Interaction of thioredoxins *h* with maize endosperm protein fractions

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Resumen

Las biotecnologías tienen aún mucho que hacer por su parte en el esfuerzo emprendido para mejorar la calidad de los alimentos. Las tiorredoxinas se conocen por ser pequeñas proteínas reductoras, ubicuas, pudiendo modular la calidad reológica del gluten y reducir la alergenicidad de los productos que derivan de harina de cereales. Este trabajo analiza la interacción de tres Trx *h* así como la de E. coli sobre las fracciones proteicas del endospermo de maíz. Los resultados confirman la capacidad de estas proteínas para reducir los puentes disulfuros intra y/o intermoleculares que liberan así agrupaciones tioles identificables por medio de un marcaje con mBBr. Además, la Trx *h*1 que posee una cisteína suplementaria en su dominio N-terminal muestra una gran capacidad para formar redes proteicas de altos pesos moleculares. Así, hay una cierta correlación entre las propiedades de estas proteínas identificadas in vitro y las características de sus interacciones con proteínas dianas. En la medida en que este tipo de interacción puede teóricamente hacerse con cualquier tiorredoxina, cloroplastidica, animal, de levadura, bacteriana o de cualquier origen, este trabajo abre perspectivas debido a las numerosas posibilidades de intervenciones sobre la materia prima in vivo o in planta para mejorar la calidad y la seguridad de los alimentos.

Summary

Biotechnologies have still much work to do on their side in the effort made to improve food quality. Trx are known to be small ubiquitous reductases, being able to modulate gluten rheological quality and to lower allergenicity of products deriving from cereals flour. This present report analyzes the interaction of three Trx *h* as well as E coli Trx on maize endosperm protein fractions. The data confirm the capacity of these proteins to reduce intra or / and intermolecular disulphides bridges, thus releasing free thiol groups which are identifiable by mBBr labelling. Trx *h*1 which has an additional cysteine in its N-terminal domain shows a great capacity to form high molecular weights protein network. There is thus a correlation between these proteins properties underlined in vitro and their interactions characteristics with target proteins. Because these interactions can theoretically be done with any Trx, from chloroplast, animal, yeast, bacterial or whatever, this opens many possibilities to intervene on raw material in vivo or in planta to improve food quality and safety.

Abbreviations: PMSF, phenylmethylsulfonyl chloride; mBBr, monobromobimane; TCA, trichloroacetic acid; DTT, dithiothreitol.

Introduction

Food insecurity and malnutrition continue to be the major developmental challenge in the beginning of the 21st century. Nearly 200 million children younger than five years are undernourished for protein, leading to a number of health problems, including stunted growth, weakened resistance to infection and impaired intellectual development. In addition, malnutrition in the form of obesity is also increasing in several developing and developed countries. Efforts to overcome poverty must take into account various factors as the food consumption habits, food nutritional status, cropping system practiced, public policies and countless other factors. In spite of the significant advances made in genetic enhancement of crop plants for nutritional value, science and technology have to play a great role in the long-term global strategy for the fight against malnutrition.

Since the impact of cereal in worldwide nutrition, increases in the productivity of cereal grain crops have helped many developing countries achieve self-sufficiency in meeting the energy requirements. Maize (*Zea mays* L) (C_4 plant), is world's one of the three most popular cereal crops for both livestock feed and human nutrition. With its high content of carbohydrates, fats, proteins, some of the important vitamins and minerals, maize is the main food of the lower-socio-economic populations and in some regions it represents more than 80% of daily food consumption.

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The maize kernel, like that of other cereal grains, includes pericarp (6%), endosperm (82%) and germ (12%) (Watson, 1987). The main structural component of the endosperm is starch, a complex carbohydrate that constitutes on an average 71% of the grain and is a source of concentrated energy. Most of the protein in a mature maize kernel is contained in the endosperm and the germ, but the endosperm protein is low in quantity as well as quality. In contrast the germ protein is sufficiently superior in quantity and quality. In normal maize, proportions of various endosperm storage protein fractions, on an average, are: albumins (3%), globulins (3%), zeins (60%) and glutelins (34%) (Salamini and Soave 1982). Zeins are characterized by a high content of glutamine, leucine and proline and are devoid of lysine and tryptophan. Since zeins are the main protein in normal genotypes, they considerably dilute the contribution of these essential amino acids witch are in balanced content in the non-zeins proteins. The need to ameliorate the nutrition value of cereal grains and maize ones in particular was well known (Osborne and Mendel, 1914)

Serious efforts to improve the nutritional quality of maize endosperm protein began in the mid-60 and find a breaking point with the discovery of the biochemical effects of mutant alleles opaque-2 and floury-2 by the Purdue university researchers (Mertz *et al.,* 1964; . Nelson *et al.,* 1965), witch made grain protein in the endosperm nearly twice as nutritious as those found in normal maize. A continuing search for identifying new and better mutants has been underway during the past several decades. In spite of the discovery of new mutants such as opaque–7, opaque-6, floury-3, mucronate and defective endosperm, none offered any additional advantage over opaque-2 in maize breeding program.

Various strategies to improve quality protein maize were underway, but all them require long-term efforts to achieve lysine levels matching the specific mutants which give a big boost in lysine content. One of the approaches exploits double mutant combination; other is based on the use of two genetic systems involving opaque-2 gene and the genetic modifiers of the opaque-2 locus (Vassal, 2000; Prasanna *et al.,* 2001), and has been quite successful.

Genetic modification involving artificial altering of the genetic material of a species was one the alternative strategies developed in the 80. Crops have been engineered to confer various benefits, such as increased yield and pest and weed resistance, resulting in reduced reliance on pesticides and herbicides, enhanced taste, drought resistance and aesthetic appeal, improved nutritive value, and longer shelf life (Falk *et al.,* 2002). In this area, different transgenic cereals with elevated thioredoxin *h* (Trx *h*) levels in seeds were obtained showing value-added traits (Cho et al, 2007). Among these transgenic plants, wheat seed products appear less allergenic and more digestible whereas in barley seed, the appearance of alpha-amylase is accelerated and its abundance and activity are increased. In the same way, an advance of germination rate is observed with barley seeds (Cho *et al.,*1999; Wong *et al.,* 2002).

Plant Trx *h* is involved in a wide variety of biological functions and the presence of its multiple forms has also been reported in plant seeds (Bestermann *et al.,* 1983). Trx *h* belongs to the redoxins family of small (12-14 kD) thermostable proteins with catalytically active disulfide groups. This class of proteins has been found in virtually all organisms, and has been implicated in myriad biochemical pathways (Buchanan *et al.,* 1994). The active site of Trx has two redox-active cysteine residues in a highly conserved amino acid sequence; when oxidized, these cysteines form a disulfide bridge (-S—S-) that can be reduced to the sulfhydryl (-SH) level through a variety of specific reactions. In physiological systems, this reduction may be accomplished by reduced ferredoxin, NADPH, or other associated thioredoxin-reducing agents.

Because of the quality of the reduced form, the addition of Trx promotes the formation of a protein network that produces flour with enhanced baking quality. Furthermore, Kobrehel *et al.,* (1994) have shown that the addition of Trx to flour of non-glutenous cereal such as rice, maize, and sorghum promotes the formation of a dough-like product.

Although Trx *h* have a homogeneous conformation, the particular microstructures of each protein are able to modulate determining effects in protein-protein interactions. It is therefore possible to modulate either dough rheological qualities or to obtain more targeted effects within the framework of an overexpression of a specific Trx in the grain. In this present report, we analyze the behaviour of different Trx against protein fractions from maize endosperm.

Materials and methods

Protein extraction

Seed wheat Trx*h* were obtained as previously described (Cazalis *et al.,* 2006). E. coli Trx was purchase from Promega. Maize (*Zea mays* L.) seeds from inbred line, FG89, were ground in a M20 mill (IKA-WERKE) to pass 0.5 mm sieve. An Osborne fractionation was performed on 200 mg meal. Each extraction step was carried out with 1 mL of solvent by mixing vigorously and placing the microfuge tubes on a rotator for 2h. Between each fraction, the remaining meal was washed with water and the supernatant disregarded. The F1 fraction was extracted with 50 mM Tris, pH 7.9, and 1mM PMSF (Sigma) buffer. F2 fraction was extracted with 70% (v/v) ethanol. Centrifugations were all performed at 12 000 *g* for 5 min in a microcentrifuge and samples then stored at -20°C. Fraction F2 was concentrated at 4 °C on Ultracel 5K centrifugal filter unit (Millipore) according manufacturer instructions. Protein concentrations were determined by the Bradford method (Bradford, 1976), with Bio-Rad reagent and BSA as a standard.

Protein interaction

Protein fractions were stepwise reduced with DTT (Lindsay and Skerrit, 1998). For the maize seed endosperm protein interaction with Trx, 1mM Trx *h*, previously reduced by 2 mM DTT was added to 60 µg protein extract and incubed at 30 °C for 30 min. samples were then applied to the gels for electrophoresis in non-reducing condition. In vitro protein mBBr labelling was carried out according Khobrehel et al (1992) with some adaptations. Protein interaction was performed as previously described. 400 nmol of mBBr was then added and the reaction was continued for another 15 min. 40 µl of a buffer (Tris 0.5M; pH 6.8; 4% SDS; 5 mM 2-mercaptoethanol; 10% glycerol; 0.005% bromophenol blue) was added to stop the reaction and derivatize excess mBBr , and the reaction continued for another 30 min at room temperature. Samples were then directly applied to gel. Following electrophoresis, gels were placed in 12% (w/v) TCA and soaked for 1 hr. Gels were then transferred to a solution of 40% methanol and 10% acid acetic overnight to remove excess mBBr. Fluorescence was visualized under UV light (365 nm). Gels were then stained with coomassie brilliant blue R-250 in 40% methanol/ 10% acetic acid for 2 h and destained overnight in the previous buffer without coomassie.

Results and discussion

Thioredoxins

The Trx *h* used are characterized by different molecular mass (fig.1 A) due principally to the extension of the N-terminal domain of the protein. Trx *h*1, in addition to exibit a low molecular weight, is characterized by a supplementary cysteine residue (Cys 11) in this domain upstream of the very typical active site WGGPC (Fig.1 B). The presence of this additional cysteine residue was reported in Trx *h* from monocots as well as from dicots (Ishiwatari *et al.,* 1995; Gelhaye *et al.,* 2003; Maeda *et al.,* 2003). About Trx *h*1, it was shown that Cys-11 residue was more implied in dimer formation events than in reductase activity (Cazalis et al, 2006). Trx *h*1 is also characterized by having a phenylalanine residue (Phe17) at the N-terminus instead of the typical tryptophan residue which was usually seen as a Trx *h* protein signature (Stein et al, 1995). Trx *h*2 has a standard profile. Trx *h*3 on the other hand, is characterized by its hydrophobic N-terminal domain and especially by the fact to appear exclusively in monomeric form, even in absence of a reducing agent such as DTT (Cazalis et al, 2006).

Protein fractions reduction

The stepwise reduction of protein fraction F1, made up albumins and globulins, is characterized by the variation in the molecular masses with the increase in the DTT concentration range. We observe the appearance of bands within molecular masses lower than 50 kD, which is concomitant with the disappearance of the higher molecular weight (fig.2 A).

The fraction F2 is mainly represented by zeins groups, the most relevant prolamins in the maize storage proteins. An (α)-zein represents the most important member of this complex group, characterized by molecular masses of 19kD and 22 kD, which are alcohol-soluble.

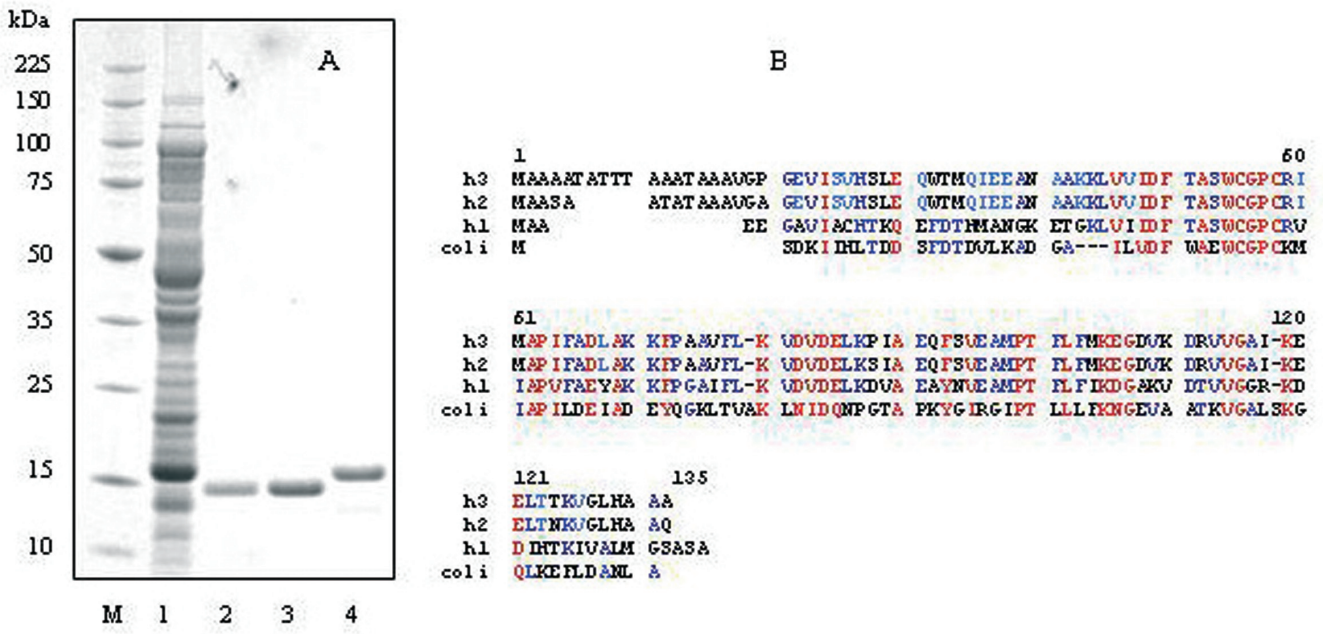

Figure 1. Overexpression and alignment of thioredoxins. (A) Gradient SDS-PAGE (4-12%) gels were loaded with the soluble fraction of *E. coli* BL21(DE3) transformed with the corresponding pET-Trx *h* construct and stained with Coomassie Brillant Blue R-250. His-tagged protein was purified by Ni-chelate affinity chromatography. Lane1: Trx *h*3 crude cell lysates; lane 2 to 4 eluates of the purified proteins of Trx*h*1, Trx*h*2, and Trx*h*3, respectively. Molecular markers (M) were loaded and their molecular mass in kDa is indicated on the left. (B) Alignment of wheat and *E. coli* thioredoxins. The accession numbers are as follows: Trx*h1*, AY072771; Trx*h2*, AF286593; Trx*h3*, AF420472; *E. coli*, NP418228.

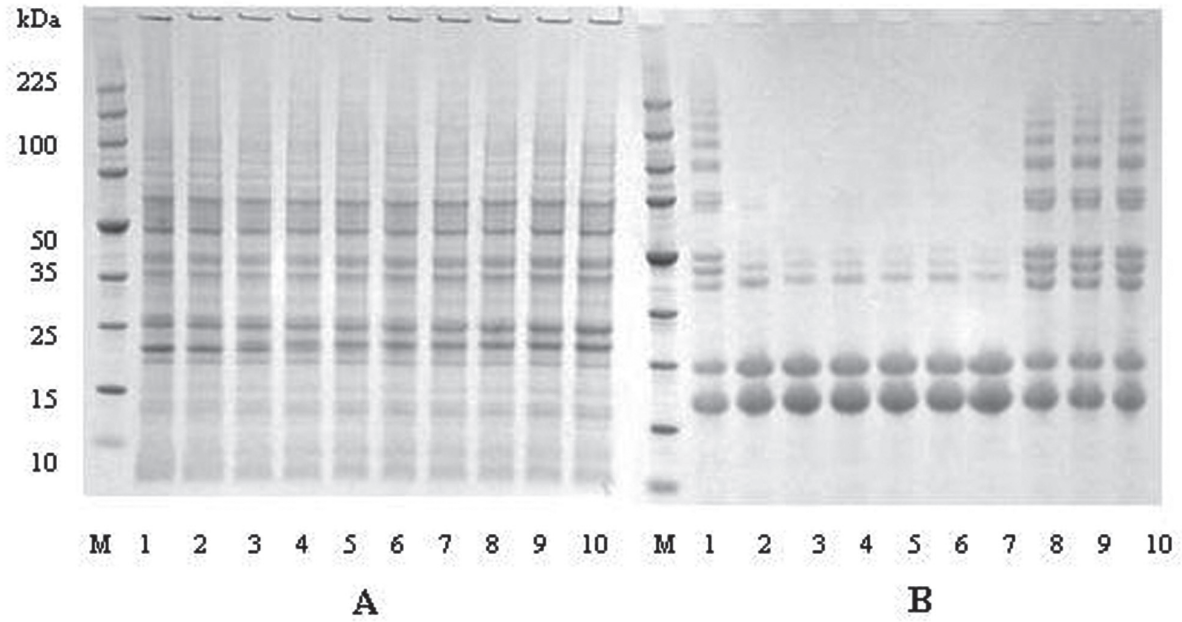

Figure 2. Protein fractions stepwise reduction. (A) Gradient SDS-PAGE (4-12%) analysis of protein samples from different stepwise reduction of soluble fraction F1 and (B) alcohol-soluble fraction F2. The DTT concentration range used was 0; 0.5; 2; 3; 4; 5; 10; 20 and 50 mM from lane 1 to 10 respectively. Molecular markers (M) were loaded and their molecular mass in kDa is indicated on the left.

Zeins of b (beta) and d (delta)-type, with a molecular mass of 14 kD and 10 kD respectively, are extracted in the presence of a reducing agent in alcoholic solution. A reducing agent is also required for extracting g (gamma)-zein, with molecular masses of 16 and 27 kD.

The reduction of F2 fraction confirms available sulfhydryl groups linking polymeric proteins. This is able to highlight oligomerization events. Indeed, as the reducing agent concentration increases, we observe initially, an increase in the a (alpha)-zein fraction. At 10 mM of DTT, the concentration is enough to break particular molecular sulfur-sulfur bonds and a stochastic cross-link occurs among monomers giving place to high molecular weight polymers formation (Fig.2 B). This phenomenon simulates what could occur in dough by addition of reducing agent during kneading.

Thioredoxin– proteins interactions

Bromobimanes in solution react with small thiols and with reactive protein thiol groups to form the corresponding S-labelled products which are stable and suitable for qualitative and quantitative determination (Crawford *et al.,* 1989). In this aim, maize endosperm proteins were extracted from full mature grain, labelled with mBBr and then subjected to SDS–PAGE. Qualitative analysis of sulfhydryl status shows either in F1 or in F2 that, in spite of the late storage mechanism during which, most proteins are oxidized, a significant number of SH groups remain free (Fig.3 A2; B2), as it was observed with wheat endosperm proteins (Rhazi *et al.,* 2003).

Trx interaction with the protein fractions underlines the two phenomena above mentioned, disulfide bridge reduction and network formation. With fraction F1, we observe that the greatest reducing capacity deals with *E. coli* Trx for which free thiol residues appear within the high molecular weights (Fig.3, A1). The electrophoretic pattern generate by others Trx *h* shows the appearance of a banding, which underline a marked tendency to network formation with F1 proteins fraction (Fig. 3, A2), as shown with mBBr fluorescence and Coomassie staining.

With fraction F2, one can underline the reducing action of Trx *h*2 by the appearance of the free sulfhydryl residues, especially between 50 and 75 kD, and characterized beyond by the fluorescent smear (Fig.3 B2), while this fluorescence of the fully reduced proteins sample appears for molecular masses higher than 75 kD. Trx *h*2 reduction capacity allows reformation of protein polymers of high molecular weigh as it occurred with the same fraction reduced by high DTT concentration (Fig.3, B2). We note in a less extent the

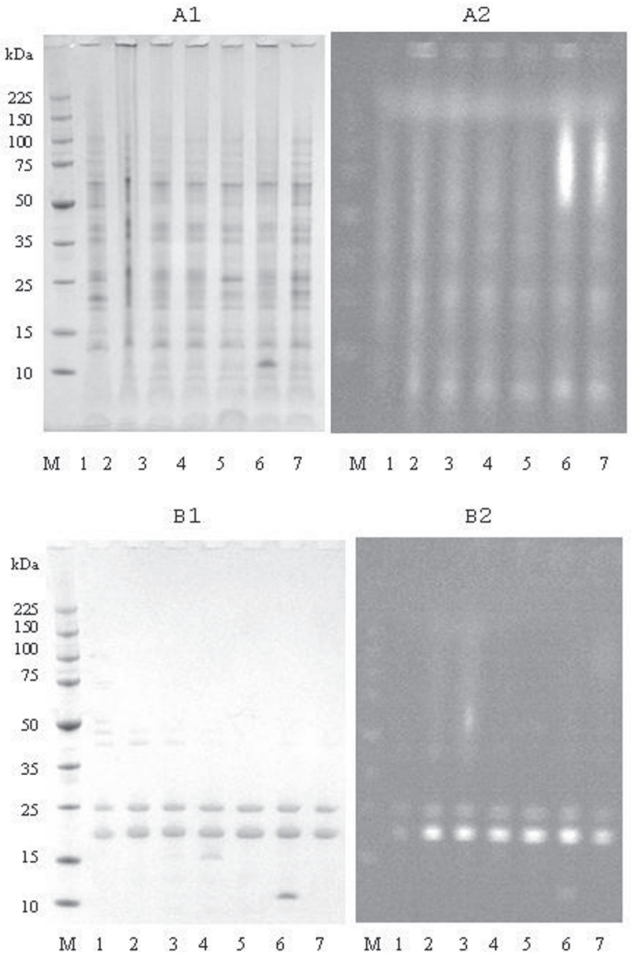

FIGURE 3. Thioredoxin-linked reduction of endosperm protein fractions. (A) Soluble protein fraction F1 and (B) alcohol-soluble protein fraction F2. Following incubation with the indicated Trx, the protein fractions were derivatized with mBBr, and fluorescence was visualized after SDS-PAGE (4-12%) (A2, B2). Gels were then stained with Coomssie R250 (A1, B1). Lane 1: protein alone; lane 2: DTT reduced protein; lanes 3 to 6, interaction with Trx*h*2, *h*1, *h*3 and *E. coli* respectively; lane 7: fully reduced protein.

reducing activity of *E. coli* Trx on fraction F2 proteins. Trx *h*1 and Trx *h*3 for their part, generate a banding similar to what it is found with stepwise reduction of the á-zeins fraction with a DTT concentration ranging from 1 to 5mM (Fig.2, B).

It was shown, in addition to activate enzymes, to reduce intramolecular disulfide bonds of storage proteins of the starchy endosperm, leading them to proteolysis (Kobrehel *et al.,* 1992; Lozano et al.1996; Besse et al.,1997), Trx alters the structure of 'chloroform-methanol' like proteins of the albumin/globulin fraction of wheat endosperm so they become more soluble (Wong *et al.,* 2004). On the basis of our results, we shows that the Trx *h* used in these experiments, interact

differently according to the protein fractions. Furthermore, we confirm that the third cysteine residue of Trx *h1* confers cross-linking capacities. Analysis of pea Trx *h* isoforms expression exhibited divergent patterns and levels of accumulation, suggesting non redundant functions (Montrichard et al, 2003; Traverso et al, 2007). Our results contribute to the unravelling of the biological functions of these Trx *h* in seed and suggest that these functions are furthermore related to the protein microstructures differences. This kind of reaction can be made virtually with any Trx, including the chloroplast Trx *f* and *m*, yeast and animal. This Trx pool constitutes an incomparable tool in biotechnology because one can choose the precise factor needed or can build it from these data base resources.

Concluding remarks

Trx have a great potential to improve food quality, for the developing countries as well as the developed ones where people is more and more sensitive to allergens and where hypoallergenic foods from cereals and legumes seeds are more than suitable. This potential is not only related to in vitro applications to modulate dough rheological characteristics for bread and bread-like made of wheat flour or non-glutenous flours such as maize, including from dicot such as pea. These proteins still have a great potential in transgenic plants generation, overexpressing specific Trx. Research programs in metabolisms regulation involving change in the redox state of thiol groups undertaken by many laboratories around the world, and especially on these redoxins (Buchanan and Balmer, 2005; Sahrawy, 2005) are in the point of reaching elegant applications in food and health fields.

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