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# Identification of thioredoxin target disulfides in proteins released from barley aleurone layers

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ABSTRACT

Thioredoxins are ubiquitous disulfide reductases involved in a wide range of cellular processes including DNA synthesis, oxidative stress response and apoptosis. In cereal seeds thioredoxins are proposed to facilitate the germination process by reducing disulfide bonds in storage proteins and other targets in the starchy endosperm. Here we have applied a thiol-specific labeling approach to identify specific disulfide targets of barley thioredoxin in proteins released from barley aleurone layers incubated in buffer containing gibberellic acid.



## 1. Introduction

Thioredoxins (Trxs) contain a conserved redox-active dicysteine motif (CGPC) and reduce disulfide bonds in target proteins (Fig. 1). Trxs thus regulate activities of target proteins and provide electrons for various reduction pathways, e.g. ribonucleotide reductase [1]. Reducing equivalents for recycling of Trx are provided from the NADPH-dependent flavoenzyme thioredoxin reductase (NTR). In relation to other organisms, plants contain a remarkably large number of Trxs, e.g. approximately 20 Trx genes in *Arabidopsis thaliana* [2]. In cereal seeds, Trxs play a particular role in the starchy endosperm during germination by modulating the activity of enzymes involved in degradation of energy reserves and inactivating their corresponding inhibitors [3–5]. Proteomics techniques have been used extensively to screen for novel targets of plant Trxs [6,7]. We have recently developed such a strategy based on labeling with isotope-coded affinity tags (ICAT) reagents [8]. The advantage of using ICAT reagents in this context is that it enables identification of thiol groups representing site-specific Trx target disulfides. Furthermore, quantification of ratios of peptides labeled with "light" $^{12}C$ ($ICAT_L$) and "heavy" $^{13}C$ ($ICAT_H$) reagents makes it possible to estimate the extent of reduction of individual target disulfides by quantitative mass spectrometry. This strategy was previously used to identify targets of barley Trx in intracellular proteins from germinating embryos, and is here applied to proteins released from aleurone layers maintained in culture medium.

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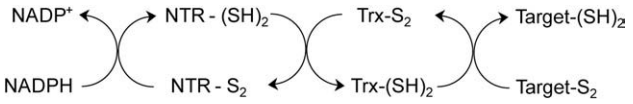


**Fig. 1 – The NADPH-dependent thioredoxin system.**

## 2. Experimental

### 2.1. *Plant material*

Aleurone layers were isolated from barley seeds essentially as described previously [9]. The embryo-containing half of the seeds (cultivar Himalaya) was removed using a scalpel and the remaining half-grains were soaked for four days in sterile water supplemented with 50 μg/mL ampicillin and 5 μg/mL nystatin. The endosperm was scraped away and 100 mg aleurone layers (fresh weight) were incubated in 2 mL buffer (20 mM $CaCl_2$, 20 mM Na succinate pH 4.2, 50 μg/mL ampicillin, 5 μg/mL nystatin, 5 μM gibberellic acid) at RT for 48 h with continuous gentle shaking. Culture supernatants were collected, centrifuged for 10 min at 12,000 rpm to remove debris, transferred to fresh tubes, and stored at −80 °C until use.

### 2.2. *Thioredoxin treatment and ICAT labeling*

Supernatants from aleurone layer cultures were dialyzed against 50 mM Tris pH 8.0 and the protein content was determined by using the Popov amido black assay [10] with bovine serum albumin as standard. The dialysed sample (100 μL, 11.2 μg protein) was incubated in 10 mM EDTA, 0.7 mM NADPH, 2 μM barley NTR (HvNTR2 [9]) and 8 μM HvTrxh2 [11] in a total volume of 170 μL for 1 h at RT. HvTrxh2 was omitted in control experiments. Reactions were stopped by addition of iodoacetamide (IAM) to a final concentration of 10 mM and samples were dialyzed against 50 mM Tris pH 8.0, followed by addition of 8 μL 1% SDS and 2 μL 50 mM tris(2-carboxyethyl) phosphine (TCEP) to 72 μL dialyzed sample and incubation at 99 °C for 10 min. Free thiol groups were blocked by addition of 20 μL 11.4 mM ICAT reagent to the thioredoxin treated sample ($ICAT_L$) and the control ($ICAT_H$). Samples were incubated 80 min at RT in the dark, followed by quenching of excess ICAT reagent by 10 μL 100 mM N-acetylcysteine (in 500 mM Tris/HCl pH 8.0) incubated for 10 min at RT. The samples were mixed 1:1, 45 μL 100 mM $CaCl_2$ was added and the volume was adjusted to 438 μL with water. Finally, 10 μL sequencing grade modified trypsin (0.1 μg/μL) (Promega, Madison, WI) was added followed by incubation at 37 °C overnight. Peptides were purified on cation exchange and avidin cartridges (Applied Biosystems) and subsequently incubated with cleaving reagents A and B (Applied Biosystems) in a 95:5 ratio (100 μL total volume) as recommended by the manufacturer.

### 2.3. *Mass spectrometry*

Purified peptides were analysed by Nano-LC-$MS^2$ performed on an LTQ Orbitrap XL (Thermo Fisher Scientific) coupled to an Agilent 1200 nano-flow system with a nanoelectrospray ion source (Proxeon, Odense). The nano-scale reversed phase HPLC column (ReproSil-Pur® 3 μm) was packed in a 15 cm long fused silica emitter with 75 μm inner diameter. The peptides were analyzed in the Orbitrap with a survey scan mass range of 350–1600 *m/z* with a resolution of R=60,000 at *m/z* 400 and a target value of $1 \cdot 10^6$ ions followed by fragmentation of the 5 most intense ions in the LTQ by CID with a target value of 10,000 ions. The raw spectra were processed to Mascot generic format files using DTA-supercharger (http://www.msquant.sourceforge.net). SwissProt 57.5 (20090707), NCBInr (20090826), and a barley EST database (barley gene index [HvGI] release 10.0: http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=barley) were queried using the Mascot Search Engine v2.2 (Matrix-Science) with 10 ppm and 0.6 Da mass tolerance in MS and MS/MS respectively, one missed cleavage site allowed, variable modifications: $ICAT_H$ (cysteine), $ICAT_L$ (cysteine), carbamidomethyl (cysteine), and oxidation (methionine). Decoy database searches yielded false discovery rates above identity threshold between 1.57–2.02%, 0–2.22% and 0.96–1.67% for Swiss-Prot, NCBInr and HvGI, respectively. False discovery rates above homology or identity threshold were between 3.45–3.60%, 2.17–8.28% and 5.45–5.62% for Swiss-Prot, NCBInr and HvGI, respectively. ICAT labeled peptide pairs were quantified using the software MSQuant (http://www.msquant.sourceforge.net). Identification and quantification of all peptides were validated by manual inspection.

## 3. Results and discussion

Germination of cereal seeds is initiated by the uptake of water by the embryo and the subsequent release of the plant hormone gibberellic acid that is detected by the aleurone layer surrounding the starchy endosperm. This signal triggers synthesis and secretion of hydrolytic enzymes and other proteins involved in the germination process from the aleurone layers. Here we have used a quantitative proteomics technique based on labeling with ICAT reagents to screen for targets of barley Trx in proteins released to the incubation medium from dissected aleurone layers. Briefly, aleurone layers were dissected from barley seeds and incubated in buffer containing gibberellic acid. Samples of proteins released from the aleurone layer were subjected to incubation in the presence of the thioredoxin system followed by labeling with ICAT reagents containing $^{12}C$ ($ICAT_L$) and $^{13}C$ ($ICAT_H$) essentially as described previously [8]. Proteins released from aleurone layers, NTR and NADPH were incubated in the presence or absence of barley thioredoxin HvTrxh2, followed by addition of IAM to quench Trx activity and block cysteine thiols released from target disulfides (Fig. 2). Subsequently remaining disulfides were completely reduced and ICAT-labeled (+Trx, $ICAT_L$; −Trx, $ICAT_H$). The samples were then mixed in a 1:1 ratio and digested by trypsin. Peptides containing non-target disulfide bonds are thus expected to yield identical amounts of thiol groups and $ICAT_H/ICAT_L$ peptide ratios of 1. In contrast, peptides containing cysteine residues released from thioredoxin target disulfide bonds are blocked by IAM and exhibit $ICAT_H/ICAT_L$ ratios >1 (Fig. 2).