disrupt the disulfide bridge of target proteins. Instead of the canonical sequence, the polypeptide that is encoded by the cloned cDNA has Trp-Cys-Pro-Pro-Cys (from amino acid 38 to amino acid 42). Recently, *h*-type thioredoxins containing the same sequences have been reported in *Arabidopsis thaliana* (Rivera-Madrid *et al.* 1995) and *Brassica napus* (Bower *et al.* 1996).

Thioredoxins have also been shown to possess a second conserved cluster of amino acids (Gly33, Pro34, Pro76, Gly92, and Ala93 in *E. coli* thioredoxin (M54881)) that is thought to mediate the interaction of thioredoxins with other proteins (Eklund *et al.* 1984, Holmgren 1985). The polypeptide encoded by the cDNA contains all the amino acids at the proper corresponding positions except for Gly in the canonical redox sequence, which is substituted by Pro as described above. These findings all strongly suggest that the polypeptide is a pumpkin *h*-type thioredoxin. In nucleotide BLAST similarity searching for the pumpkin thioredoxin *h*, rice thioredoxin *h* had the highest score (129) and E-value (3e-29) among plant *h*-type thioredoxins registered,

indicating that the structural characteristics of thioredoxin *h* had been formed before the appearance of monocots and dicots (Rivera-Madrid *et al.* 1995). The pumpkin thioredoxin *h* was predicted to have no signal for organelles by PSORT (prediction of protein sorting signals and localization sites) in ExPASy Proteomics Tools, though *Brassica rapa* thioredoxin *h* has been reported to have 15 amino acids of a signal peptide (Toriyama *et al.* 1998).

The spectrum of the purified brownish protein has an absorption shoulder at around 280 nm as described above. *Chlamydomonas reinhardtii* (Stein *et al.* 1995) and *Triticum aestivum* (Gautier *et al.* 1998) *h*-type thioredxins have also been reported to have absorption shoulders

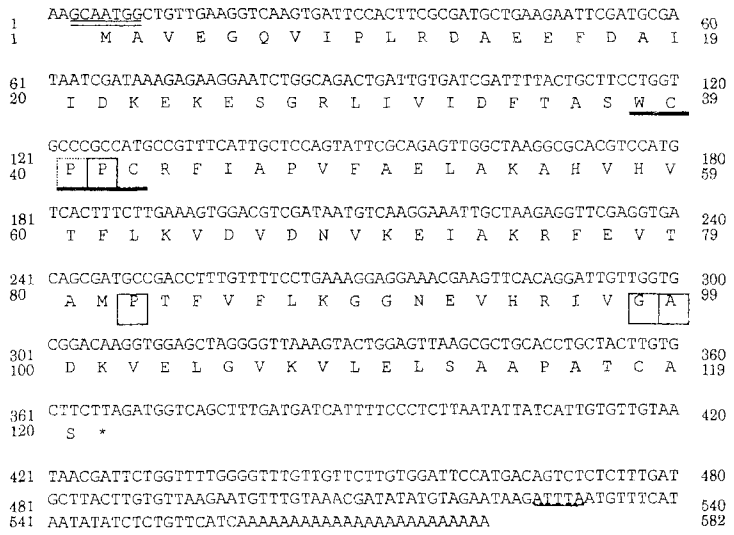

Fig. 3. Nucleotide sequence of the cDNA immunoscreened with antiserum against the brownish protein and deduced primary structure of protein encoded by the cDNA. The region that coincides with one of the Kozak arrangements (GNNATGG), which are thought to be a characteristic structure to initiation site of translation in eukaryotes, is indicated by double underlining. The ATTTA sequence, which possibly confers mRNA instability, is underlined. The sequence that is thought to work as a redox-active site of thioredoxin *h* is indicated by bold underlining. The amino acids that are thought to mediate the interaction of thioredoxins with other proteins are boxed.

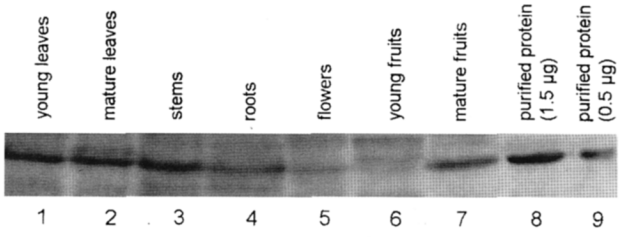

Fig. 4. Western blotting analysis of content of the brownish protein in different organs of pumpkin plants. Soluble protein was extracted from young leaves (*lane 1*), fully expanded mature leaves (*lane 2*), stems (*lane 3*), roots (*lane 4*), flowers (*lane 5*), young fruits (*lane 6*), and fully expanded mature fruits (*lane 7*). Each lane received 55 µg of the soluble protein except for *lane 8* and *9* (1.5 and 0.5 µg of the purified brownish protein, respectively).

around 280 nm. Both pI values, which were determined by isoelectric focusing and calculated from the predicted thioredoxin *h*, suggested that the expected protein is acidic. However, the relative molecular mass of the purified brown protein estimated from SDS-PAGE was almost three-fold greater than the predicted molecular mass of the thioredoxin *h* encoded by the cDNA. This contradiction suggests that the thioredoxin *h* has formed a trimer oxidatively during extraction and purification steps. Rey *et al.* (1998) reported that a thioredoxin-like protein in potato chloroplast, which is inducible by water deficit, had a Mr of 33 500, which is more than two-fold greater than the molecular masses of general thioredoxins. Based on the results of sequence comparison with thioredoxin, they suspected that the gene of the protein occurred through duplication of the thioredoxin gene. In our study, the cDNA encoding thioredoxin *h* was obtained from 12 000 pfu of λ phages. Therefore, the possibility that the number of phages used for the primary screening was not sufficient to lift the phages that contained the cDNA encoding the true target protein can not be ruled out. This thing might suggest that the purified protein is a novel thioredoxin-like protein, not a trimer that was formed by oxidative polymerization of thioredoxin *h*.

The organ specificity of the purified brownish protein was examined by Western blot analysis (Fig. 4). It was found that the protein is present at relatively high

concentrations in leaves, stems, and fully expanded mature fruits. We will further examine the organ specificities in more detail and the inducibilities against a variety of stresses.

References

Bower, M.S., Matias, D.D., Fernandes-Carvalho, E., Mazzurco, M., Gu, T., Rothstein, S.J., Goring, D.R.: Two members of the thioredoxin-*h* family interact with the kinase domain of a *Brassica* S locus receptor kinase. - Plant Cell **8**: 1641-1650, 1996.

Bradford, M.M.: A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Brugidou, C., Marty, I., Chartier, Y., Meyer, Y.: The *Nicotiana tabacum* genome encodes two cytoplasmic thioredoxin genes which are differently expressed. - Mol. gen. Genet. **238**: 285-293, 1993.

Cabrillac, D., Cock, J.M., Dumas, C., Gaude, T.: The *S*-locus receptor kinase is inhibited by thioredoxins and activated by pollen coat proteins. - Nature **410**: 220-223, 2001.

Crawford, N.A., Droux, M., Kosower, N.S., Buchanan, B.B.: Evidence for function of the ferredoxin thioredoxin system in the reductive activation of target enzymes of isolated intact chloroplasts. - Arch. Biochem. Biophys. **271**: 223-239, 1989.

Droux, M., Miginiac-Maslow, M., Jacquot, J.P., Gadal, P., Crawford, N., Kosower, A., Buchanan, B.B.: Ferredoxin-thioredoxin reductase: a catalytically active dithiol group links ferredoxin to thioredoxin functional in photosynthetic enzyme regulation. - Arch. Biochem. Biophys. **256**: 372-380, 1987.

Eklund, H., Cambillau, C., Sjoberg, B.M., Holmgren, A., Jornvall, H., Hoog, J.O., Branden, C.I.: Conformational and functional similarities between glutaredoxin and thioredoxins. - EMBO J. **3**: 1443-1449, 1984.

Fujita, M., Adachi, Y., Hanada, Y.: Preliminary characterization of glutathione S-transferases that accumulate in callus of pumpkin (*Cucurbita maxima* Duch.). - Plant Cell Physiol. **35**: 275-282, 1994.

Gautier, M.-F., Lullien-Pellerin, V., De Lamotte-Guery, F., Guirao, A., Joudrier, P.: Characterization of wheat thioredoxin *h* cDNA and production of an active *Triticum aestivum* protein in *Escherichia coli*. - Eur. J. Biochem. **252**: 314-324, 1998.

Holmgren, A.: Thioredoxin. - Annu. Rev. Biochem. **54**: 237-271, 1985.

Ishiwatari, Y., Fujiwara, T., McFarland, K.C., Nemoto, K., Hayashi, H., Chino, M., Lucas, W.J.: Rice phloem thioredoxin *h* has the capacity to mediate its own cell-to-cell transport through plasmodesmata. - Planta **205**: 12-22, 1998.

Ishiwatari, Y., Honda, C., Kawashima, I., Nakamura, S., Hirano, H., Mori, S., Fujiwara, T., Hayashi, H., Chino, M.: Thioredoxin *h* is one of the major proteins in rice phloem sap. - Planta **195**: 456-463, 1995.

Li, X., Nield, J., Hayman, D., Langridge, P.: Thioredoxin activity in the C terminus of *Phalaris* S protein. - Plant J. **8**: 133-138, 1995.

Marty, I., Meyer, Y.: Nucleotide sequence of a cDNA encoding a tobacco thioredoxin. - Plant mol. Biol. **17**: 143-147, 1991.

Rey, P., Pruvot, G., Becuwe, N., Eymery, F., Rumeau, D., Peltier, G.: A novel thioredoxin-like protein located in chloroplast is induced by water deficit in *Solanum tuberosum* L. plants. - Plant J. **13**: 97-107, 1998.

Rivera-Madrid, R., Mestres, D., Marinho, P., Jacquot, J.-P., Decottignies, P., Miginiac-Maslow, M., Meyer, Y.: Evidence for five divergent thioredoxin *h* sequences in *Arabidopsis thaliana*. - Proc. nat. Acad. Sci. USA **92**: 5620-5624, 1995.

Scheibe, R.: Redox-modulation of chloroplast enzymes: a common principle for individual control. - Plant Physiol. **96**: 1-3, 1991.

Stein, M., Jacquot, J.-P., Jeannette, E., Decottignies, P., Hodges, M., Lancelin, J.-M., Mittard, V., Schmitter, J.-M., Miginiac-Maslow, M.: *Chlamydomonas reinhardtii* thioredoxins: structure of the genes coding for the chloroplastic *m* and cytosolic *h* isoforms: expression in *Escherichia coli* of the recombinant proteins, purification and biochemical properties. - Plant mol. Biol. **28**: 487-503, 1995.

Toriyama, K., Hanaoka, K., Okada, T., Watanabe, M.: Molecular cloning of a cDNA encoding a pollen extracellular protein as a potential source of a pollen allergen in *Brassica rapa*. - FEBS Lett. **424**: 234-238, 1998.

Vries, S.D., Hoge, H., Bisseling, T.: Isolation of total and polysomal RNA from plant tissues. - Plant mol. Biol. Manual **B6**: 1-13, 1988.